Effect of Particulate Biphasic Nano-calcium Phosphate with High HA/TCP Ratios on Bone Formation in Rabbit Calvarial Defects

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

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Thesis Title	Effect of Particulate Biphasic Nano-calcium Phosphate with High HA/TCP Ratios on Bone Formation in Rabbit Calvarial Defects
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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ ผลของวัสดุไบเฟสิกนาโนแคลเซียมฟอสเฟสที่มีอัตราส่วน HA/TCP สูงชนิดอนุภาคต่อการสร้างกระดูกในรอยวิการกะโหลกกระต่าย
ผู้เขียน นายพงศกร ประเสริฐธรรม
สาขาวิชา ศัลยศาสตร์ช่องปากและแม็กซิลโลเฟเชียล
ปีการศึกษา 2556

บทคัดย่อ

วัตถุประสงค์: การศึกษานี้มีวัตถุประสงค์เพื่อประเมินผลของวัสดุทดแทนกระดูกไบฟาสิกแคลเซียมฟอสเฟตที่มีสัดส่วนของไฮดรอกซีอาปาไทต์ต่อไตรแคลเซียมฟอสเฟตสูง ต่อการสร้างกระดูกในรอยวิการที่กะโหลกศีรษะของกระต่าย

วิธีการศึกษา: กระต่ายสายพันธ์นิวซีแลนด์สีขาวจำนวน 16 ตัว ถูกสุ่มแบ่งออกเป็น 2 กลุ่ม กระดูกศีรษะของสัตว์ทดลองแต่ละตัวถูกสร้างรอยวิการรูปวงกลมขนาดเส้นผ่านศูนย์กลาง 10 มม. จำนวน 2 วง กลุ่มที่1 (มีกระต่ายจำนวน 3 ตัวต่อหนึ่งระยะเวลาศึกษา) รอยวิการถูกปลูกด้วยกระดูกอัตมันหรือถูกทิ้งให้ว่างเปล่า กลุ่มที่2 (กระต่าย 5 ตัวต่อหนึ่งระยะเวลาการศึกษา) รอยวิการถูกปลูกด้วยวัสดุไบฟาสิกแคลเซียมฟอสเฟต1 (อัตราส่วนของไฮดรอกซีอาปาไทต์ต่อไตรแคลเซียมฟอสเฟต 8:2) หรือ วัสดุไบฟาสิกแคลเซียมฟอสเฟต2 (อัตราส่วนของไฮดรอกซีอาปาไทต์ต่อไตรแคลเซียมฟอสเฟต 9:1) สัตว์ทดลองถูกทำให้ตายอย่างสงบที่เวลา 2 และ 8 สัปดาห์ การประเมินปริมาณของกระดูกและวัสดุปลูกทำโดยการวัดความหนาแน่นของภาพถ่ายรังสีดิจิตอล การตรวจเอกซเรย์ไมโครคอมพิวเตอร์และการตรวจทางจุลพยาธิวิทยา

ผลการศึกษา: จากภาพทางจุลพยาธิวิทยา กลุ่มที่ปลูกด้วยกระดูกอัตมันแสดงการเชื่อมกันอย่างสมบูรณ์ในขณะที่รอยวิการว่างเปล่าแสดงลักษณะการหายเป็นเนื้อเยื่อเกี่ยวพัน รอยวิการที่ปลูกด้วยวัสดุไบฟาสิกแคลเซียมฟอสเฟต1 และ วัสดุไบฟาสิกแคลเซียมฟอสเฟต2 สามารถคงลักษณะของสันกระดูกที่ดีเหมือนก่อนสร้างรอยวิการได้ การวิเคราะห์เอกซเรย์ไมโครคอมพิวเตอร์พบว่าที่ 8 สัปดาห์ กลุ่มที่ปลูกด้วยกระดูกอัตมันมีปริมาณกระดูกต่อปริมาตร (34.58±8.85) สูงกว่ากลุ่มอื่นอย่างมีนัยสำคัญ ($P<0.05$) ไม่พบความแตกต่างอย่างมีนัยสำคัญของปริมาณวัสดุปลูกต่อปริมาตรระหว่างวัสดุไบฟาสิกแคลเซียมฟอสเฟต1 และวัสดุไบฟาสิกแคลเซียมฟอสเฟต2 การวิเคราะห์ทางจุลพยาธิวิทยาแสดงการเพิ่มขึ้นของกระดูกที่สร้างใหม่ของกลุ่มกระดูกอัตมัน (4.30±0.76, 12.83±7.74) สูงกว่ารอยวิการว่างเปล่า (2.82±1.19, 8.14±6.35) วัสดุไบฟาสิกแคลเซียมฟอสเฟต1 (3.01±2.57, 8.81±3.86) และ วัสดุไบฟาสิกแคลเซียมฟอสเฟต2 (3.24±1.09, 10.27±3.98)

สรุปผลการศึกษา: วัสดุทดแทนกระดูกไบฟาสิกแคลเซียมฟอสเฟตที่มีสัดส่วนของไฮดรอกซีอาปาไทต์สูงแสดงถึงคุณสมบัติการเป็นโครงร่างที่ดี สามารถเข้ากันได้ดีกับเนื้อเยื่อของสิ่งมีชีวิต และสามารถคงสภาพรูปร่างให้เกิดการซอมแซมรอยวิการได้เป็นอย่างดี จากการที่มีคุณสมบัติในการละลายตัวที่ช้า รวมทั้งยังส่งเสริมขบวนการสร้างกระดูกในสัตว์ทดลอง วัสดุทดแทนกระดูกไบฟาสิกแคลเซียมฟอสเฟตที่มีสัดส่วนของไฮดรอกซีอาปาไทต์สูงนี้สมควรได้รับการพิจารณาทำการศึกษาทดลองทางคลินิกต่อไป

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Major program	Oral and Maxillofacial Surgery
Academic Year	2013

Abstract

Objective: This study aimed to evaluate the effect of biphasic calcium phosphate with high HA/TCP ratios on bone formation in rabbit calvarial defects.

Materials and Methods: Sixteen New Zealand white rabbits were randomly divided into 2 groups of a control and an experiment. In each animal, bilateral circular defects (10-mm diameter) were created on the calvarium. Group I (3 rabbits per each time frame) defects were grafted with autogenous bone chips or left empty. Group II (5 rabbits per each time frame) defects were grafted with BCP1 (HA:TCP, 8:2) or BCP2 (HA:TCP, 9:1). The animals were sacrificed at 2 and 8 weeks. Bone formation and residual of grafting material were assessed by radiographic densitometry, Micro computed tomography (micro-CT) and histomorphometric analysis.

Results: Histology observation revealed autogenous bone group showed bridging defect while unfilled defects group showed connective tissue heal. BCP1 and BCP2 preserved the good contour of defect. Micro-CT analysis, at 8 weeks, autogenous group had a significantly ($p<0.05$) greater bone volume fraction (34.58±8.85) than the other groups. No statistically significant was observed for material volume fraction between BCP1 and BCP2. The histomorphometric analysis demonstrated increasing of newly formed bone in autogenous group (4.30±0.76, 12.83±7.74) was higher than unfilled defect (2.82±1.19, 8.14±6.35), BCP1 (3.01±2.57, 8.81±3.86) and BCP2 (3.24±1.09, 10.27±3.98).

Conclusions: BCP at high ratio of HA presented good osteoconductive properties, biocompatibility with the living tissue and excellent space maintaining capacity with slow biodegradation rates and enhanced bone formation in animal model. BCP with high ratio of HA should be considered for further clinical trial study.

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This thesis would not have been possible unless I had got supported, guided and helped. I'm heartily thankful to my supervisors Associate Professor Prisana Pripatnanont and Assistance Professor Srisurang Suttapreyasri, whose encouragement, supervision and support from the preliminary to the concluding level enabled me to develop an understanding all of the subject and raise me up more than I can be.

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I wish to express my love and gratitude to all my friends and coworkers. And lastly, my greatly gratitude goes to my parents for unconditioned love and untiring support.

Pongsakorn Praserttham

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List of Abbreviations and Symbols

ANOVA	=	One-way analysis of variance
BCP	=	Biphasic calcium phosphate
BVF	=	Bone volume fraction
BV/TV	=	Bone volume per total volume
CaP	=	Calcium phosphate
cm	=	Centimeter
et al	=	And others
GB	=	Grafted bone
gm	=	Gram
HA	=	Hydroxyapatite
H&E	=	Hematoxylin and eosin
kg	=	Kilogram
kVp	=	Kilovoltage peak
mg	=	Miligram
min	=	Minute
micro-CT	=	Micro computed tomography
ml	=	Milliliter
mm	=	Millimeter
MVF	=	Material volume fraction
MV/TV	=	Material volume per total volume
NB	=	Newly formed bone
OB	=	Original bone
OD	=	Optical density
ROI	=	Region of interest
SD	=	Standard deviation
sec	=	Second
SEM	=	Scanning electron microscope

List of Abbreviations and Symbols (Continued)

TCP	=	Tricalcium phosphate
W	=	Watt
µA	=	Microampere
µl	=	Microliter
µm	=	Micrometer
μm^3	=	Cubic micrometer

List of Papers and Proceedings

1. Pripatnanont Prisana, Prasertthamm Pongsakorn, Suttapreyasri Srisurang, Leepong Narit, Monmaturapoj Naruporn. Bone regeneration potential of biphasic nano-calcium phosphate with high HA/TCP ratios in rabbit calvarial defects. ***JBMR Part B, Applied Biomaterials***.
2. Prasertthamm P, Pripatnanont P, Suttapreyasri S, Leepong N, Monmaturapoj N. In vivo biocompatibility of porous BCP in two difference ratios. ***The 11th Dental Faculty Consortium of Thailand Academic Meeting and Research Presentation (DFCT2013)*** May; 118-123.

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23-Dec-2013

Manuscript number: JBMR-B-13-0745

Dear Dr. Pripatnanont:

We are pleased to receive your manuscript entitled "Bone regeneration potential of biphasic nano-calcium phosphate with high HA/TCP ratios in rabbit calvarial defects" by Pripatnanont, Prisana; Praserttham, Pongsakorn; Suttapreyasri, Srisurang; Leepong, Narit; Monmaturapoj, Naruporn. We will be sending it out for review shortly.

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Thailand

To whom it may concern :

This is to certify that Mr. Pongsakorn Praserttham who presented the article"In vivo biocompatibility of porous BCP in two different ratios" in the 11th Research conference of the Dental Faculty Consortium of Thailand (DFCT2013), on 7-9 May 2013 at Pullman Pattaya Hotel, Chonburi, Thailand, organized by the Faculty of Dentistry, Thammasat University was given permission to submit this article in thesis.

Given on this 1 day of November 2013.

(Professor Dr.Sittichai Koontongkaew)
Dean

Introduction

The use of grafts to repair bone loss is challenged in modern dentistry. Bone could be lost as a result of physiological resorption caused by dental loss, trauma, bone pathology or infection.[1] The development and popularization of dental implants have stimulated the study of bone substitutes because the stability and position of the implant depend on the quality and quantity of bone.[2] In situations of poor quality and quantity of bone, the placement of dental implants in optimum position is often not feasible without bone reconstruction.[3]

Ideal bone substitutes should be osteoconductive, osteoinductive, biocompatible and bio-resorbable.[4] In addition, this material should be able to withstand initial inflammation phase and allow penetration of host vascular tissue, which leads to proper revascularization and induction of osteoblast and osteoclast permitting osseointegration.[2]

Although autograft is considered as the "gold standard" bone grafting material that enhances bone regeneration and repairs bone defects, its harvesting is associated with donor site morbidity and restricted availability.[5] Allograft bone harvested from cadaver sources, is also frequently used due to its ''off the shelf'' availability, in various shapes, sizes, and endless quantity. However, the risk of disease transmission and immunological reaction was concerned.[6] Because of limitation of autografts and allografts, there is a great need to develop synthetic alternative biomaterials for bone replacement, repair, and augmentation.

Currently, several options of bone grafting materials are commercialized available for bone regeneration procedures. These include animal bones (processed cow bones), corals and coral derived, polymers (natural or synthetic), synthetic ceramics (calcium phosphates, calcium sulfates, calcium carbonate, bioactive glasses), and composites.[7]

One of the most popular groups of bone substitutes is calcium phosphate (CaP) bioceramics. CaP has been used for bone reconstruction in orthopedic and oral-maxillofacial surgery since the beginning of the 1970s.[8] The rational for the development of CaP biomaterial is their similarity in composition to the bone mineral and the properties of biodegradability, bioactivity, and osteoconductivity. Also, interconnected porosity, another important property of bone, can be introduced during the manufacturing process.[9] CaP can be classified to several types based on chemical properties such as calcium to phosphate ratio, crystallinity, and physical forms.[10] It has to be noted that subtle different in these parameters can have a crucial effect on the

biological outcome. For biomedical applications, hydroxyapatite (HA) and tricalcium phosphate (TCP) or the combination, termed biphasic calcium phosphate (BCP), are most frequently use.[11]

HA is one of the most widely used calcium phosphate because of its chemical similarities to the inorganic component of bone which chemical formula of $Ca_{10}(PO_4)_6(OH)_2$ has a theoretical composition of 39.68 wt% Ca, 18.45 wt% P; Ca/P wt ratio of 2.151 and Ca/P molar ratio of 1.667.[11] Synthetic HA has exhibited strong affinity to host tissue by forming a chemical bond with host tissue that offers HA a greater advantage in clinical applications.[12] However, despite chemical similarities, disadvantages of HA are weakness under tensile stress and very slow resorbability.[13]

TCP is degradable bioceramic with the chemical formula of $Ca_3(PO_4)_2$ and Ca/P molar ratio is lower than 1.50. Generally, there are two form of TCP, β-tricalcium phosphate (β -TCP) and α-tricalcium phosphate (α -TCP).[11] The dissolution rated of TCP was 3-12 times faster than HA.[14]

The term biphasic calcium phosphate (BCP) was first used by Nery et al to describe the bioceramic that consisted of mixture of HA and β-TCP.[15] The concept is to combine an optimum balance of the more stable phase HA and more soluble TCP to control the dissolution rate of materials to be corresponded to the rate of bone regeneration.[10] This property depends on the proportion of HA/β-TCP, crystallinitily, particle sizes, pore size, porosity, sintering temperature and conditions.[9]

There are various HA/TCP ratios published in the literatures, which possess different physical and biological properties. These various ratios, from *in vitro* and *in vivo* studies, with HA/TCP ranged from 20/80 to 85/15 and each ratio claimed its good properties.[16-20]

In rabbit calvarial models, Hwang et al reported BCP with HA/TCP ratio of 60/40 showed highest amount of newly formed bone than pure HA and pure β-TCP at the 4 and 8 week of healing periods.[21] This result was consistence with the study from Park et al that used BCP with same HA/TCP ratio and found greater new bone formed when compared to anorganic bovine bone (Bio-Oss®) and β-TCP (Cerasorb®) at 8 weeks of healing period.[22] Lim et al found BCP with HA/TCP ratio of 70/30 (Osteon®) showed normal contour of augmented areas but the unfilled defect groups showed collapsed tissue profiles, suggested that BCP has effective space maintenance throughout 8 weeks of healing period.

In summary, BCP is usually used as an osteoconductive matrix that maintaining space for new bone formation which dissolution rate varies upon the dissolution of TCP. Higher HA contents lead to slow degradation rate, but ensure volume maintenance while TCP is gradually resorbed and replaced by newly formed bone.[9] BCP with high ratio of HA/TCP have a slow biodegradible properties that can act as a space maintainer may be suitable for some clinical situation such as grafting material for alveolar bone preservation techniques or for maxillary sinus augmentation procedure.

This study is one part of cooperation between the CranioMaxillofacial Hard Tissue Engineering Center (CTEC), Faculty of Dentistry, Prince of Songkla University and National Metal and Materials Technology Center (MTEC) to research and develop calcium phosphate bone substitute materials. The porous BCP were prepared in particulate form by MTEC. Briefly, porous BCP samples were fabricated by foaming technique with HA/TCP ratios ranged from 5:5 to 9:1. The porous BCP with HA/TCP ratios of 8:2 and 9:1 were selected since their showed highest cell attachment of osteoblast-like Saos-2 cells.

This study aimed to assess *in vivo* bone regenerative potency of porous BCP in two different HA/TCP ratios (8:2 and 9:1) in calvarial defects of the rabbit.

Objective of the Study

Research Question

Does high proportion of HA/TCP enhance new bone formation in rabbit calvarial defects?

General objectives

To investigate biological effect of porous BCP with high ratios HA/TCP as a grafting material in rabbit calvarial defects.

Specific objectives

1. To evaluate new bone formation after grafting with two different ratios of porous BCP (HA/TCP; 90/10 and 80/20) in rabbit calvarial defects.
2. To compare the new bone formation among using porous BCP with high HA/TCP ratios and autogenous bone for grafting in rabbit calvarial defects.

Hypothesis

The porous BCP with high HA/TCP ratios will bridge defect and enhance new bone formation not differ from autogenous bone when used as bone substitute materials in rabbit calvarial defects.

Materials and Methods

Scope of Study

Sixteen rabbits were randomly divided into 2 groups. Bilateral circular defects (10-mm diameter) were created on the calvarium in each animal. Group I (3 rabbits per each time frame) defects were grafted with autogenous bone chips or left empty. Group II (5 rabbits per each time frame) defects were grafted with BCP1 (HA80/TCP20) or BCP2 (HA90/TCP10). Digital radiograph, micro computed tomography and histomorphometric analysis were performed at 2 and 8 weeks after implantation. The results within each group and between groups at different investigation time were compared. Significant difference were set at $P<0.05$ (Figure.1)

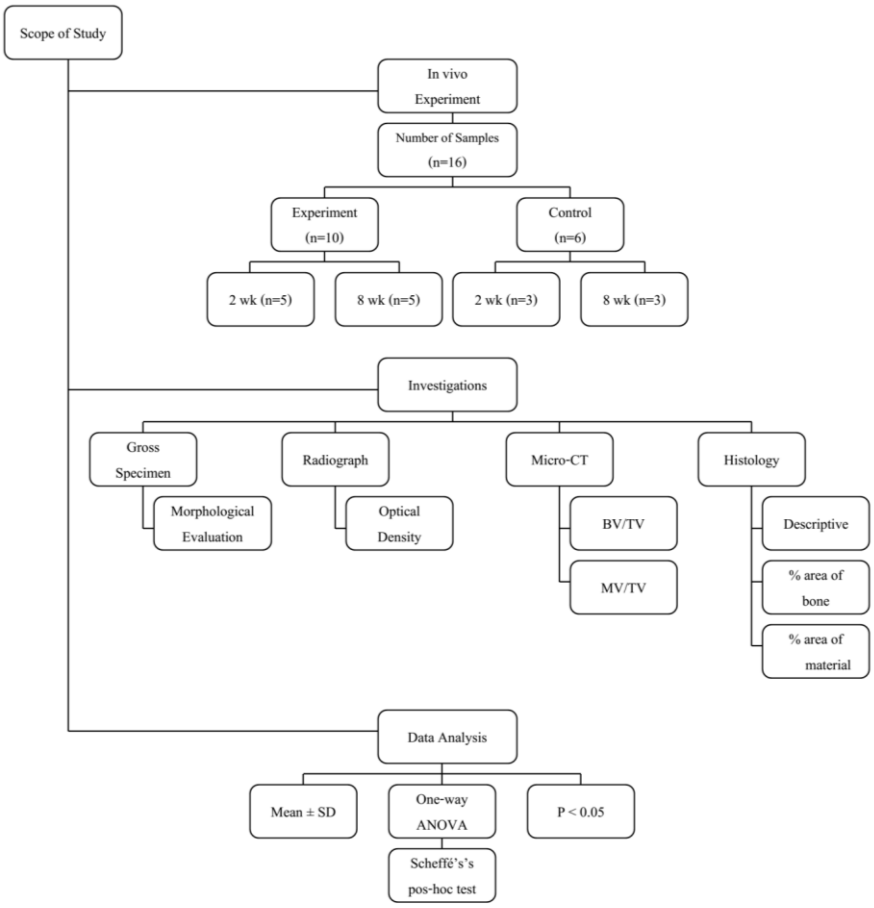

Figure.1 Experimental overviews

Materials

The porous BCP with HA/TCP ratios of 8:2 and 9:1 were obtained from MTEC. There are 80% porosity, well-interconnected pore structure and a pore size of 100 to 300 μm in both ratios of BCP. (Figure.2)

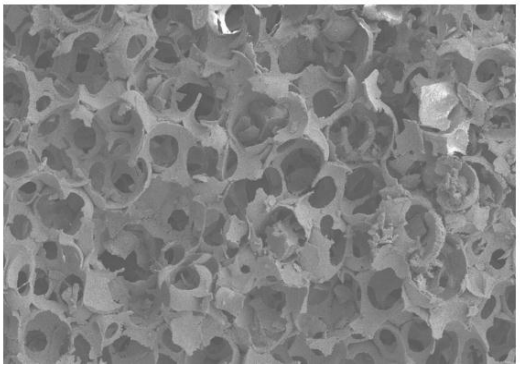

Figure.2 SEM photographs of BCP granules showing the pore structure, size100 - 300 μm, original magnification x 50

Animal Preparation

Sixteen male adult (10-12 months old) New Zealand white rabbits each weighing 3 - 4 kg were divided into 2 groups, with 10 animals in the experimental group and 6 in the control group. Each animal was kept in a single cage and fed a standard laboratory diet and water.

Surgical Procedure

The procedures were performed according to the regulations of and with the approval of the Animal Experiment Ethics Committee of Prince of Songkla University. Anesthesia was induced using ketamine 25 mg/kg and diazepam 5 mg/kg intramuscularly 30 min before surgery. Thiopental 5 mg/kg was administered intravenously and then titrated at the rate of 2 mg/kg every 15 min (with a maximum dose of 30 mg/kg) until unconsciousness was achieved.[23]

The surgical field was disinfected with povidone-iodine 10%. A mid-sagittal incision was made after local infiltration of 2% lidocaine hydrochloride with 1:100,000 epinephrine 1.8 ml. Subperiosteal dissection was carried out, and two identical bicortical bone defects diameter of 10 mm were carefully created using a trephine bur with saline irrigation.

The rabbits were randomly divided into 2 groups. In control groups (n=6), defects were filled with autogenous bone chips 0.15 gm by weight, that were minced with a bone morselizer (Salvin Dental Specialtie Inc, Charlotte, NC, USA), or left empty. In experiment groups (n=10), defects were randomly filled with porous BCP1 (8:2) or BCP2 (9:1) at 0.10 gm by weight plus 0.9% normal saline solution until total weight equal to the minced autogenous bone chips. The periosteum, muscle and skin were sutured using vicryl® 4/0. (Figure.3)

The rabbits were sacrificed at 2 and 8 weeks after surgery with 1.2-1.3 ml overdose of 200 mg/ml pentobarbital sodium administered intravenously via the marginal ear vein. The calvarial bone was harvested using a small sharp fissure bur and then fixed in 10% formalin.

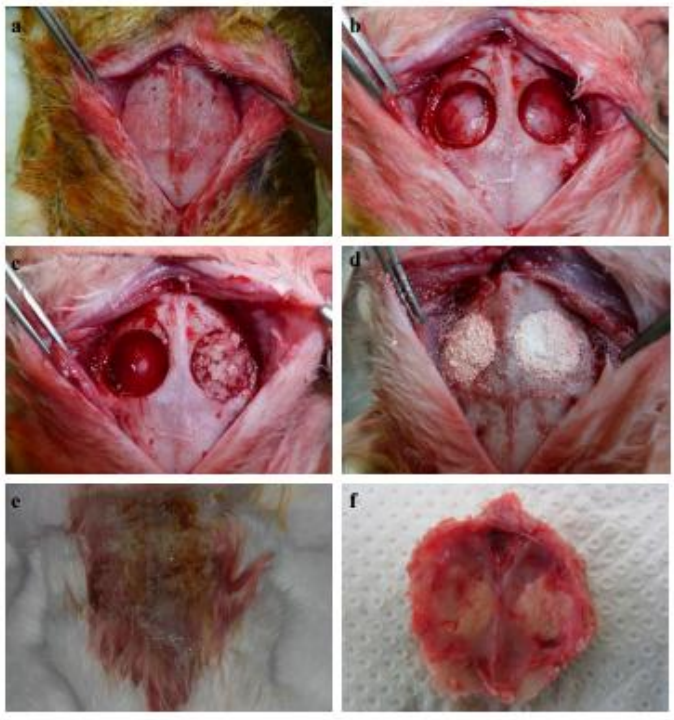

Figure.3 Rabbit's calvarial defects (a). Two identical bicortical defects diameter of 10 mm created by a trephine bur (b). Group I defects filled with autogenous bone chips or left empty (c). Group II defects filled with porous BCP1 (8:2) or BCP2 (9:1) (d). Wound closure (e). Gross specimen after dissected (f)

Digital Radiograph

To obtain radiographic images of the specimens, the digital radiographs were taken by hand-held portable X-ray devices. (NOMAD, Aribex Inc., Utah, USA) with digital sensor size 0 attaching to digital sensor holder (XCP-DS, Rinn, Densply, IL, USA) to control the vertical distance at 10 cm. The setting for all exposure was 60 kVp, 2.3 mA and 0.3sec. Images were captured on receptor (Sopix CMOS, Instrumentarium Dental, Tuusula, Finland). The mean optical density (OD) of the defect was calculated and analyzed by using Image Pro Plus 7.0 software (Media Cybernetics Inc., Silver Spring, MD, USA)

Specimen Processing

After radiography, each specimen was trimmed and cut mid-coronally along the center of two circular defects into 2 halves; one half was used for micro-CT analysis, and the other half was used for histomorphometric analysis. (Figure.4)

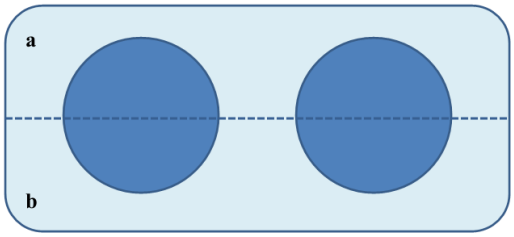

Figure.4 Schematic drawing of cutting specimen into 2 pieces for micro-CT analysis and Histomorphometric analysis

Micro computed tomography (Micro-CT) analysis

A high resolution micro-CT system (Micro-CT80, Scanco, Medica AG, Basseersdorf, Switzerland) was used. After calibration the specimens were scanned perpendicularly to cranium vault at 55 kVp, 72 μA and 4W in high-resolution mode (18.5μm^3/voxel). Scanned data were reconstructed by built-in software.

Before analysis, the grayscale threshold values were determined to discriminate bone and ceramic from soft tissue.[24] The threshold value of "bone-ceramic" was specified. The lower threshold was selected by identifying the lowest threshold of bone voxels within the defects. The upper threshold referred to the highest threshold of ceramic voxels given that the total setup volume divided by total volume was greater than 0.95. The threshold value for the "ceramic" was determined by tracing 8 clearly identified BCP particles then the summation of the lowest threshold of each particle divided by the total volume greater than 0.95 was set as lower threshold of the ceramic threshold. The "bone" threshold was calculated by subtracting the ceramic threshold from the bone-ceramic threshold.

After determination of the threshold values, the margins were traced to specify ROI the defect. (Figure 4) The percent of bone volume fraction (BVF, BV/TV), percentage of radio-opaque voxels (as bone threshold range) divided by the total defect volume, and percent of material volume fraction (MVF, MV/TV), percentage of radio-opaque voxels (ceramic threshold range) divided by the total defect volume, were determined.

Histology Processing

Following radiography, one half of the specimens were processed to obtain thin ground sections using undecalcified techniques, according to the technique of Donath and Breune[25] with minor modifications. Briefly, the specimens were dehydrated in an ascending series of alcohol rinses and embedded in a glycolmethacrylate resin (Technovit 7200 VLC, Kulzer, Wehrheim, Germany). After polymerization the specimens were serial sectioned along their longitudinal axis with a high-precision diamond disc at approximately 150 μm and ground down to approximately 15-30μm with a specially designed grinding machine (EXAKT® cutting and grinding system, EXAKT® Apparatebau, Norderstedt, Hamburg, Germany). Three sections contained the central portion were selected and stained with Goldner's Masson trichrome. All slides were examined descriptively before histomorphometric analysis.

The other half of specimens, after finished the micro-CT analysis, were decalcified in formic acid and then embedded in paraffin. Serial sections in 5 μm were cut from the center and stained with hematoxylin and eosin for descriptively examination.

Histomorphometric Analysis

All slides were then loaded into an Aperio ScanScope XT (Aperio ePathology Solutions, California, USA) and scanned at 40x magnifications. Digital histologic images were captured with special software form same company (Aperio ImageScope 9.0, Aperio ePathology Solutions, California, USA). Three undecalcified sections contained the central portion were selected for histomorphometric analysis. The quantity of new bone formation was calculated as the percentage of newly formed bone area to the total defect area and the amount of grafting material particle area that were calculated as the percentage of each grafting material particle area to the total area using Image Pro Plus 7.0 (Media Cybernetics, MD, USA).

$$\text{Percentage of new bone area} = \frac{\text{new bone area} \times 100}{\text{total area}}$$

$$\text{Percentage of material particle} = \frac{\text{grafted material area} \times 100}{\text{total area}}$$

Statistical Analysis

Statistical analysis was performed using statistical analysis software (SPSS ver15.0, SPSS Inc., Chicago, USA). Data were tested for normality and presented as means ± SD. One-way analysis of variance and multiple comparison by Scheffé's post-hoc test ($P<0.05$) were used to compare the differences between the mean optical densities, the percentage of newly formed bone and percentage of BCP particles in each groups.

Results

Animal

All rabbits well tolerated with the surgical procedure and the anesthesia and recovered after surgery without any evidence of infection or wound dehiscence. They were able to eat the food and drink water ad libitum. Wound healing was uneventful and the sutures were removed on the tenth post-operative day.

Gross Specimen Observation

Specimens were harvested at 2 and 8 weeks in one piece before trimmed and cut into 2 halves. At 2 weeks, autogenous group showed normal contour of defect with firm to soft in consistency while unfilled defect presented thin and collapsed profile with vary soft consistency. (Figure.5a, 6a) BCP1 and BCP2 showed better contour of defects with hard consistency. (Figure.5c, 6c)

At 8 weeks, autogenous group showed normal contour of bone defect with hard in consistency while unfilled defect still appeared collapsed with soft to firm consistency. (Figure.5b, 6b) Both BCP1 and BCP2 presented well incorporated of BCP particles to host bone, hard consistency and good contour of the defects. (Figure.5d, 6d)

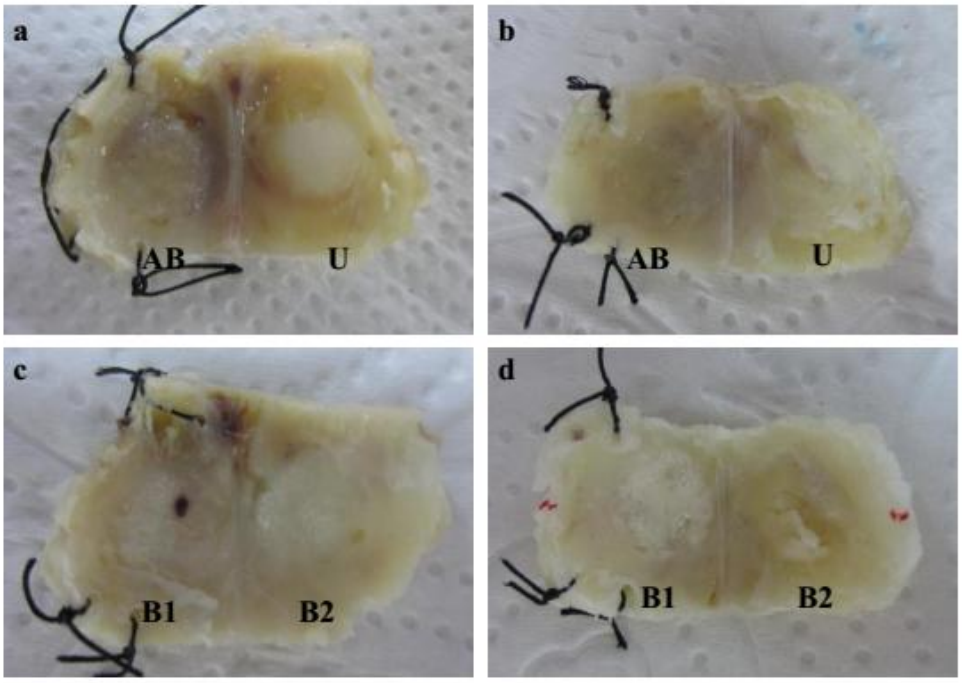

Figure.5 Specimen before cutting into 2 halves at 2 weeks (a, c) and 8 weeks (b, d) of healing periods; AB = Autogenous group, U = Unfilled defect, B1 = BCP1 (8:2), B2 = BCP2 (9:1)

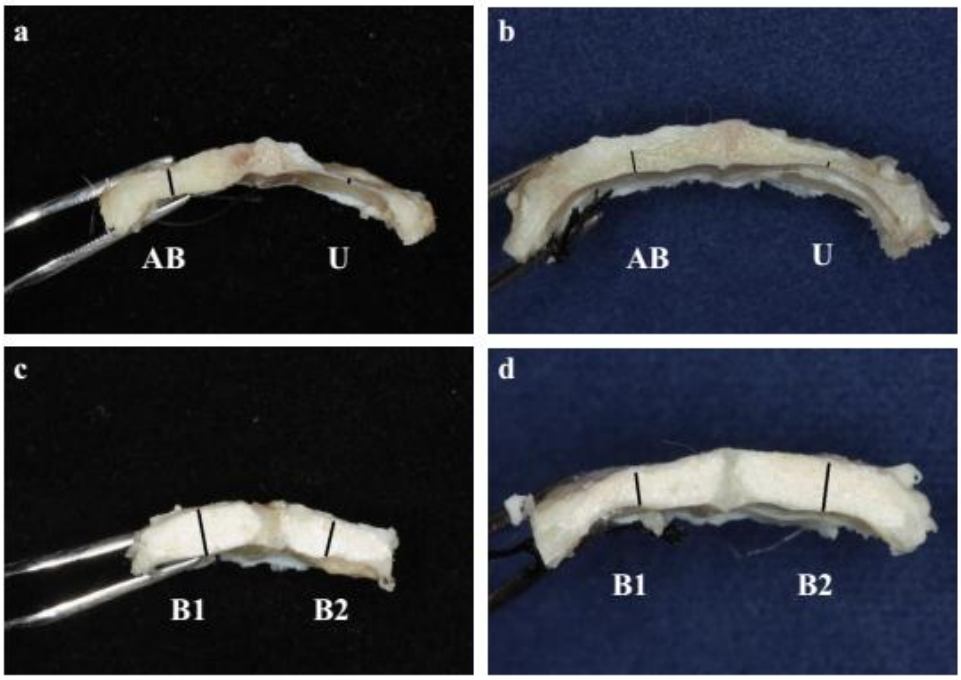

Figure.6 Specimen after mid-coronally cut at 2 weeks (a, c) and 8 weeks (b, d) of healing periods; AB = Autogenous group, U = Unfilled defect, B1 = BCP1 (8:2), B2 = BCP2 (9:1), Black line = Thickness of defect

Radiographic Features

The specimens were radiographed before processing for micro-CT and histology analysis. At 2 weeks, autogenous group showed radiopaque masses of bone chips of varying sizes and densities while unfilled defect groups presented circular homogeneous radiolucent area with clear border. (Figure.7a) BCP1 and BCP2 showed well-delineated radiopaque areas which the bone defects were filled with the distinct radiopaque particles of BCP (Figure.7c)

At 8 weeks, autogenous group showed homogeneous radiopaque mass distributed along the defect.(Figure.7b) Unfilled defect groups presented circular homogenous radiolucent area with irregular border (Figure.7b) BCP1 and BCP2 defects still showed distinct radiopaque particles of BCP, however density and diameter of the defects seem to less than at 2 weeks. (Figure.7d)

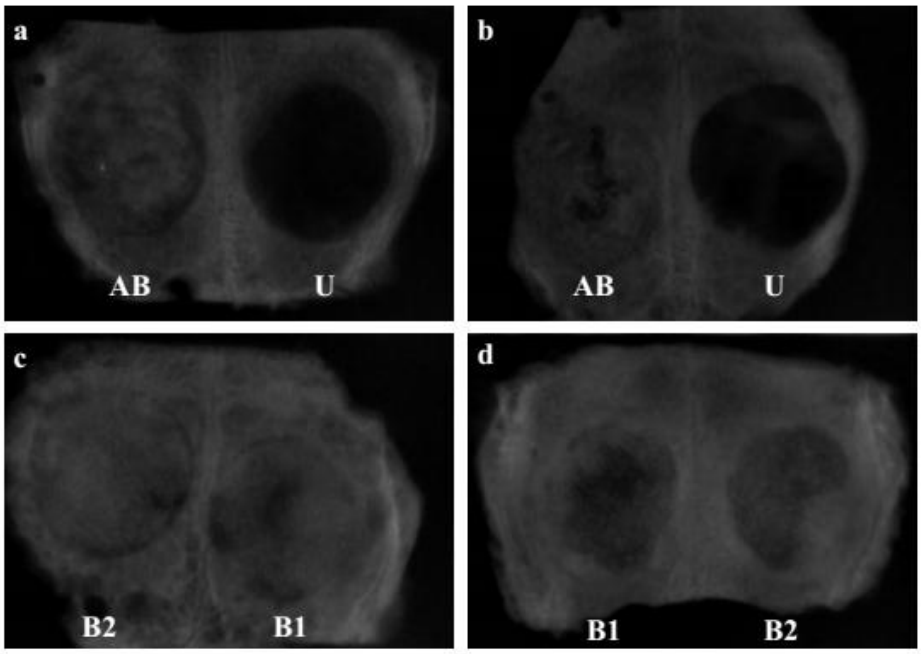

Figure.7 Radiograph of the rabbits' calvarium at 2 weeks (a, c) and 8 weeks (b, d) of healing periods; AB = Autogenous group, U = Unfilled defect, B1 = BCP1 (8:2), B2 = BCP2 (9:1)

Radiomorphometric Analysis

The results of radiomorphometric analysis are presented in Table.1 and Figure.8. At 2 weeks, the mean optical density (OD) of autogenous bone chip group (0.305±0.069), BCP1 (0.324±0.080) and BCP2 (0.333±0.061) were significantly ($p<0.05$) higher than unfilled defect (0.098± 0.048).

At 8 weeks, the mean OD of autogenous bone chip group (0.210±0.015) and unfilled defect (0.122±0.059) were significantly ($p<0.05$) lower than BCP1 (0.389±0.096) and BCP2 (0.415±0.090). In addition, the mean OD of BCP2 was also significantly ($p=0.031$) higher than autogenous bone chip group.

Table.1 Data of radiomorphometric in each group after 2 and 8 weeks

Group	2 weeks	8 weeks
Autogenous bone chip	0.305 ± 0.069*	0.210 ± 0.015
Unfilled defect	0.098 ± 0.048	0.122 ± 0.059
BCP1 (8:2)	0.324 ± 0.080*	0.389 ± 0.096*
BCP2 (9:1)	0.333 ± 0.061*	0.415 ± 0.090*£

Values are present as mean ± SD
* = Statistical significant difference from unfilled defect (P< 0.05)
*£ = Statistical significant difference from autogenous bone chip at p = 0.031

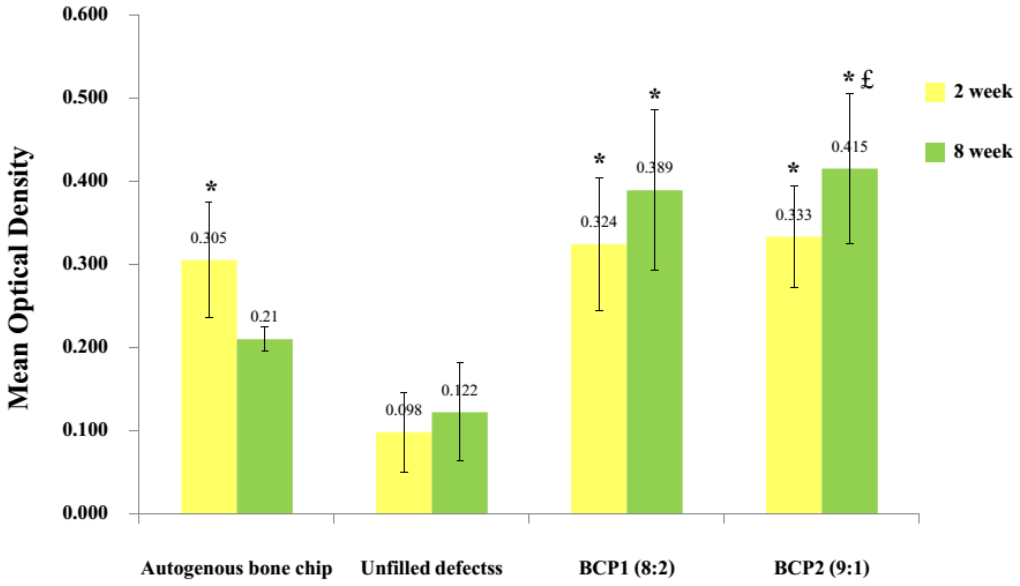

Figure.8 Mean optical density in each group after 2 and 8 weeks of healing

Micro-CT Analysis

The total volumes of newly formed bone within the ROI (bone volume fraction or BV/TV), as well as residual of grafting materials (material volume fraction or MV/TV), were summarized in Table.2.

At 2 weeks, percentage of bone volume fraction for autogenous group, unfilled defects, BCP1 and BCP2 were 29.48±9.84, 4.23±2.08, 9.90±0.75 and 10.57±0.85 respectively. Percentage of material volume fraction for BCP1 and BCP2 were 28.64±2.31 and 29.92±4.39 respectively.

At 8 weeks, percentage of bone volume fraction for autogenous group, unfilled defects, BCP1 and BCP2 were 34.58±8.85, 8.40±5.37, 20.70±2.76 and 20.72±3.97 respectively. Percentage of material volume fraction for BCP1 and BCP2 were 25.02±3.51 and 24.71±3.91 respectively.

Only autogenous group at 8 weeks of healing period had a significantly greater bone volume fraction than the other groups ($p<0.05$) (Figure.9a) Nevertheless, no statistically significant was observed for material volume fraction between BCP1 and BCP2 at both time frames and also each material between the 2 time frames (Figure.9b) And 3D-reconstruction of bone volume fraction and material volume fraction of each groups at both 2 timeframes were show in Figure.10 and Figure.11.

Table.2 Bone volume fraction and material volume fraction of micro-CT result at 2 and 8 weeks

Timing	Group	BV/TV (%)	MV/TV (%)
2 weeks			
	Autogenous bone chip	29.48 ± 9.84	
	Unfilled defect	4.23 ± 2.08	
	BCP1 (8:2)	9.90 ± 0.75	28.64 ± 2.31
	BCP2 (9:1)	10.57 ± 0.85	29.92 ± 4.39
8 weeks			
	Autogenous bone chip	34.58 ± 8.85*	
	Unfilled defect	8.40 ± 5.37	
	BCP1 (8:2)	20.70 ± 2.76	25.02 ± 3.51
	BCP2 (9:1)	20.72 ± 3.97	24.71 ± 3.91

Values are present as mean ± SD
BV/TV: Bone volume per total volume
MV/TV: Residual materials per total volume
* = Statistical significant difference from other groups at each time frame ($P< 0.05$)

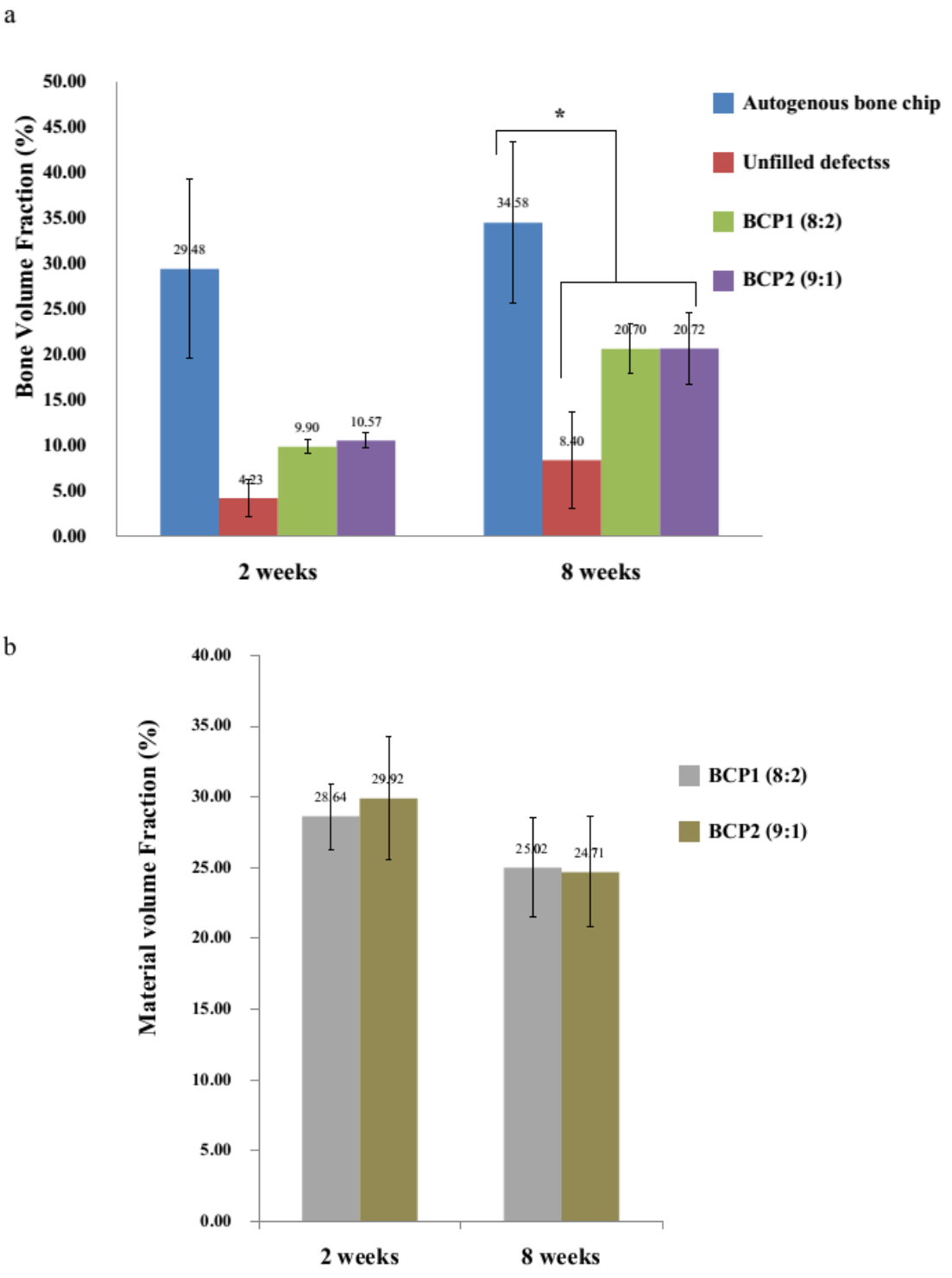

Figure.9 Micro-CT analysis; Bone volume fraction (a) and Material volume fraction (b)

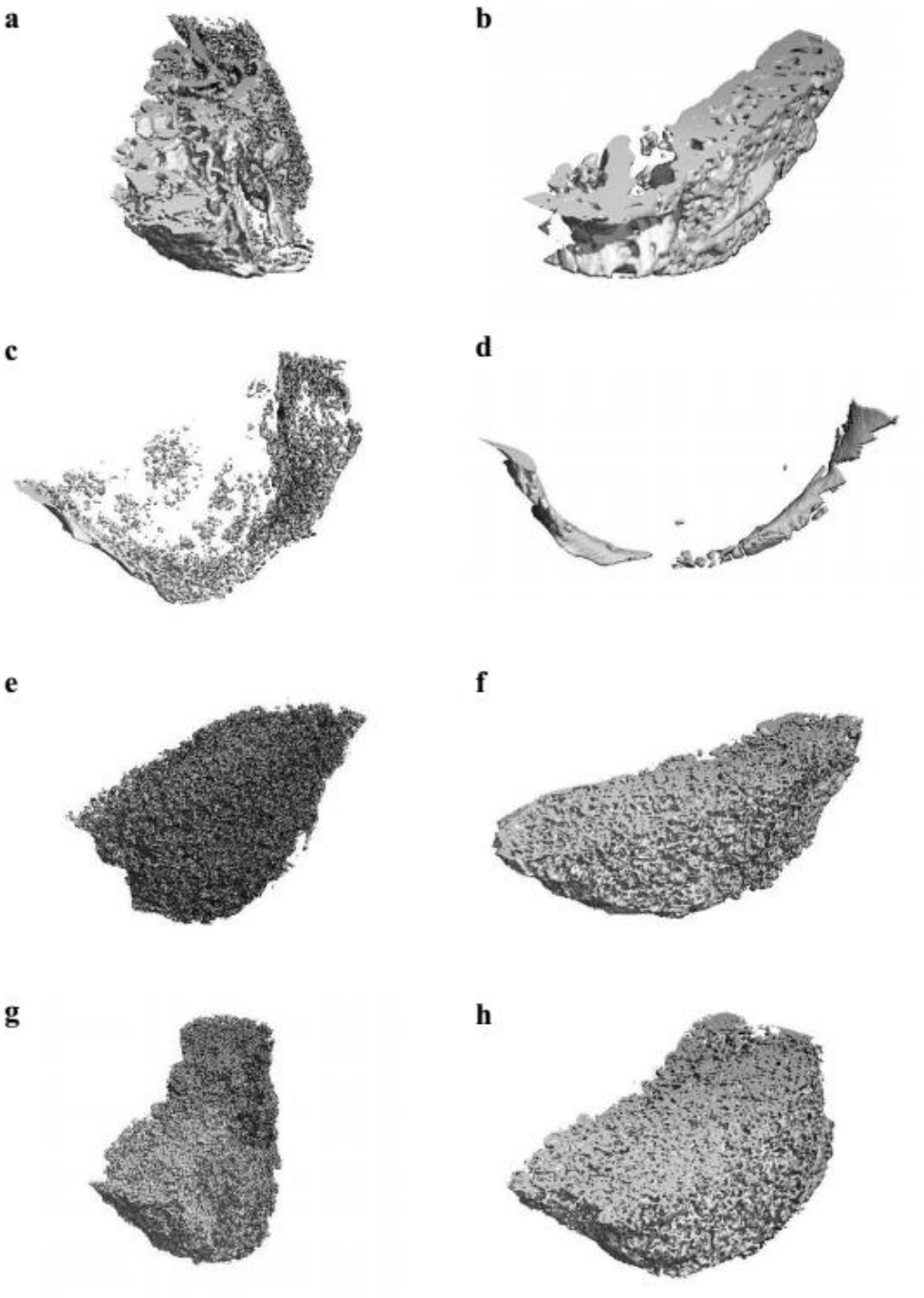

Figure.10 3D-reconstruction of bone volume fraction at 2 weeks (a, c, e, g) and 8 weeks (b, d, f, h) of healing period; Autogenous group (a, b), Unfilled defect (c, d), BCP1 (e, f) and BCP2 (g, h)

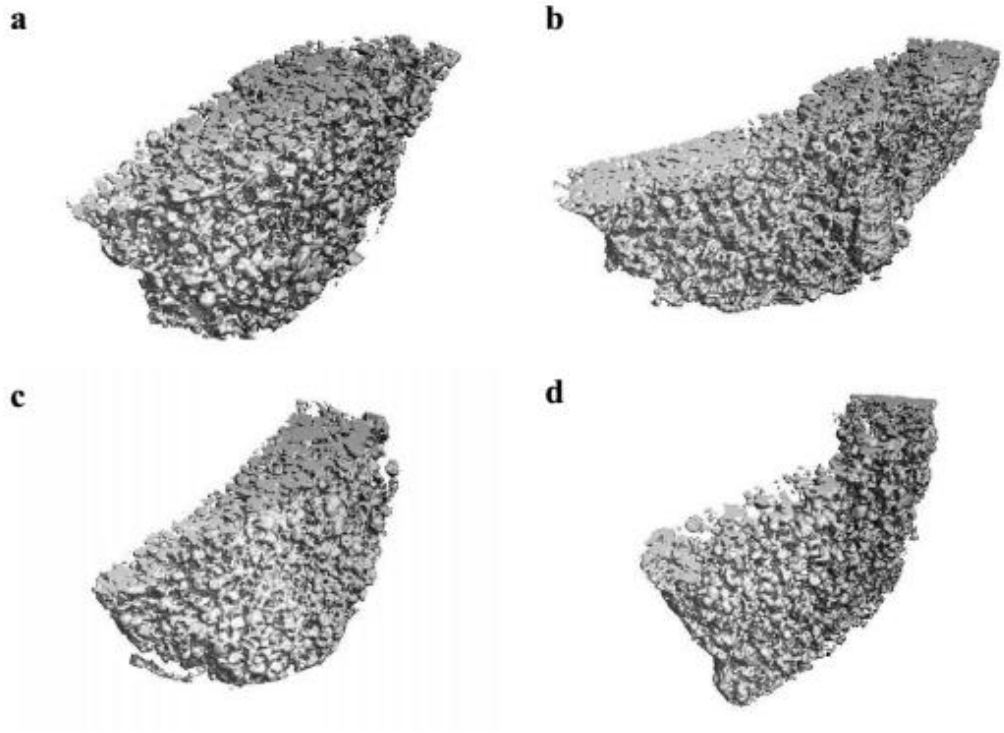

Figure.11 3D-reconstruction of material volume fraction at 2 weeks (a, c) and 8 weeks (b, d) of healing periods; BCP1 (a, b) and BCP2 (c, d)

Histology

Picture of undecalcified specimens were show in Figure.12-13 and picture of decalcified specimens were show in Figure.14-16.

Undecalcified specimens at 2 weeks, autogenous group showed autogenous bone chip filled in defects, and the connective tissue was infiltrated from the periphery of the defect. (Figure.12a, 12e) Unfilled defects presented collapsed contour that filled with loosely connective tissue. (Figure.12b, 12f) BCP1 and BCP2 groups showed normal contour defects, and dense BCP particles were found all over the defect. (Figure.12c-d, 12g-h)

At 8 weeks, autogenous group showed completely bridging defects with good continuity, and the newly formed bone was well incorporated with the grafts and the host bone. (Figure.13a, 13e) Unfilled defect presented some bony island but the defect were not bridged and still lost of continuity. (Figure.13b, 13f) Both BCP1 and BCP2, the BCP particles were visually less than at 2 weeks; newly formed bone was projected from the defect edge, extended in a

centripetal direction and corporate well with the BCP particles. However, little amount of bone was observed in the central part and the gap between materials was filled with loose connective tissue. It was also noted that grafting material preserved the good contour of the defects. (Figure. 13c-d, 13g-h)

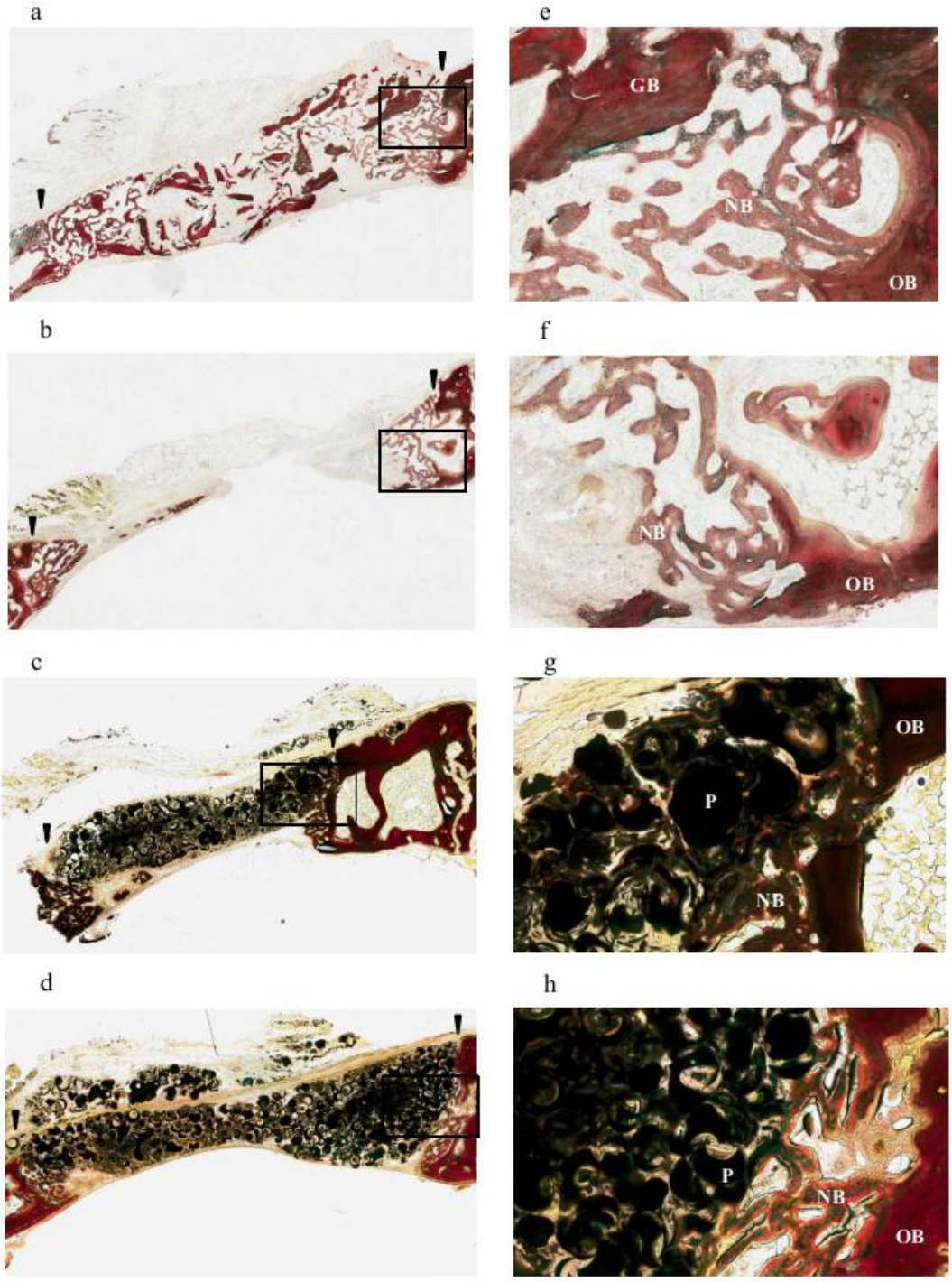

Figure.12 Undecalcified specimens at 2 weeks of healing periods; Autogenous group (a, e),

Unfilled defect (b, f), BCP1 (c, g) and BCP2 (d, h). Arrowhead = defect margin, GB = grafted bone, NB = newly formed bone, OB = original bone, P = BCP particle. Goldner's Masson trichrome, original magnification (a, b, c, d) and Goldner's Masson trichrome, original magnification x 5 (e, f, g, h)

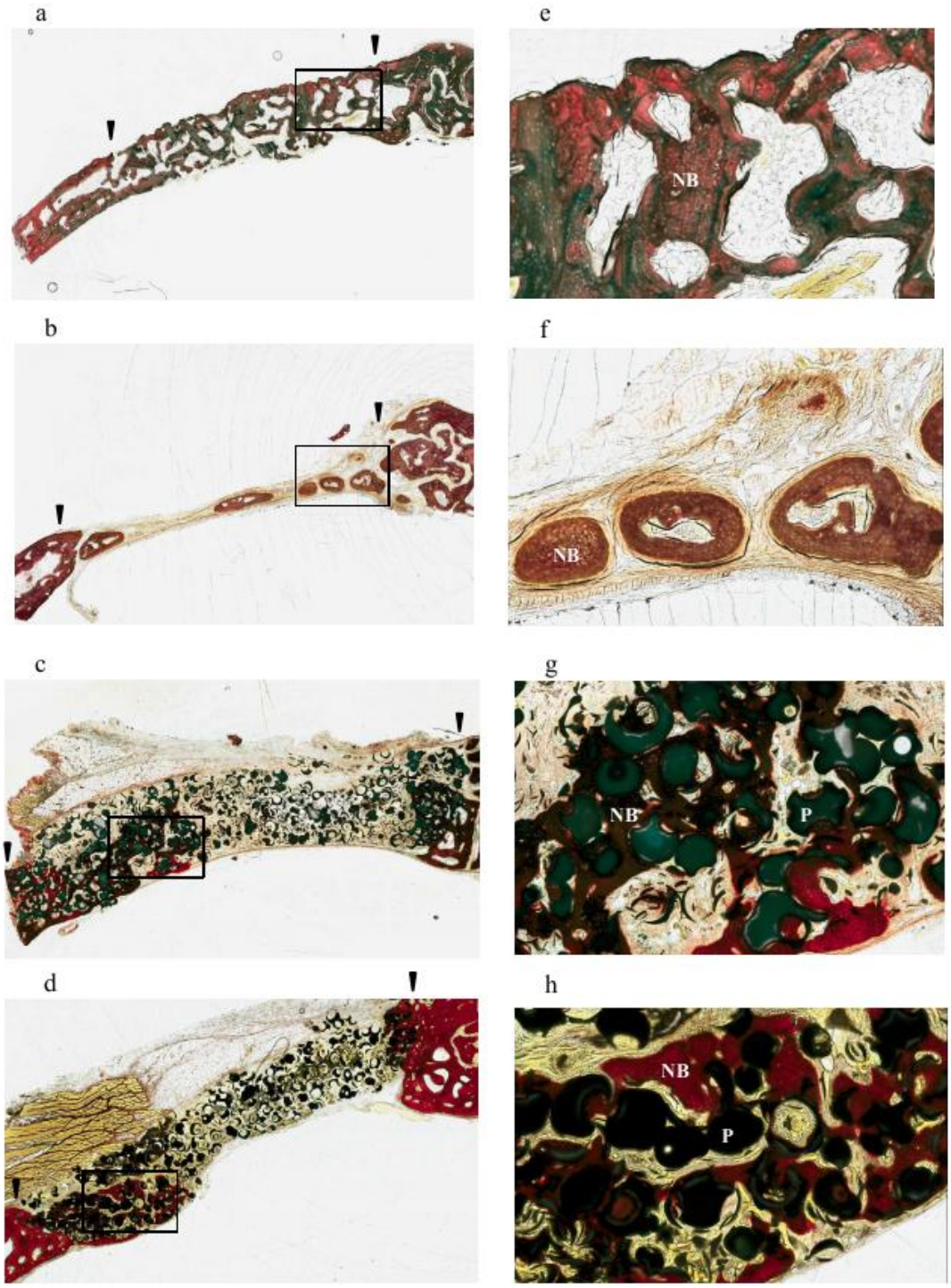

Figure.13 Undecalcified specimens at 8 weeks of healing periods; Autogenous group (a, e),

Unfilled defect (b, f), BCP1 (c, g) and BCP2 (d, h). Arrowhead = defect margin, GB = grafted bone, NB = newly formed bone, OB = original bone, P = BCP particle. Goldner's Masson trichrome, original magnification (a, b, c, d) and Goldner's Masson trichrome, original magnification x 5 (e, f, g, h)

The decalcified specimens were not different from undecalcified specimens. At 2 weeks, autogenous group showed autogenous bone chip filled in defects, and the newly formed bone projected from the edge of the defect. (Figure.14a, 16a) Unfilled defects presented collapsed contour with loosely connective tissue. (Figure.14b, 16b) BCP1 and BCP2 groups showed normal contour defects that filled with the distinct particles of BCP. (Figure.14c-d, 16c-d)

At 8 weeks, autogenous group showed bridging defects with completely bone remodeling process. (Figure.15a, 16e) Unfilled defect presented only connective tissue healing. (Figure.15b, 16f) Both BCP1 and BCP2, the BCP particles were smaller than at 2 weeks; newly formed bone was projected from the defect edge, extended in a centripetal direction and corporate well with the BCP particles. Little amount of bone was observed in the central part of defects (Figure. 15c-d, 16g-h)

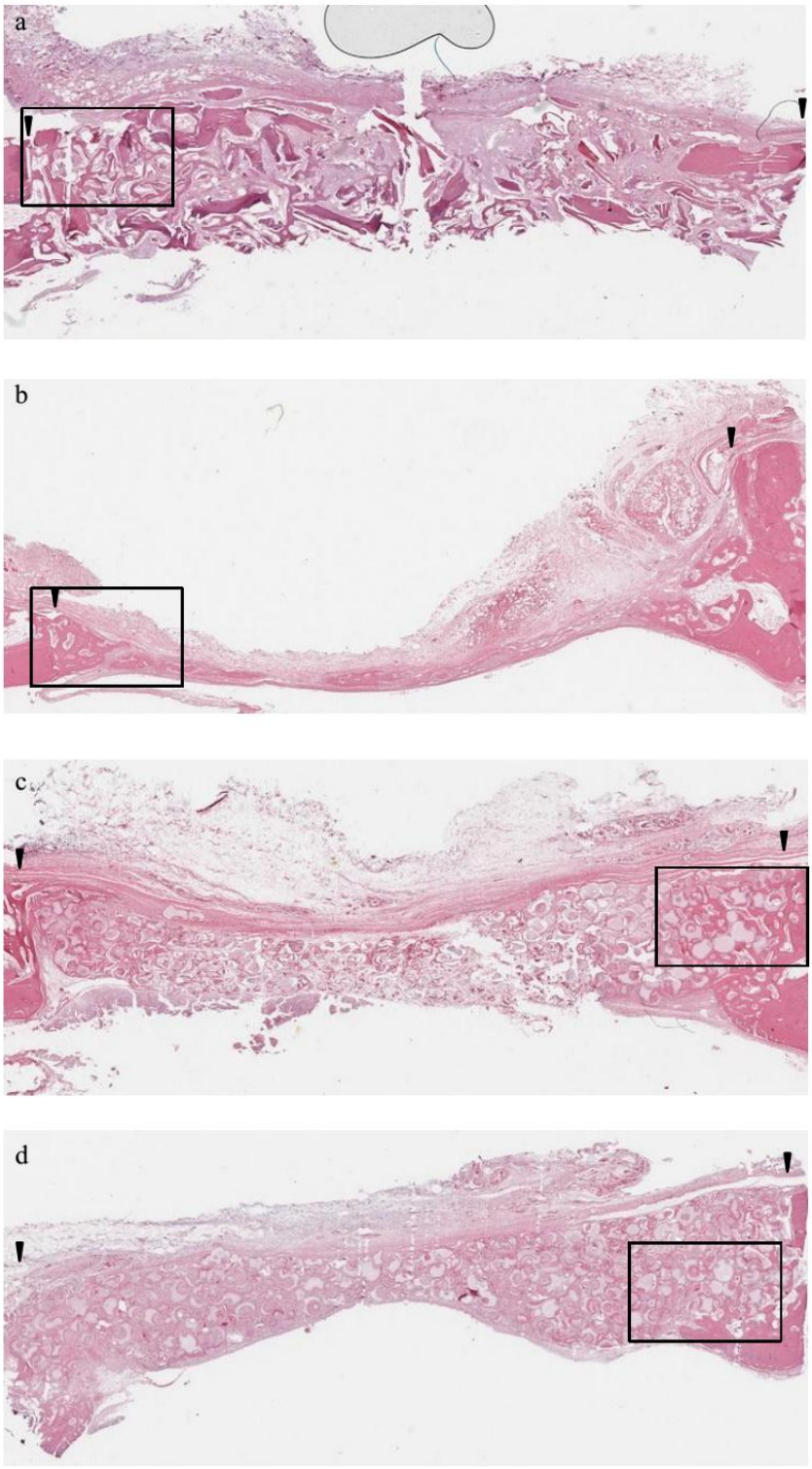

Figure.14 Decalcified specimens at 2 weeks of healing periods; Autogenous group (a), Unfilled defect (b), BCP1 (c) and BCP2 (d). Arrowhead = defect margin. Hematoxylin and eosin, original magnification

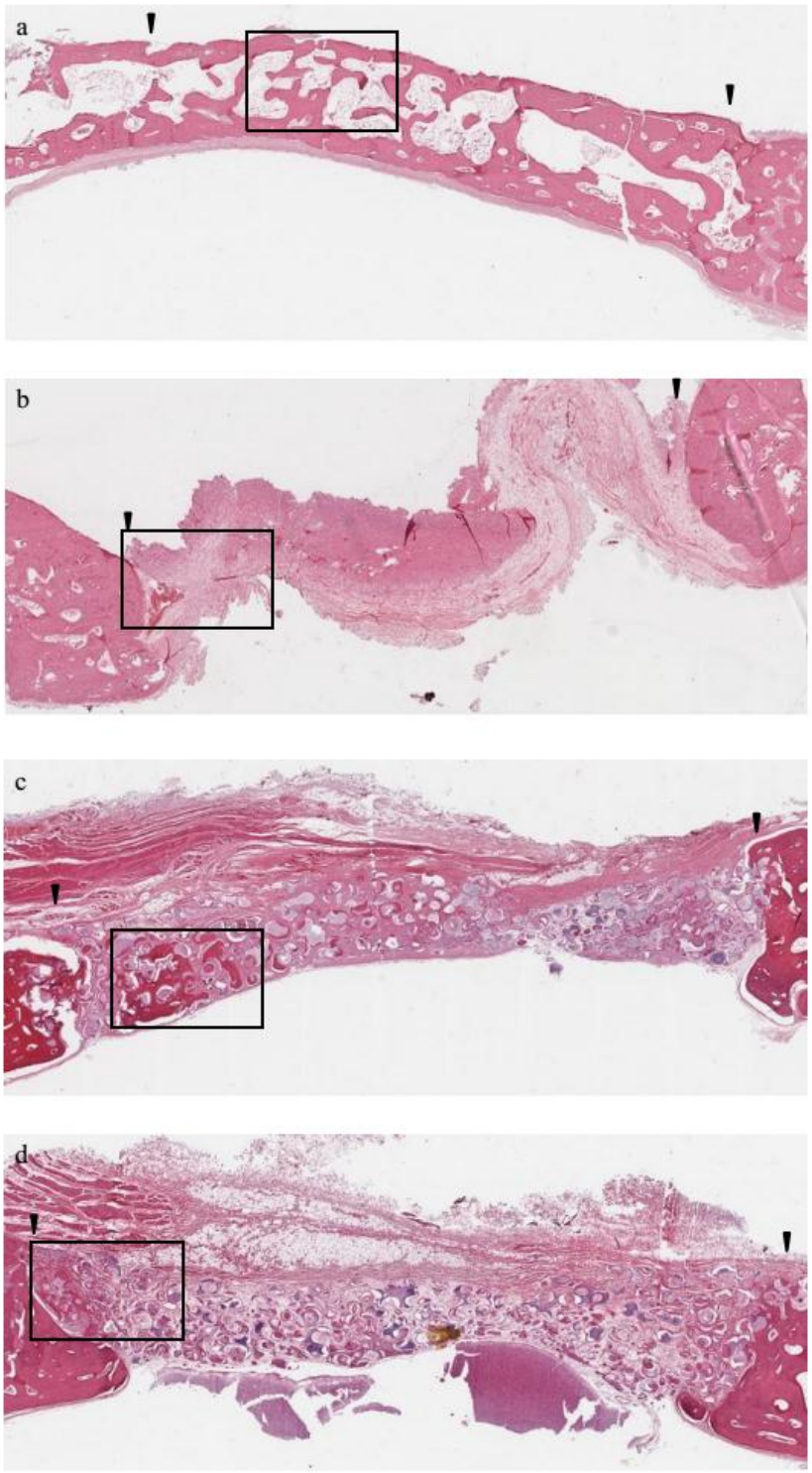

Figure.15 Decalcified specimens at 8 weeks of healing periods; Autogenous group (a), Unfilled defect (b), BCP1 (c) and BCP2 (d). Arrowhead = defect margin. Hematoxylin and eosin, original magnification

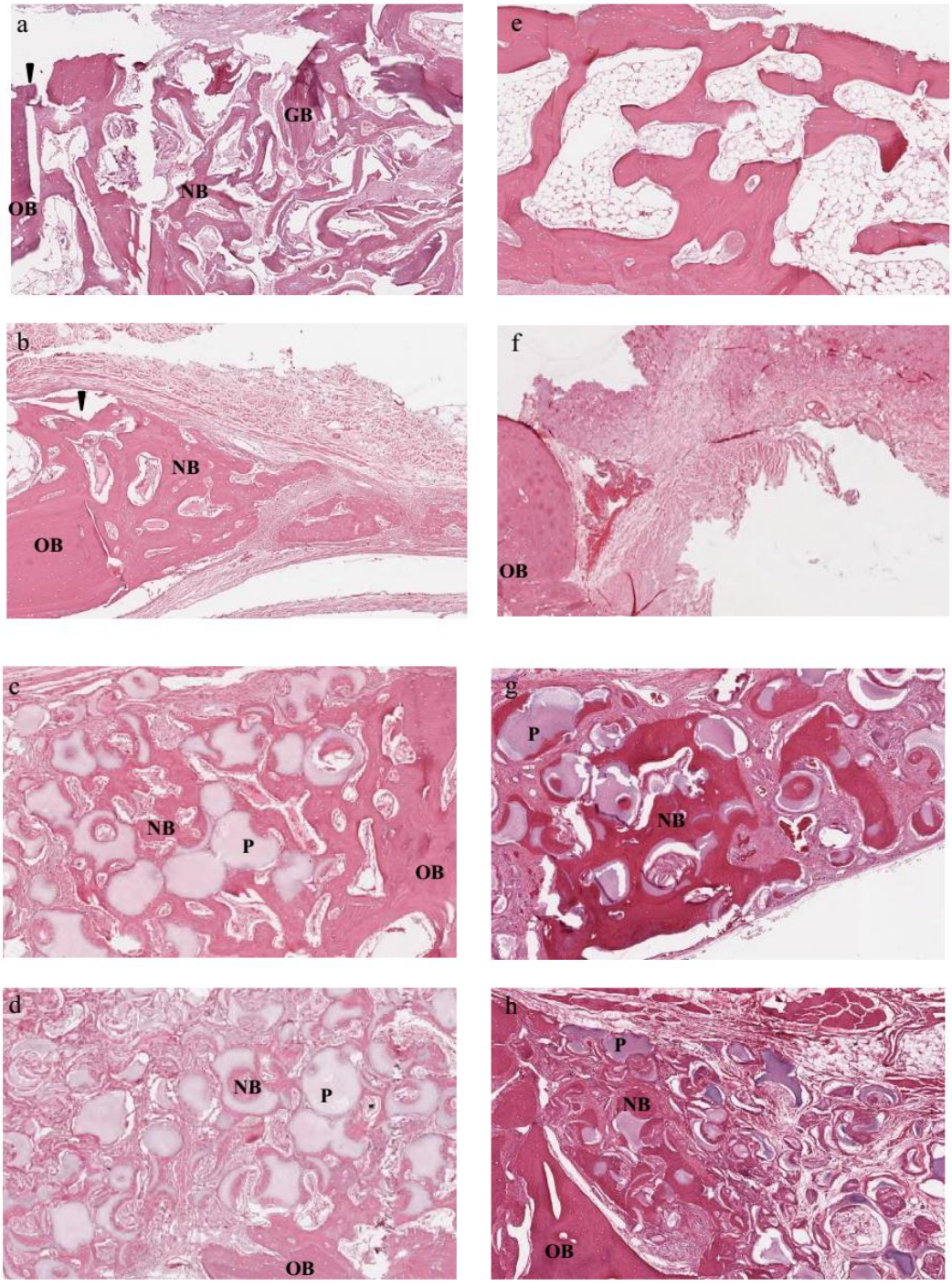

Figure.16 Decalcified specimens at 2 weeks (a, b, c, d) and 8 weeks (e, f, g, h) of healing periods; Autogenous group (a, e), Unfilled defect (b, f), BCP1 (c, g) and BCP2 (d, h). Arrowhead = defect margin; GB = grafted bone; NB = newly formed bone; OB = original bone; P = BCP particle. Hematoxylin and eosin, original magnification x 5

Histomorphometric Analysis

The percentage of new bone area and percentage of grafting material particle area were presented in Table.3 and Figure.17.

At 2 weeks, percentage of new bone area for autogenous group, unfilled defects, BCP1 and BCP2 were 4.30±0.76, 2.82±1.19, 3.01±2.57 and 3.24±1.09 respectively. Percentage of grafting material area for autogenous group, BCP1 and BCP2 were 24.85±7.07, 21.76±7.07 and 21.70±4.86 respectively.

At 8 weeks, percentage of new bone area for autogenous group, unfilled defects, BCP1 and BCP2 were 12.83±7.74, 8.14±6.35, 8.81±3.86 and 10.27±3.98 respectively. Percentage of grafting material area for autogenous group, BCP1 and BCP2 were 18.61±8.43, 22.36±2.80 and 20.42±3.33 respectively.

From 2 to 8 weeks, increasing of newly formed bone was found in all groups. Nevertheless, only autogenous group showed decreasing in grafting material area.

Table.3 Histomorphometry result after at 2 and 8 weeks

Timing	Group	Bone (%)	Material (%)
2 weeks			
	Autogenous bone chip	4.30 ± 0.76	24.85 ± 7.07
	Unfilled defect	2.82 ± 1.19	-
	BCP1 (8:2)	3.01 ± 2.57	21.74 ± 2.31
	BCP2 (9:1)	3.24 ± 1.09	21.70 ± 4.86
8 weeks			
	Autogenous bone chip	12.83 ± 7.74	18.61 ± 8.43
	Unfilled defect	8.14 ± 6.35	-
	BCP1 (8:2)	8.81 ± 3.86	22.36 ± 2.80
	BCP2 (9:1)	10.27 ± 3.98	20.42 ± 3.33

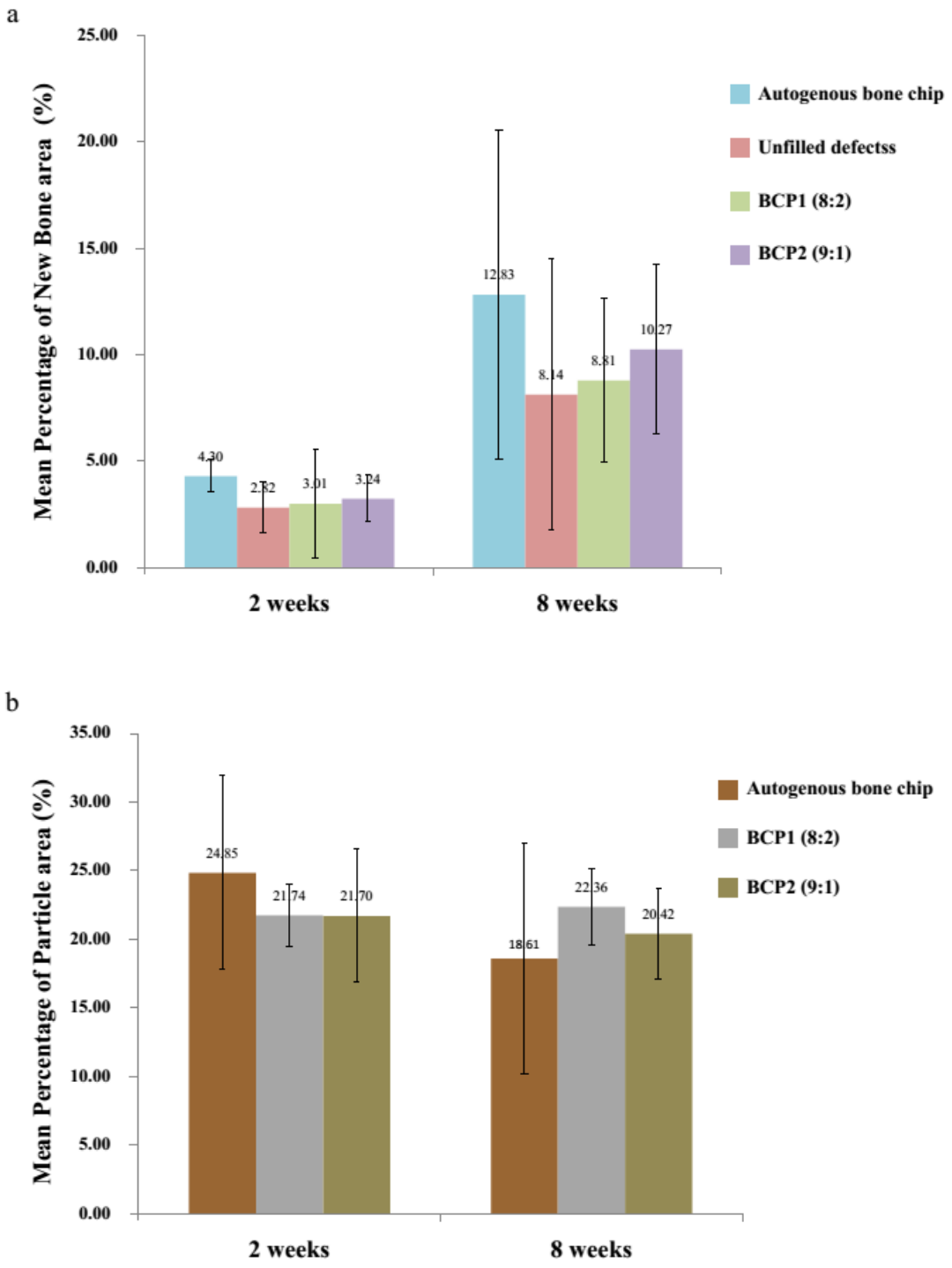

Figure.17 Histomorphometric analysis; Percentage of new bone are (a) and Percentage of grafting material particle area (b)

Moreover, the results of amount of bone from histomorphometry was significantly correlated with the results from micro-CT only in autogenous group at 2 week (r = 1.00, P = 0.01), unfilled defect group at 2 weeks (r = 1.00, p = 0.01), BCP1 at 2 weeks (r = -1.00, p=0.04) and BCP2 at 8 weeks (0.996, 0.04), as well as, the amount of BCP particles was significantly correlated only in BCP1 at 2 weeks (r=-1.00, p=0.01).

Table.4 Pearson's correlation coefficient of data between micro-CT and histomorphometry

Data	Group	Pearson's correlation	
		2 weeks	8 weeks
Bone			
	Autogenous bone chip	1.000*	-0.553
	Unfilled defect	1.000*	0.602
	BCP1 (8:2)	-1.000*	-0.298
	BCP2 (9:1)	-0.162	0.996£
Material			
	BCP1 (8:2)	-1.000*	0.752
	BCP2 (9:1)	0.765	-0.309

* = Statistical significant difference at P < 0.01
£ = Statistical significant difference at P < 0.05

Discussions

A large number of bone grafting materials have been developed and generally used in dentistry for rehabilitation of maxillofacial and periodontal defects. The development of highly effective bone materials, which achieve the most predictable regenerative results, is a major challenge for researchers and clinicians. In this study, the osteoconductive effect of newly developed biphasic calcium phosphate with high HA/TCP ratio was evaluated in rabbit calvarial defects.

The Experimental Model

Rabbit model has been used successfully in previous studies at Prince of Songkla University concerning grafting materials.[23, 26, 27] The rabbit model has several advantages, such as standardization of experimental conditions and experiment repeatability, ease of handling and size, inexpensiveness, and rapid bone turnover rate.[28]

Although critical-size defect in adult rabbit has been defined as at least 15 mm in circular diameter[29, 30], some studies reported that 8-mm diameter defect is critical because it could not heal without the use of bone graft.[31] This present study used 10-mm diameter circular defect because it was possible to create two defects in one animal. Sohn et al. showed no differences in bone healing between the circular defect created by a trephine sized 11 mm and 15 mm in diameters at each study period of 2, 6, 8 and 12 weeks and there was also no difference of bone formation between 2 and 4 or between 8 and 12 weeks.[32] However, the actual diameter of the created defect in Sohn's study was 10 mm because the diameter of trephine was the outer diameter.

In rabbits, the bone metabolism is approximately three times faster than in humans.[33] Therefore, a healing period of 2 weeks was chosen for evaluating the early phase of the healing response, such as the stability of the materials or the host reactions. And a healing period of 8 weeks was selected since it is appropriate for evaluating the late phase of the healing response, such as bone incorporation, resorption of materials, bone remodeling, or the amount of bone regeneration.[32]

In this study, number of animals in control and experiment groups were not equal (n=6 in control groups and n=10 in experiment groups) according to the suggestion from the animal ethic committee, Prince of Songkla University, to used minimal number of animals as necessary that result in 3 animals per each timeframe in control groups. Hence, the result of BVF from micro-CT analysis and percentage of new bone area from histomorphometry had a wide range of standard deviation, then there was no significant statistically differences even the values seem to be different. (Table.2 and Table.3)

Gross Specimen Observation

BCP1 and BCP2 groups showed full and better contour of augmented areas than the unfilled defect group that showed collapsed tissue profiles particularly at the central portion. These suggested that porous BCP has favorable space maintaining capacities at both 2 and 8 weeks. This result was consistence with the study from Lim et al that used BCP with HA/TCP ratio of 70/30 (Osteon™, Genoss. Co. Ltd., Suwon, Korea) and found effective space maintenance throughout 8 weeks of healing period.[34] If the space collapses, the blood clot cannot stabilized and epithelial down growth cannot be prevented, consequently impairing new bone formation.[35]

Radiographic Analysis

Within the study periods, the experimental group presented higher radio-density than the control group. The high density is related to the inorganic composition of BCP that possesses more density than the organic and inorganic part in the autogenous bone.[36]

From 2 to 8 weeks, increasing of mean OD was found in unfilled defect group, BCP1 and BCP2 which indicated the more accumulation of new bone formation after time passed. Nevertheless, autogenous group showed decreasing density which possibly due to the resorption of bone chip in the remodeling process. BCP1 and BCP2 showed less degradation because of the small ratios of TCP.

Micro-CT Analysis

Micro-CT imaging data has limitation in discrimination of materials that show similar density such as bone chip and new bone. Results from Micro-CT revealed that at 8 weeks of healing periods, autogenous bone chip group had the significantly highest bone volume ($p<0.05$) but this data did not reflect the actual bone regeneration because the bone volume included both the grafted bone chip and new mineralized bone. The bone volume in the experimental groups represented actual new bone formed voxels that could be discriminated from BCP particles. BCP1 and BCP2 group had higher bone volume than the unfilled defect groups, but the difference was not statistically significant. Moreover, from 2 to 8 weeks, increasing of BVF for BCP1 (10.8±2.00%) and BCP2 (10.15±3.12%) were higher than the empty defects (4.17±3.29%), suggesting the osteoconductivity of BCP.

In addition, some study found more bone being formed in defects filled with BCP with higher TCP ratios, Jan et al reported percentage of BVF were up to 64% when used BCP with HA/TCP ratio of 60/40 (Straumann AG, Basel, Switzerland) mixed with rabbit's own blood filled in 15-mm defects[37], the higher BVF most probably due to resorption of the TCP providing more space for new bone formation.

At 2 weeks, both BCP1 and BCP2 has MVF approximately 20% but the material was used by weight not by volume, therefore the less volume was possibly due to loosely pack of material and material porosity. However, measurement amount of residual materials by the micro-CT was depended on setting of grayscale threshold values. In this study, the ceramic threshold values were measured by tracing only obvious BCP particles and cannot detect the lower threshold values of partially dissolved material voxels. Therefore, the estimate amount of material by micro-CT is sensitive and the result depended on the setting of grayscale threshold that could be under or overmeasured.

Histological Analysis

The histological result confirmed the critical-size nature in adult rabbit model since the defects were not completely bridged in unfilled defects. (Figure 13b, 15b) The newly

formed bone was in close contact with the BCP1 and BCP2 particles, proving that these BCP with high ratio of HA have good osteoconductive properties. (Figure 13g-h, 16g-h)

The histomorphometry result supported the micro-CT evaluation that BCP1 and BCP2 group has higher bone regeneration than the unfilled defect groups. At 8 weeks of healing periods BVF of BCP1 (20.70±2.76) and BCP2 (20.72±3.97) were two folds higher than the percentage of bone area from histomorphometry (8.81±3.86 and 10.27±3.98 respectively) that possibly due to micro-CT measured volume of bone in 3-dimension while histomorphometry represented only a section of 15-30 μm at the center of circular defect in 2-dimension.

In addition, the result from of this study was slightly better than the previous study, used synthetic BCP block (8mm in diameter and 3-mm thick) with HA/TCP ratio of 60/40 filled in 8-mm circular defects which new bone formation was only 9.03±3.39.[21] However, the other study used BCP with HA/TCP ratio of 60/40 (MBCP; Biomatlante, Nantes, France) reported newly formed bone was 17.6±5.2[22]

Grafting Material

Bone substitutes degradation was assessed by comparing the material content at two time points. From visual observation, particle size of BCP1 and BCP2 were reduced. Moreover, from 2 and 8 week, decreasing of MVF of BCP1 (2.62±1.2) and BCP2 (5.21±0.48) was not significant different which probably due to small ratio of TCP. Theoretically, the TCP part will be dissolved into Ca^{2+} and PO_4^{3-} ions, whereas HA will retain its form and structure and will not be resorbed. [38]

The micro-CT and histology results showed that BCP1 and BCP2 had effective space maintaining capacity since the unfilled defect group were pressed and flatten while both BCP group maintained the space of the defect during the 2 and 8 weeks. This probability due to structural support from high ratios of HA can be effective to against the pressure of surrounding soft tissue.

In this study, total porosity of BCP1 and BCP2 were 80% and a pore size ranging from 100 to 300 μm. It is hypothesized that the interconnecting large pore size and high porosity of these BCP provide proper space necessary for vascular invasion and enhance bone in-growth within the material. The minimum recommended pore size for a bone substitute is 100

μm. Smaller pores (75-100 μm) showed an in-growth of unmineralized osteoid tissue or were penetrated by fibrous tissue only (10-44 and 44-75 μm)[39] . Some studies have recommended pore sizes more than 300 μm are more favorable for direct osteogenesis, since they allow vascularization and high oxygenation.[40-42] Interconnected pore are more useful than dead-end types, since a continuous connection of the pore system has provided the way for cell distribution and migration, as well as it allows an efficient blood vessel formation which suitable for sustaining bone tissue neo-formation and possibly remodeling.[43]

In summary, results from this study showed favorable outcomes of BCP with high ratios of HA/TCP in promotion of bone healing of the defects, which could be an effective material in various clinical conditions that space maintaining is important and slow degradation is a requirement such as in an atrophic alveolar ridge augmentation, a large osseous defect grafting, a cystic cavity and a sinus floor augmentation.

Conclusions

In conclusion, BCP1 (8:2) and BCP2 (9:1) presented good osteoconductive properties, biocompatibility with the living tissue and excellent space maintaining capacity with slow biodegradation rates and enhanced bone formation in animal model. BCP with high ratio of HA should be considered for further clinical trial study.

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Appendix

Journal of Biomedical Materials Research: Part B - Applied Biomaterials

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Bone regeneration potential of biphasic nano-calcium phosphate with high HA/TCP ratios in rabbit calvarial defects

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Bone regeneration potential of biphasic nano-calcium phosphate with high HA/TCP ratios in rabbit calvarial defects

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Abstract

This study aimed to evaluate the effect of biphasic calcium phosphate with high HA/TCP ratios on bone formation in rabbit calvarial defects. Sixteen New Zealand white rabbits were randomly divided into 2 groups of a control and an experiment. In each animal, bilateral circular defects (10-mm diameter) were created on the calvarium. Group I (3 rabbits per time frame) defects were grafted with autogenous bone chips or left empty. Group II (5 rabbits per time frame) defects were grafted with BCP1 (HA:TCP, 8:2) or BCP2 (HA:TCP, 9:1). The animals were sacrificed at 2 and 8 weeks. Bone formation and residual grafting material were assessed by radiographic densitometry, micro computed tomography (micro-CT) and histomorphometric analysis. Histology observation revealed the autogenous bone group demonstrated bridging defect while the unfilled defect group showed connective tissue healing. BCP1 and BCP2 preserved good contour of the defect. Micro-CT analysis, at 8 weeks, demonstrated the autogenous group had a significantly ($p<0.05$) greater bone volume fraction (34.58±8.85) as compared with the other groups. No statistical significance was observed for material volume fraction between BCP1 and BCP2. The histomorphometry demonstrated a higher increase in newly formed bone in the autogenous group (4.30±0.76, 12.83±7.74) than in the unfilled defect (2.82±1.19, 8.14±6.35), BCP1 (3.01±2.57, 8.81±3.86) and BCP2 (3.24±1.09, 10.27±3.98). In conclusion, BCP at high ratio of HA presented good osteoconductive properties, and space maintaining capacity with slow biodegradation.

Keyword: Biphasic calcium phosphate, histomorphometry, micro-CT, nanohydroxyapatite, rabbit

Introduction

Calcium phosphate ceramics (CaP) are the most popular ceramics used for bone substitution. The most commonly used CaPs are hydroxyapatite (HA), tricalcium phosphate (TCP) and biphasic calcium phosphate (BCP); a combination of the two. HA exhibits strong osteoconductive property and excellent biocompatibility due to its chemical similarity to the mineralized phase of bone, but the disadvantages are weakness under tensile stress and very slow resorbability[1]. TCP is generally recognized as resorbable bioceramics that has gradually been replaced with bone tissue. However, a faster rate of resorption than regeneration of newly formed bone was observed[2]. BCP, the incorporation of slow resorbable HA and rapidly resorbable TCP, is preferred as an osteoconductive matrix which maintains space for new bone formation with a dissolution rate that varies upon the dissolution of TCP. Higher HA content leads to a slow degradation rate, but ensures volume maintenance – while TCP is gradually resorbed and replaced by newly formed bone[3].

There are various HA/TCP ratios published in the literature, all of which possess different physical and biological properties. These various ratios, from *in vitro* and *in vivo* studies, with HA/TCP ranging from 20/80 to 85/15 and each ratio claimed having good properties[4-8].

In rabbit calvarial models, Hwang et al. 2012, reported that BCP with an HA/TCP ratio of 60/40 showed higher amounts of newly formed bone than pure HA and pure β-TCP at 4 and 8 weeks of healing[9]. This result was consistent with the study from Park et al 2010, that used BCP with the same HA/TCP ratio and found an increase in new bone formed when compared to anorganic bovine bone (Bio-Oss®) and β-TCP (Cerasorb®) at 8 weeks of healing[10]. Lim et al 2010, found that BCP with an HA/TCP ratio of 70/30 (Osteon®) showed normal contour of augmented areas but the unfilled defect groups showed collapsed tissue

profiles, suggesting that BCP has effective space maintenance throughout 8- week period of healing[11].

Some clinical situation - such as sinus lift grafting, socket preservation for undetermined treatment plan and repairing of peri-implant defects - require material with slow or transitional degradation and space maintenance properties. BCP with a high ratio of HA is preferred in those conditions. This study aimed to assess *in vivo* bone regenerative and degradation capacity of porous BCP in high HA/TCP ratios (8:2 and 9:1) in calvarial defects of rabbits.

Materials and Methods

Materials

The porous BCP was prepared by the National Metal and Materials Technology Center (MTEC) of Thailand. The BCP with HA/TCP ratios of 8:2 and 9:1 were selected from 5:5 to 9:1 ratio which gained the best results from *in vitro* biocompatibility testing. They contained 80% porosity, well-interconnected pore structures and a pore size of 100 to 300 µm in both ratios of BCP (Fig. 1).

Animal Preparation

Sixteen adult male (12 months old) New Zealand white rabbits each weighing 3 - 4 kg were used in this study. The animals were randomly divided into 2 groups, 6 animals for a control (autogenous group versus unfilled group) and 10 for an experimental group (BCP1 versus BCP2) as in Table I. Each group was divided into 2 time frames; 2 and 8 weeks for evaluation. Each animal was kept in a single cage and fed a standard laboratory diet and water.

Surgical Procedure

The procedures were performed according to the regulations of and with the approval of the Animal Experiment Ethics Committee of Prince of Songkla University, Ref. No.

01/2011. Anesthesia was induced using ketamine 25 mg/kg and diazepam 5 mg/kg intramuscularly 30 min before surgery. Thiopental 5 mg/kg was administered intravenously and then titrated at the rate of 2 mg/kg every 15 min (with a maximum dose of 30 mg/kg) until unconsciousness was achieved[12].

The surgical field was disinfected with 10% povidone-iodine. A mid-sagittal incision was made after local infiltration of 2% lidocaine hydrochloride with 1:100,000 epinephrine 1.8 ml. A subperiosteal dissection was performed, and two 10-mm-diameter bicortical bone defects were carefully trephined with a 10-mm internal diameter trephine bur and saline irrigation on the parietal bone bilaterally.

After random allocation, in the control group (n=6), defects were filled with autogenous bone chips 0.15 g by weight in one side. Bone chips were minced with a bone morselizer (Salvin Dental Specialtie Inc, Charlotte, NC, USA). The other side defect was left empty. In the experiment group (n=10), defects were randomly filled with porous BCP1 (8:2) or BCP2 (9:1) at 0.10 g by weight plus 0.9% normal saline solution until the total weight was equal to the minced autogenous bone chips (Fig. 2). The periosteum, muscle and skin were sutured layer by layer using Vicryl® 4/0.

The rabbits were sacrificed at 2 and 8 weeks after surgery with 1.2-1.3 ml overdose of 200 mg/ml pentobarbital sodium administered intravenously via the marginal ear vein. The calvarial bone was harvested using a small sharp fissure bur and then fixed in 10% formalin.

Digital Radiograph

The calvarial specimens were radiographed by using hand-held portable X-ray devices (NOMAD, Aribex Inc., Utah, USA) with digital sensor size 0 attach to digital sensor holder (XCP-DS, Rinn, Densply, IL, USA). The setting for all exposures was 60 kVp, 2.3 mA and 0.3sec at the distance of 10 cm. Images were captured on receptor (Sopix CMOS, Instrumentarium Dental, Tuusula, Finland). The mean optical density (OD) of the defect was

calculated and analyzed by using Image Pro Plus 7.0 software (Media Cybernetics Inc., Silver Spring, MD, USA)

Specimen Processing

After radiography, each specimen was trimmed and cut mid-coronally along the center of two circular defects into 2 halves; one half was used for micro-CT analysis, and the other half was used for histomorphometric analysis (Fig. 3).

Micro computed tomography (Micro-CT) analysis

A high resolution micro-CT system (Micro-CT80, Scanco, Medica AG, Basseersdorf, Switzerland) was used. After calibration the specimens were scanned perpendicularly to the cranium vault at 55 kVp, 72 µA and 4W in high-resolution mode (18.5µm^3/voxel). Scanned data were reconstructed by built-in software.

Initially, the grayscale threshold values were determined to discriminate bone and ceramic from soft tissue[13]. After determination of the threshold values, the region of interest (ROI) was traced to specify the defect margin. The percentage of bone volume fraction (BVF, BV/TV), calculated as a percentage of radio-opaque voxels in a bone threshold range divided by the total defect volume, and the percentage of the material volume fraction (MVF, MV/TV), calculated as a percentage of radio-opaque voxels in a ceramic threshold range divided by the total defect volume, were determined.

Histology Processing

One half of the circular defect specimens were processed to obtain thin ground sections using undecalcified techniques, according to the technique of Donath and Breune[14] with minor modifications. Briefly, the specimens were dehydrated in an ascending series of alcohol rinses and embedded in a glycolmethacrylate resin (Technovit 7200 VLC, Kulzer, Wehrheim, Germany). After polymerization the specimens were serially sectioned along their longitudinal axis with a high-precision diamond disc at approximately 150 µm and ground

down to approximately 15-30μm with a specially designed grinding machine (EXAKT® cutting and grinding system, EXAKT® Apparatebau, Norderstedt, Hamburg, Germany). Three sections were stained with Goldner's Masson trichrome. All slides were examined descriptively before histomorphometric analysis.

The other half of the circular defect specimens, after the micro-CT analysis, were decalcified in formic acid and then embedded in paraffin. Serial sections in 5 μm were cut from the center and stained with hematoxylin and eosin for descriptive examination.

Histomorphometric Analysis

All slides were then loaded into an Aperio ScanScope XT (Aperio ePathology Solutions, California, USA) and scanned at 40x magnification. Digital histologic images were captured with special software from the same company (Aperio ImageScope 9.0, Aperio ePathology Solutions, California, USA). The undecalcified sections containing the central portion were used for histomorphometric analysis. The quantity of new bone formation was calculated as the percentage of newly formed bone area to the total defect area and the amount of grafting material particle area was calculated as the percentage of each grafting material particle area to the total area using Image Pro Plus 7.0 (Media Cybernetics, MD, USA).

$$\text{Percentage of new bone area} = \frac{\text{new bone area} \times 100}{\text{total area}}$$

$$\text{Percentage of material particle} = \frac{\text{grafted material area} \times 100}{\text{total area}}$$

Statistical Analysis

Statistical analysis was performed using statistical analysis software (SPSS ver15.0, SPSS Inc., Chicago, USA). Data were tested for normality and presented as means ± SD. One-way analysis of variance and multiple comparison by Scheffé's post-hoc test ($P<0.05$)

were used to compare the differences among groups of the mean optical densities, the percentage of bone volume fraction (BVF, BV/TV), the percentage of material volume fraction (MVF, MV/TV), the percentage of newly formed bone and the percentage of BCP particles in each groups. Difference between the two times points of each group were analyzed using the Paired T-Test.

Results

Animal

All rabbits tolerated the surgical procedure and the anesthesia well. All recovered after surgery without any complications. Wound healing was unremarkable.

Radiographic Features

At 2 weeks, the autogenous group showed various sizes and densities of radiopaque masses in the defects while the unfilled defect group presented a circular homogeneous radiolucent area with clear border (Fig. 4a). BCP1 and BCP2 showed distinct radiopaque particles with well-delineated borders (Fig. 4c).

At 8 weeks, the autogenous group showed a non-distinct border which blended with the homogeneous radiopaque mass inside the defects (Fig. 4b). The unfilled defect group presented an irregular border of the defect with an infiltrating hazy density mass (Fig. 4b). BCP1 and BCP2 defects showed distinct radiopaque particles with less density than at 2 weeks and there were blended radiopaque density along the border of the defects (Fig. 4d).

Radiomorphometric Analysis

The results of radiomorphometric analysis are presented in Table II and Figure 5. At 2 weeks, the mean optical density (OD) of the autogenous bone chip group (0.305 ± 0.069), BCP1 (0.324 ± 0.080) and BCP2 (0.333 ± 0.061) were not different but significantly ($p<0.05$) higher than the unfilled defect (0.098 ± 0.048).

At 8 weeks, the mean OD of the autogenous bone chip group (0.210±0.015) was lower than at 2 weeks while the others were contradictory. Moreover the autogenous bone chips group and the unfilled defect (0.122±0.059) were also significantly ($p<0.05$) lower than BCP1 (0.389±0.096) and BCP2 (0.415±0.090).

Specimen Observation

At 2 weeks, the defect of the autogenous group was dense while the unfilled defect was loose and soft (Fig. 6a). BCP1 and BCP2 showed a normal contour of calvarium curvature with dense and packed particles (Fig. 6c).

At 8 weeks, the autogenous group showed a full contour of the defect filled with seamless dense tissue while the unfilled defect showed loose tissue within the defect (Fig. 6b). Both BCP1 and BCP2 presented a full contour of densely packed BCP particles and tissue (Fig. 6d). ***Micro-CT Analysis***

The total volumes of newly formed bone within the ROI (bone volume fraction or BV/TV), as well as the residual of grafting materials (material volume fraction or MV/TV), were summarized in Table III and Figure 7.

At both time frames the BV/TV in the autogenous group was highest (29.48±9.84, 34.58±8.85), but significantly higher ($p<0.05$) than other groups only at the 8-week period. The MV/TV of BCP1 and BCP2 were not different at both time frame and the material volume of both groups was lower at the 8-week period (25.02±3.51, 24.71±3.91) than at the 2-week period (28.64±2.31, 29.92±4.39) but not significantly.

Histology

Undecalcified specimens at 2 weeks, the autogenous group showed autogenous bone chips filled in the defects, and the connective tissue was infiltrated from the periphery of the defect (Fig. 8a, 8e). The unfilled defects presented thin loosely connective tissue bridging the defects (Fig. 8b, 8f). BCP1 and BCP2 groups showed normal contour defects, and dense

BCP particles were evenly distributed inside the defects (Fig. 8c-d, 8g-h). At 8 weeks, the autogenous group showed completely bridged defects with good contour, and an increase in new bone formation than at 2 weeks. The newly formed bone was well incorporated with the grafts and the host bone (Fig. 9a, 9e). The unfilled defect presented a small amount of bony island and loose connective tissue (Fig. 9b, 9f). For both BCP1 and BCP2, the BCP particles were less visible than at 2 weeks; newly formed bone was projected from the defect margin, extended in a centripetal direction and incorporated well with the BCP particles. However, a scattered small amount of new bone was observed in the central part and the gap between materials was filled with loose connective tissue. It was also noted that the grafting material preserved a good contour of the defects (Fig. 9c-d, 9 g-h).

The histological observation of H&E staining of the decalcified specimens were similar to the undecalcified specimens (Fig.10) where bone regeneration was most pronounced in the autogenous group and least pronounced in the unfilled defect group whereas BCP1 and 2 were not different in terms of bone formation and grafting material.

Histomorphometric Analysis

The percentage of new bone area and the percentage of grafting material particle area were presented in Table IV and Figure 11.

The percentage of new bone in all groups increased from 2 weeks to 8 weeks and was highest in the autogenous group at both time frames (4.30±0.76, 12.83±7.74). On the contrary, the percentage of grafting material area decreased with time and was more pronounced in the autogenous group (24.85±7.07, 18.61±8.43) while BCP1 and BCP2 only slightly changed (BCP1:21.76±7.07, 22.36±2.80; BCP2: 21.70±4.86, 20.42±3.33).

Discussion

A large number of bone grafting materials have been developed and generally used in dentistry for rehabilitation of maxillofacial and periodontal defects. The development of

highly effective bone materials to - achieve the most predictable regenerative results - is a major challenge for researchers and clinicians. In this study, the osteoconductive effect of newly developed biphasic calcium phosphate with high HA/TCP ratios were evaluated in rabbit calvarial defects.

The Rabbit models have been used successfully in previous studies concerning grafting materials[12, 15, 16]. Although the critical-size defect in adult rabbits has been defined as at least 15 mm in circular diameter[17, 18], many studies used a 10-mm defect because it could be created bilaterally in the cranium and less than 20 percent of bone formation was found in the unfilled defect[12, 15, 16]. Sohn et al. in 2012, showed no differences in the bone formation between the circular defects created by 11-mm and 15-mm external diameters trephines at each study period of 2, 6, 8 and 12 weeks. There was also no difference of bone formation between 2 and 4 or between 8 and 12 weeks[19]. The healing period of 2 and 8 weeks were chosen to demonstrate early and late phases of bone regeneration and also to detect the resorption rate of materials.

Function of bone substitute material is to be a scaffold for vessel and new bone growth. The material should be strong enough to withstand the pressure and present in the defect when new bone is being formed[20]. BCP1 and BCP2 served this function and showed full and better contour of augmented areas than the unfilled defect group that showed collapsed loose connective tissue. BCP with a high ratio of HA could maintain space throughout 8 weeks of healing and was effective for bone ingrowths as well as being compatible with the surrounding tissue.

This study used both radiographs and micro CT for evaluation. Conventional radiographs and optical density represented only radiodensity of the inorganic part of the grafting material and natural bone. Increasing density from 2 to 8 weeks in the unfilled defect group was directly related to bone accumulation but it was not applicable to other

groups since both autogenous bone and BCP underwent a resorption process simultaneously with bone formation process. Nevertheless, the autogenous group showed decreasing density which implied that the resorption of the bone chips in the remodeling process was more advanced than bone formation and the density of bone formation in this stage was not as mature as the grafting bone.

In this study, at each time frame, a number of animals in the control groups (n=3) were small and less than the experiment groups (n=5), therefore, it influenced the statistical analysis of the BVF and the percentage of new bone area where significant difference cannot be detected.

Micro-CT imaging data has limitation in discriminating similar density materials such as bone chip and new bone. Although the autogenous bone chip group showed significantly higher bone volume at 8 weeks of healing, this volume did not reflect the actual bone regeneration because it counted both the grafted bone chips and new mineralized bone together. In contrast, the bone volume in the experimental groups and unfilled group represented actual new bone formed voxels and the BCP groups showed higher bone formation than the unfilled group in both time frames. When the 2 time frames were compared, BCP groups showed approximately 2 times the increase of bone formation. The rate of material degradation detected from the reduced volume of material over 6 weeks (from 2 to 8 weeks) was 12.63% (BCP1) and 17.41% (BCP2) which is possibly due to the degradation of TCP. Although the study period is not long enough to detect complete degradation of the material, it shows degradation capacity during the bone healing phase.

The histomorphometry result supported the micro-CT evaluation that the BCP1 and BCP2 group had higher bone regeneration than the unfilled defect groups, but the rate of bone increase from 2 to 8 weeks of each group was comparable. A previous study used BCP disks (8mm in diameter and 3-mm thick) with an HA/TCP ratio of 60/40 in 8-mm circular

defects and found less new bone formation (9.03±3.39 at 8 weeks)[9] than the present study. While another study used BCP particles at the same HA/TCP ratio of 60/40 (MBCP; Biomatlante, Nantes, France) and found higher bone formation (17.6±5.2)[10]. This study used BCP in the particle form but a higher ratio of HA, yet found a similar result.

In summary, BCP with high ratios of HA/TCP showed favorable outcomes in promotion of bone formation in the calvarial defect of rabbits. BCP could be effective for conditions where space maintaining and slow degradation are required such as in an atrophic alveolar ridge augmentation, a large osseous defect grafting, a cystic cavity, long term socket preservation and for a sinus floor augmentation.

Conclusion

In conclusion, BCP1 (8:2) and BCP2 (9:1) presented good osteoconductive properties, biocompatibility with the living tissue and space maintaining capacity with slow biodegradation rates in an animal model. BCP with a high ratio of HA should be considered for a further clinical trial study.

Acknowledgements

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Captions to Figures

Fig. 1 SEM photographs of BCP granules showing the pore structure, size100 - 300 µm, original magnification x 50

Fig. 2 Bilateral calvarial defects filled with different materials; AB = Autogenous group, U = Unfilled defect, B1 = BCP1 (8:2), B2 = BCP2 (9:1)

Fig. 3 Schematic drawing of cutting specimen into 2 pieces for micro-CT analysis and histomorphometric analysis

Fig. 4 Radiograph of the rabbits' calvarium at 2 weeks (a, c) and 8 weeks (b, d) of healing; AB = Autogenous group, U = Unfilled defect, B1 = BCP1 (8:2), B2 = BCP2 (9:1)

Fig. 5 Mean optical density in each group after 2 and 8 weeks of healing

Fig. 6 Mid-coronally cut specimen at 2 weeks (a, c) and 8 weeks (b, d) of healing AB = Autogenous group, U = Unfilled defect, B1 = BCP1 (8:2), B2 = BCP2 (9:1), Black line = Thickness of defect

Fig. 7 Micro-CT analysis; Bone volume fraction (a) and Material volume fraction (b)

Fig. 8 Undecalcified specimens at 2 weeks of healing; Autogenous group (a, e), Unfilled defect (b, f), BCP1 (c, g) and BCP2 (d, h). Arrowhead = defect margin, GB = grafted bone, NB = newly formed bone, OB = original bone, P = BCP particle. Goldner's Masson trichrome, original magnification (a, b, c, d) and Goldner's Masson trichrome, original magnification x 5 (e, f, g, h)

Fig. 9 Undecalcified specimens at 8 weeks of healing; Autogenous group (a, e), Unfilled defect (b, f), BCP1 (c, g) and BCP2 (d, h). Arrowhead = defect margin, GB = grafted bone, NB = newly formed bone, OB = original bone, P = BCP particle. Goldner's Masson trichrome, original magnification (a, b, c, d) and Goldner's Masson trichrome, original magnification x 5 (e, f, g, h)

Fig. 10 Decalcified specimens at 2 weeks (a, b, c, d) and 8 weeks (e, f, g, h) of healing;

Autogenous group (a, e), Unfilled defect (b, f), BCP1 (c, g) and BCP2 (d, h). Arrowhead = defect margin; GB = grafted bone; NB = newly formed bone; OB = original bone; P = BCP particle. Hematoxylin and eosin, original magnification x 5

Fig. 11 Histomorphometric analysis; (a) Percentage of new bone areas and (b) Percentage of grafting material particle area

Captions to Tables

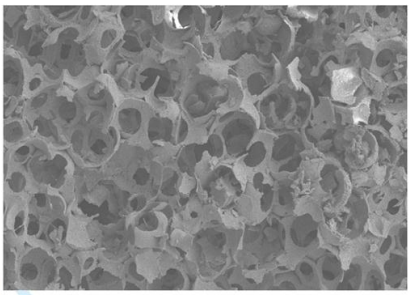
Table I Groups of study

Table II Results of radiomorphometric in each group after 2 and 8 weeks

Table III Bone volume fraction and material volume fraction of micro-CT results at 2 and 8 weeks

Table IV Histomorphometry results at 2 and 8 weeks

Captions to Figures



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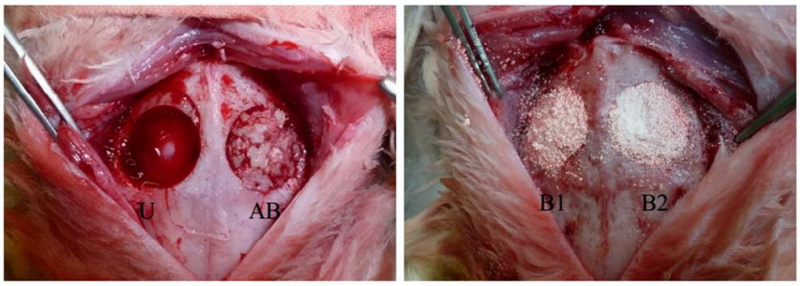

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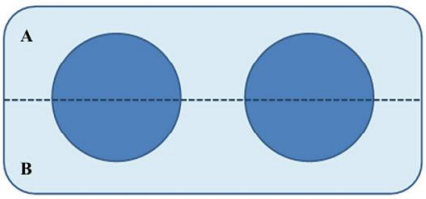

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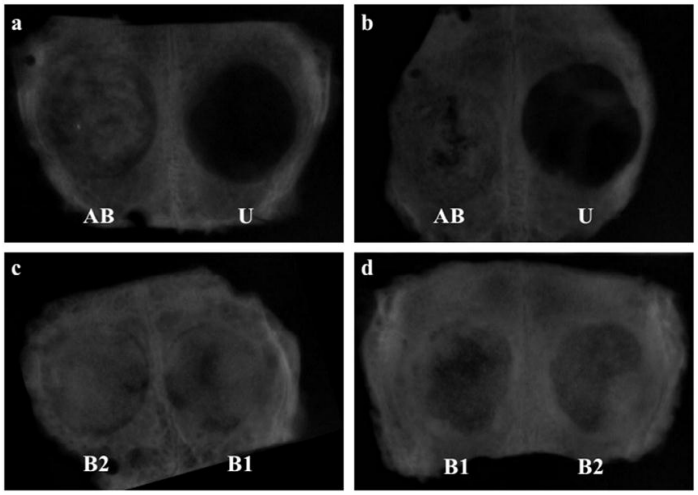

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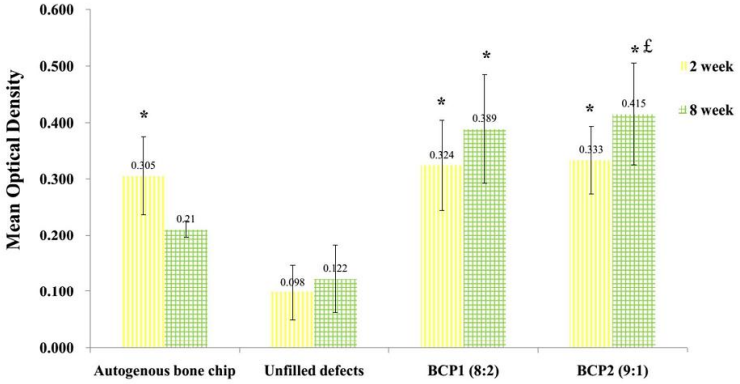

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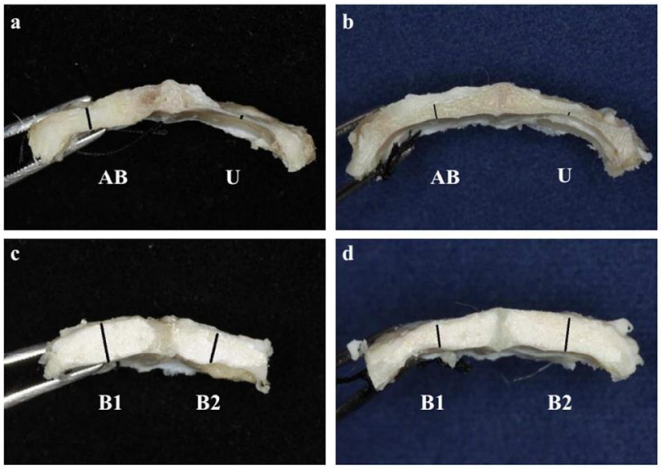

Fig. 6 Mid-coronally cut specimen at 2 weeks (a, c) and 8 weeks (b, d) of healing

AB = Autogenous group, U = Unfilled defect, B1 = BCP1 (8:2), B2 = BCP2 (9:1),

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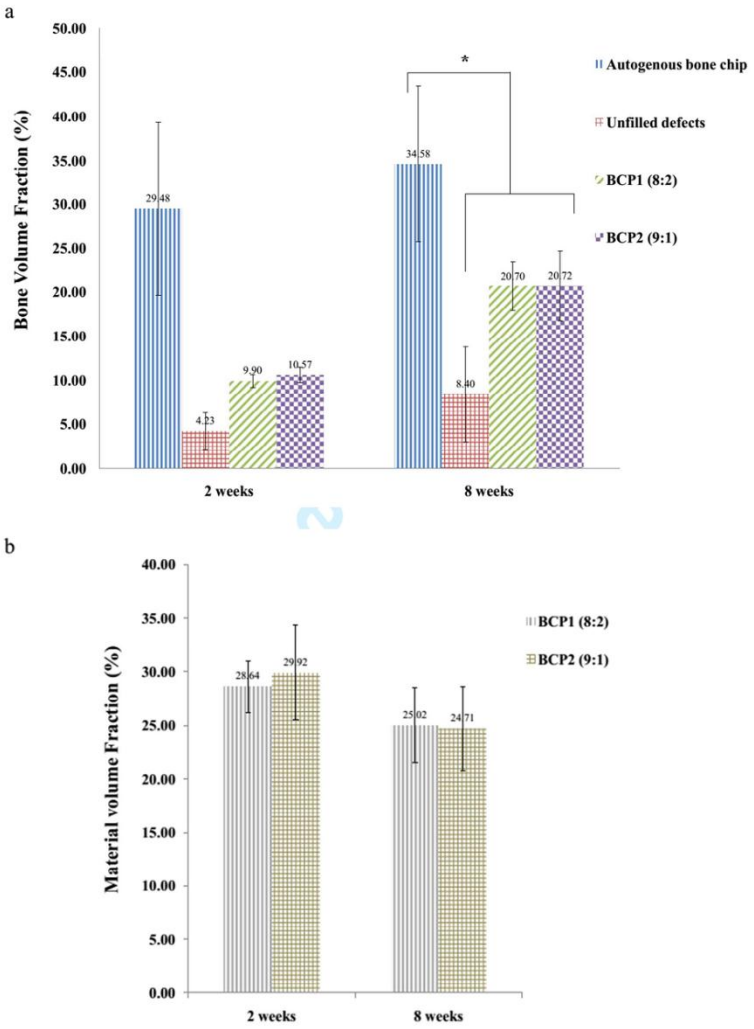


Fig. 7 Micro-CT analysis; Bone volume fraction (a) and Material volume fraction (b)

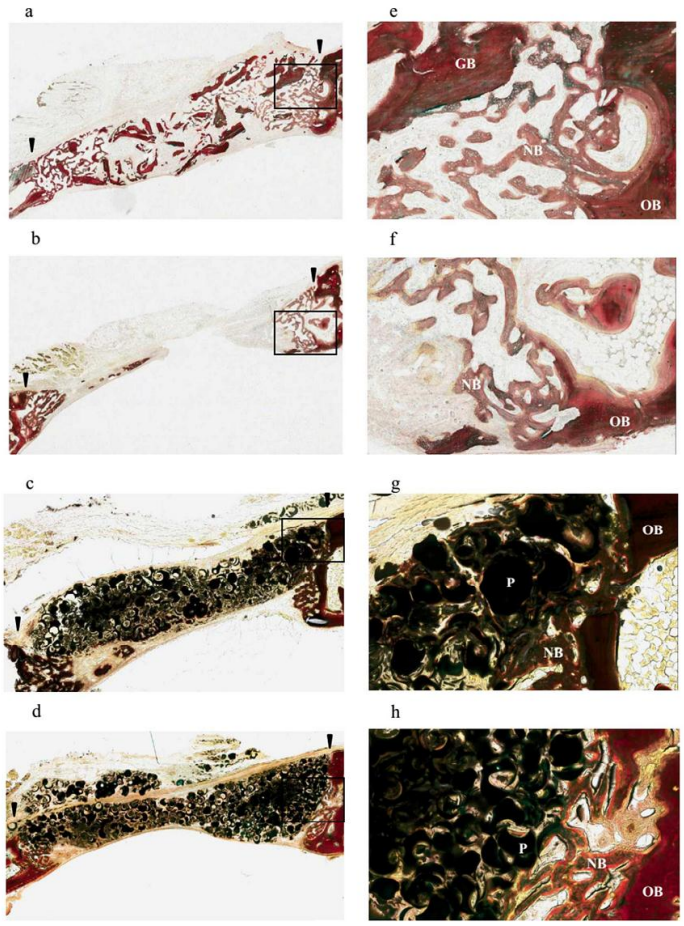
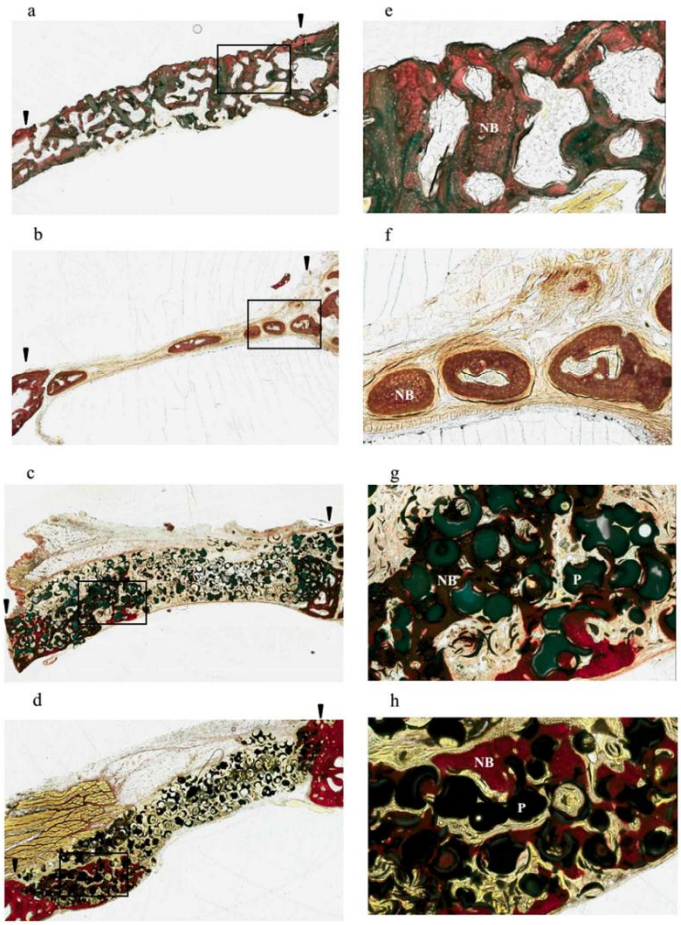


Fig. 8 Undecalcified specimens at 2 weeks of healing; Autogenous group (a, e), Unfilled defect (b, f), BCP1 (c, g) and BCP2 (d, h). Arrowhead = defect margin, GB = grafted bone, NB = newly formed bone, OB = original bone, P = BCP particle. Goldner's Masson trichrome, original magnification (a, b, c, d) and Goldner's Masson trichrome, original magnification x 5 (e, f, g, h)



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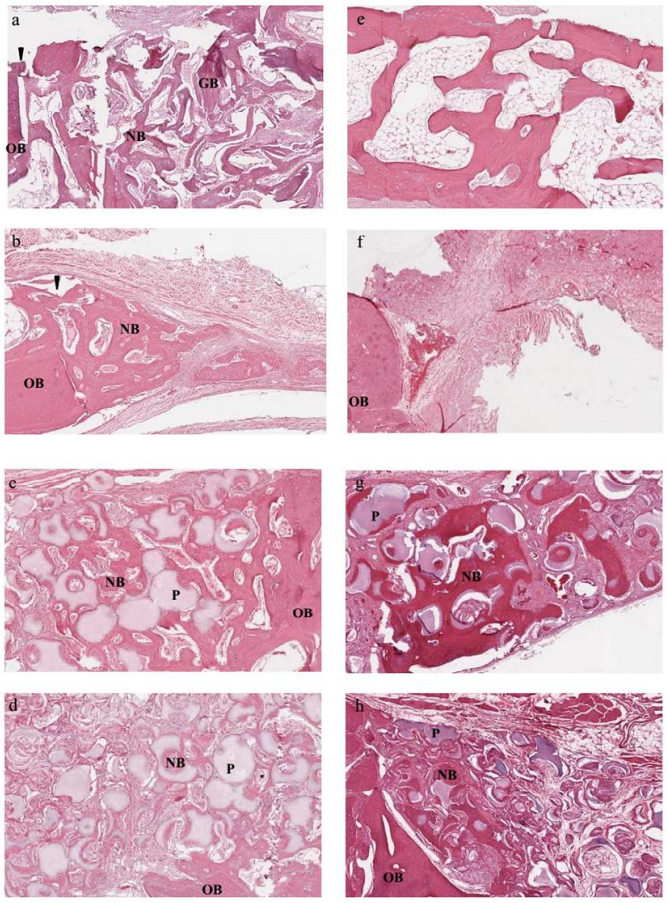

Fig. 10 Decalcified specimens at 2 weeks (a, b, c, d) and 8 weeks (e, f, g, h) of healing; Autogenous group (a, e), Unfilled defect (b, f), BCP1 (c, g) and BCP2 (d, h). Arrowhead = defect margin; GB = grafted bone; NB = newly formed bone; OB = original bone; P = BCP particle. Hematoxylin and eosin, original magnification x 5

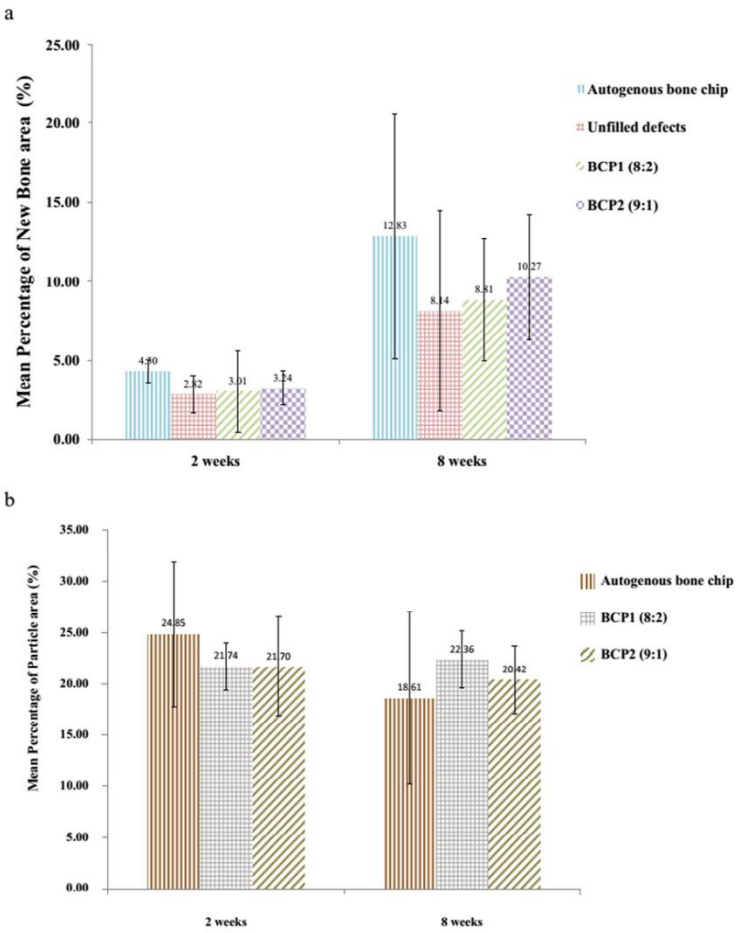

Fig. 11 Histomorphometric analysis; (a) Percentage of new bone areas and (b) Percentage of grafting material particle area

Captions to Tables

Table I Groups of study

Study Group	Detail	Number of rabbits		
		2 weeks	8 weeks	Total
Control	Autogenous bone chip / Unfilled defects	3	3	6
Experiment	BCP1 (8:2) / BCP2 (9:1)	5	5	10
Total				16

Table II Results of radiomorphometric in each group after 2 and 8 weeks

Group	2 weeks	8 weeks
Autogenous bone chip	0.305 ± 0.069*	0.210 ± 0.015
Unfilled defects	0.098 ± 0.048	0.122 ± 0.059
BCP1 (8:2)	0.324 ± 0.080*	0.389 ± 0.096*
BCP2 (9:1)	0.333 ± 0.061*	0.415 ± 0.090*†

Values are present as mean ± SD

*= Statistical significant difference from unfilled defect (P<0.05)

† = Statistical significant difference from autogenous bone chip at P=0.0031

Table III Bone volume fraction and material volume fraction of micro-CT results at 2 and 8 weeks

Timing	Group	BV/TV (%)	MV/TV (%)
2 weeks	Autogenous bone chip	29.48 ± 9.84	
	Unfilled defects	4.23 ± 2.08	
	BCP1 (8:2)	9.90 ± 0.75	28.64 ± 2.31
	BCP2 (9:1)	10.57 ± 0.85	29.92 ± 4.39
8 weeks	Autogenous bone chip	34.58±8.85*	
	Unfilled defects	8.40 ± 5.37	
	BCP1 (8:2)	20.70 ± 2.76	25.02 ± 3.51
	BCP2 (9:1)	20.72 ± 3.97	24.71 ± 3.91

Values are present as mean ± SD

BV/TV: Bone volume per total volume

MV/TV: Residual materials per total volume

* = Statistical significant difference from other groups at each time frame (P<0.05)

Table IV Histomorphometry results at 2 and 8 weeks

Timing	Group	Bone (%)	Material (%)
2 weeks	Autogenous bone chip	4.30 ± 0.76	24.85 ± 7.07
	Unfilled defects	2.82 ± 1.19	-
	BCP1 (8:2)	3.01 ± 2.57	21.74 ± 2.31
	BCP2 (9:1)	3.24 ± 1.09	21.70 ± 4.86
8 weeks	Autogenous bone chip	12.83 ± 7.74	18.61 ± 8.43
	Unfilled defects	8.14 ± 6.35	-
	BCP1 (8:2)	8.81 ± 3.86	22.36 ± 2.80
	BCP2 (9:1)	10.27 ± 3.98	20.42 ± 3.33


30th Anniversary
DFCT
The Dental Faculty Consortium of Thailand
The Best of Thai Dental Research
11th
Dental Faculty Consortium of Thailand Academic Meeting and Research Presentation (DFCT2013)
7-9 May, 2013
Pullman Pattaya Hotel, Chonburi, Thailand
The Dental Faculty Consortium of Thailand
Faculty of Dentistry ,Thammasat University

Welcome Message

by Conference Chair

Faculty of Dentistry, Thammasat University would like to welcome you to the 11th Dental Faculty Consortium of Thailand Academic Meeting and Research Presentation (DFCT2013), at the Pullman Pattaya Hotel, Chonburi. The theme for the meeting is "The Best of Thai Dental Research". This is an opportunity to bring together the dental leaders, educators, researchers, as well as students to exchange ideas and contribute to the latest developments, innovations in this rapidly advancing, multidisciplinary field of research in dentistry. The scope of DFCT2013 has expanded to cover dental education administration, research, as well as national policy and strategy in the dentistry profession. Our scientific program is rich and varied with 1 keynote speech, 1 special lecture from Prof. JM ten Cate (Global President of International Association for Dental Research), 9 invited talks and around 80 scientific abstracts split between 3 parallel 2 oral sessions and 1 poster session. There are 16 full papers submitted by graduate students as part of their graduation requirement. Besides, there is a Dean forum on research trend in dentistry in Thailand. This meeting will also mark a very special occasion, the 30th anniversary of DFCT (established since 1983). DFCT has members from eight universities, Chiang Mai, Chulalongkorn, Khon Kaen, Mahidol, Naresuan, Prince of Songkla, Srinakarinwirot, and Thammasat.

As a conference chair of DFCT2013, I know that the success of the conference depends ultimately on the many people who have worked with us in planning and organizing both the scientific program and supporting social arrangements. In particular, we thank the Scientific Program Committee Chair for an international arrangement of the abstract/paper submission and reviewing system; the Award Judging Committee Chair for advice and brilliant suggestion on organizing junior and senior research competitions; the Scientific Program Committee for their thorough and timely reviewing of the abstracts/papers, and our sponsors who have helped us to keep down the costs of DFCT2013 for all participants. Recognition should go to the Local Organizing Committee members who have all worked extremely hard for the details of important aspects of the conference programs and social activities.

On behalf of the DFCT2013 organizing committee, I am honored and delighted to welcome you to the conference and believe we have chosen a venue that guarantees a successful scientific conference amid the scenery of Pattaya beach.

Warm Regards

Prof.Dr. Sittichai Koontongkaew
Dean, Faculty of Dentistry, Thammasat University
Conference Chair

PP20

In vivo biocompatibility of porous BCP in two different ratios

Pongsakorn Praserttham, Prisana Pripatnanont, Srisurang Suttapreyasri, Narit Leepong, Naruporn Monmaturapoj*

Objectives:This study aimed to evaluate the effect of biphasic calcium phosphate with high HA/TCP ratios on bone formation in rabbit calvarial defects. Methods: Sixteen New Zealand white rabbits were randomly divided into 2 groups. Bilateral circular defects (10-mm diameter) were created on the calvarium in each animal. Group I (3 rabbits per each time frame) defects were grafted with autogenous bone chips or left empty. Group II (5 rabbits per each time frame) defects were grafted with BCP1 (HA:TCP, 8:2) or BCP2 (HA:TCP, 9:1). Micro computed tomography (micro-CT) and histological analysis were performed at 2 and 8 weeks after implantation. Results: Micro-CT analysis, at 2 and 8 weeks, autogenous bone chip group had a significantly greater bone volume fraction (29.48±9.84 and 34.58±8.85) than other groups ($p<0.05$). BCP1 (9.90±0.75 and 20.70±2.76) and BCP2 (10.57±0.85 and 20.72±3.97) showed higher percent of bone volume than unfilled defect groups (4.23±2.08 and 8.40±5.37), but no statistically significant difference was found. The material volume fraction of BCP1 (28.64±2.31 and 25.02±3.5) and BCP2 (29.92±4.39 and 24.71±3.91) at both time frames were not significant different and also no difference of each material between the 2 time frames was found. Histological observation, autogenous bone group showed bridging defect with bone formed along the defect. The unfilled defect showed soft tissue healing defect. BCP1 and BCP2 showed more bone formation at 8 weeks than at 2 weeks. Conclusions: BCP at high ratios of HA showed osteoconductive properties and biocompatibility with the living tissue and could be considered for further clinical trial.

In vivo biocompatibility of porous BCP in two different ratios

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Abstract

Objectives: This study aimed to evaluate the effect of biphasic calcium phosphate with high HA/TCP ratios on bone formation in rabbit calvarial defects.

Methods: Sixteen New Zealand white rabbits were randomly divided into 2 groups. In each animal, bilateral circular defects (10-mm diameter) were created on the calvarium. Group I defects were grafted with autogenous bone chips or left empty. Group II defects were grafted with BCP1 (8:2) or BCP2 (9:1). Micro-CT and histological analysis were performed at 2 and 8 weeks after implantation.

Results: Micro-CT analysis, at 2 and 8 weeks, autogenous bone chip group had significantly ($p<0.05$) highest bone volume fraction (29.48±9.84 and 34.58±8.85). No statistically significant was observed for material volume fraction. Histological observation, autogenous bone group showed bridging defect while unfilled defects group showed connective tissue heal. BCP1 and BCP2 showed more bone formation at 8 weeks than at 2 weeks.

Conclusions: BCP at high ratios of HA showed osteoconductive properties and biocompatibility with the living tissue and could be considered for further clinical trial.

Keywords: *Biphasic calcium phosphate, Bone substitutes, Calvarium, Micro-CT, Rabbits*

Introduction

Bone grafting procedures are essential in the treatment of maxillofacial osseous defects.(1) Although autogenous bone has excellent osteogenic properties and elicits no immune response, it has several disadvantages and limitations regarding to patient morbidity, harvest quantity, and complications such as paresthesia and infection.(2) Therefore, researchers have been working to develop the material substitution for autogenous bone.

One of the most popular groups of bone substitutes is calcium phosphate bioceramic (CaP). The most commonly used CaPs are hydroxyapatite (HA), tricalcium phosphate (TCP) and biphasic calcium phosphate (BCP); a combination of the two.

HA exhibits strong osteoconductive property and excellent biocompatibility due to its chemical similarity to the mineralized phase of bone, but the disadvantages are weakness under tensile stress and very slow resorbability.(3)

TCP is generally recognized as resorbable bioceramics that gradually been replaced with bone tissue. However, faster rate of resorption than regeneration of newly formed bone was observed.(4)

BCP, the incorporation of slow resorbable HA and rapidly resorbable TCP, has advantage for new bone formation because of its controllable bioactivity.(5) Moreover, *in vivo* reactivity also depends on the ratio of HA/TCP.(6)

There are various HA/TCP ratios published in the literatures, which possess different physical and biological properties. These various ratios, from *in vitro* and *in vivo* studies, ranged from HA20/TCP80 to HA85/TCP15 and each ratio claimed its good properties.(7-11) Basically, BCP is usually used as an osteoconductive matrix that maintaining space for new bone formation which dissolution rate vary upon the dissolution of TCP. Higher HA contents lead to slow degradation rate, but ensure volume maintenance while TCP is gradually resorbed and replaced by newly formed bone.(12) BCP may be suitable for some clinical situation such as grafting material for alveolar bone preservation techniques or for maxillary sinus augmentation procedure.

This study aimed to assess *in vivo* bone regenerative potency of BCP in two different HA/TCP ratios (8:2 and 9:1) in calvarial defects of the rabbit.

Materials and Methods

Graft Materials

The porous BCP was prepared by the National Metal and Materials Technology Center of Thailand (MTEC). The BCP with HA/TCP ratios of 8:2 and 9:1 were selected from 5:5 to 9:1 ratio which had been tested for the best results of *in vitro* biocompatibility. There are 80% porosity, well-interconnected pore structure and a pore size of 100 to 300 µm in both ratios of BCP.

Animal Preparation

Sixteen male adult (10-12 months old) New Zealand white rabbits each weighing 3-4 kg were used in this study. The Experimental protocol was approved by the Animal Research Ethics Committee, Prince of Songkla University.

Surgical Procedure

Anesthesia was induced using ketamine 25 mg/kg and diazepam 5 mg/kg intramuscularly 30 min before surgery. Thiopental 5 mg/kg was administered intravenously and then titrated at the rate of 2 mg/kg every 15 min (with a maximum dose of 30 mg/kg) until unconsciousness was achieved.[13]

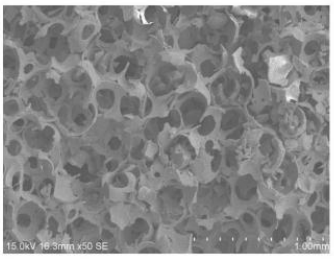

Figure 1- SEM photographs of BCP granules showing the pore structure, size100 - 300 µm, original magnification x 50

The surgical field was disinfected with povidone-iodine 10%. A mid-sagittal incision was made after local infiltration of 2% lidocaine hydrochloride with 1:100,000 epinephrine 1.8 ml. Subperiosteal dissection was carried out, and two identical bicortical bone defects diameter of 10 mm were carefully created using a trephine bur with saline irrigation.

Study Group	Detail	Number of rabbits		
		2 weeks	8 weeks	Total
Control	A : Autogenous bone chip B : Unfilled defects	3	3	6
Experimental	C : BCP1 (8:2) D : BCP2 (9:1)	5	5	10
Total				16

Table 1- Groups of study

The rabbits were randomly divided into 2 groups. (Table 1) In control groups (n=6), defects were filled with autogenous bone chips 0.15 gm by weight plus 15µl of 0.9% normal saline solution, that were minced with a bone morselizer (Salvin Dental Specialtie Inc, Charlotte, NC, USA), or left empty. In experiment groups (n=10), defects were randomly filled with porous BCP1 (8:2) or BCP2 (9:1) at 0.10 gm by weight plus 15µl of 0.9% normal saline solution. (Figure 2) The periosteum, muscle and skin were sutured using vicryl® 4/0.

The rabbits were sacrificed at 2 and 8 weeks after surgery with 1.2-1.3 ml overdose of 200 mg/ml pentobarbital sodium administered intravenously via the marginal ear vein. The calvarial bone was harvested using a small sharp fissure bur and then fixed in 10% formalin. Each specimen was trimmed and cut mid-coronally along the center of two circular defects into 2 halves; one half was used for micro-CT analysis, and the other half was used for histological analysis. (Figure 3)

Micro computed tomography (Micro-CT) analysis

A high resolution micro-CT system (Micro-CT80, Scanco, Medica AG, Basseersdorf, Switzerland) was used. After calibration the specimens were scanned perpendicularly to cranium vault at 55 kVp, 72 µA and 4W in high-resolution mode (18.5µm^3/voxel). Scanned data were reconstructed by built-in software.

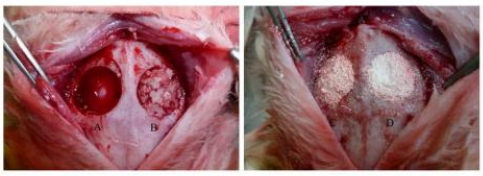

Figure 2- Bilateral calvarial defects filled with different materials. (A: Unfilled defect, B: Autogenous bone chip, C: BCP1 and D: BCP2)

Before analysis, the grayscale threshold values were determined to discriminate bone and ceramic from soft tissue.[14] The threshold value of "bone-ceramic" was specified. The lower threshold was selected by identifying the lowest threshold of bone voxels within the defects. The upper threshold referred to the highest threshold of ceramic voxels given that the total setup volume divided by total volume was greater than 0.95. The threshold value for the "ceramic" was determined by tracing 8 clearly identified BCP particles then the summation of the lowest threshold of each particle divided by the total volume greater than 0.95 was set as lower threshold of the ceramic threshold. The "bone" threshold was calculated by subtracting the ceramic threshold from the bone-ceramic threshold.

After determination of the threshold values, the margins were traced to specify ROI the defect. (Figure 4) The percent of bone volume fraction (BVF, BV/TV), percentage of radio-opaque voxels (as bone threshold range) divided by the total defect volume, and percent of material volume fraction (MVF, MV/TV), percentage of radio-opaque voxels (ceramic threshold range) divided by the total defect volume, were determined.

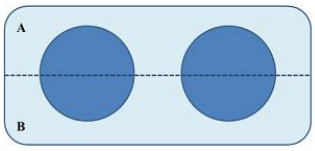

Figure 3- Specimen was cut into 2 pieces, A: For micro-CT analysis and B: For Histological analysis

Histological analysis

The specimens were processed to obtain thin ground sections, according to the technique of Donath and Breune[15] with minor modifications. Briefly, the specimens were dehydrated in an ascending series of alcohol rinses and embedded in a glycolmethacrylate resin (Technovit 7200 VLC, Kulzer, Wehrheim, Germany). After polymerization the specimens were sectioned along their longitudinal axis with a high-precision diamond disc at approximately 150µm and ground down to approximately 15-30µm with a specially designed grinding machine (EXAKT® cutting and grinding system, EXAKT® Apparatebau, Norderstedt, Hamburg, Germany).

Two sections contained the central portion were selected and stained with Goldner's Masson trichrome for light microscopic examination (Axiostar; Carl Zeiss, Göttingen, Germany).

Statistical Analysis

Statistical analysis was performed using statistical analysis software (SPSS ver15.0, SPSS Inc., Chicago, USA). Significant differences among groups were identified by one-way ANOVA with Scheffé's post-hoc test. Data was presented as means ± SD, and P-value less than 0.05 was considered statistically significant.

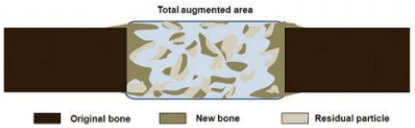

Figure 4- Schematic drawing shows the ROI for Micro-CT analysis.

Results

Animals

All rabbits were recovered and healed uneventfully during the study period.

Micro computed tomography analysis

The total volumes of newly formed bone within the ROI (bone volume fraction or BV/TV), as well as residual of grafting materials (material volume fraction or MV/TV), were summarized in Table 2.

Within the healing periods, only autogenous bone chip group had a significantly greater percent of bone volume fraction (29.48±9.84 and 34.58±8.85) than other groups ($p<0.05$). BCP1 (9.90±0.75 and 20.70±2.76) and BCP2 (10.57±0.85 and 20.72±3.97) showed higher percent of bone volume than unfilled defect groups (4.23±2.08 and 8.40±5.37), but no statistically significant difference was found. (Figure 5A) The percent of material volume fraction of BCP1 (28.64±2.31 and 25.02±3.5) and BCP2 (29.92±4.39 and 24.71±3.91) at both time frames were not significant different and also no difference of each material between the 2 time frames was found. (Figure 5B)

Timing	Group	BV/TV (%)	MV/TV (%)
2 weeks			
	Autogenous bone chip	29.48 ± 9.84*	
	Unfilled defect	4.23 ± 2.08	
	BCP1 (8:2)	9.90 ± 0.75	28.64 ± 2.31
	BCP2 (9:1)	10.57 ± 0.85	29.92 ± 4.39
8 weeks			
	Autogenous bone chip	34.58 ± 8.85*	
	Unfilled defect	8.40 ± 5.37	
	BCP1 (8:2)	20.70 ± 2.76	25.02 ± 3.51
	BCP2 (9:1)	20.72 ± 3.97	24.71 ± 3.91

Values are present as mean ± SD
BV/TV: Bone volume per total volume
MV/TV: Residual materials per total volume
* = Significant statistical difference from other groups at each time frame ($P< 0.05$)

Table 2- Micro-CT result after 2 and 8 weeks of healing

Histological analysis

Autogenous group; At 2 weeks, the defect was filled with autogenous bone chips. The connective tissue infiltrated from the periphery of the defect. (Figure 6A, 6E) The defects were completely bridged and gained good continuity at 8 weeks, and the newly formed bone was well incorporated with the grafts and the host bone. (Figure 7A, 7E)

Unfilled defects group; At 2 weeks, the contour of the defects were collapsed, and the defects were filled with connective tissue. (Figure 6B, 6F) There were some bony islands at 8 weeks but the defect were not bridged and still lost of continuity. (Figure 7B, 7F)

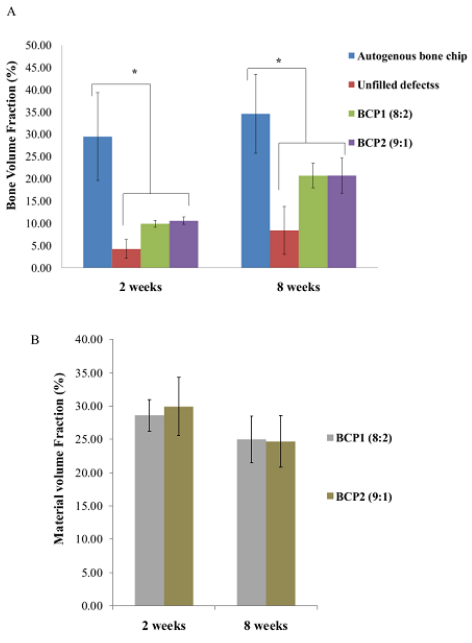

Figure 5- Micro-CT analysis, A: Bone volume fractions and B: Material volume fractions

For Experimental groups; Histological finding revealed indistinguishable between BCP1 and BCP2. At 2 weeks, dense BCP particles were found all over the defect. At 8 weeks, BCP particles were visually less than at 2 weeks; newly formed bone was projected from the defect edge, extended in a centripetal direction and corporated well with the BCP particles. However, little amount of bone was observed in the central part and the gap between materials was filled with loose connective tissue. It was also noted that grafting material preserved the good contour of the defects. (Figure 6C-D, 6G-H, 7C-D, 7G-H)

Discussion

A large number of bone grafting materials have been developed and generally used in dentistry for rehabilitation of maxillofacial and periodontal defects. In this study, the osteoconductive effect of newly developed biphasic calcium phosphate with high HA/TCP ratio was evaluated in rabbit calvarial defects.

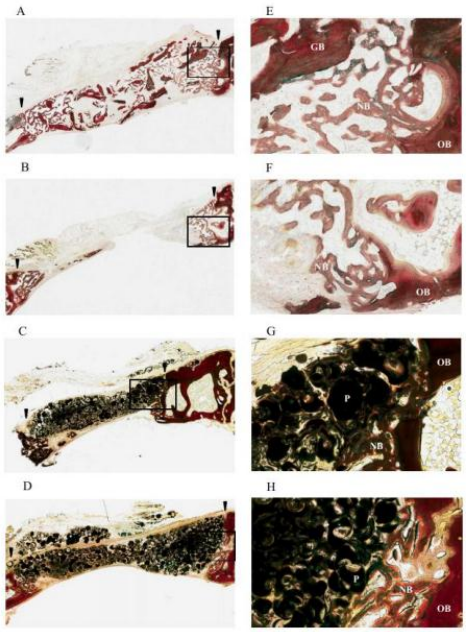

Figure 6- Histology examination at 2 weeks of healing periods: Autogenous bone chip (A, E), Unfilled defect (B, F), BCP1 (C, G) and BCP2 (D, H). Arrowhead = defect margin; GB = grafted bone; NB = newly formed bone; OB = original bone; P = BCP particle. Goldner's Masson trichrome, original magnification (A, B, C, D) and Goldner's Masson trichrome, original magnification x 5 (E, F, G, H)

Rabbit model has been used successfully in our previous studies concerning grafting materials.[13, 16, 17] The rabbit model has several advantages, such as standardization of experimental conditions and experiment repeatability, ease of handling and size, inexpensiveness, and rapid bone turnover rate.[18]

Although critical-size defect in adult rabbit has been defined as at least 15 mm in circular diameter[19, 20], some studies reported that 8-mm diameter defect is critical because it could not heal without the use of bone graft.[21] This present study used 10-mm diameter circular defect because it was possible to create two defects in one animal. Sohn et al. showed no differences in bone healing between the circular defect created by a trephine sized 11 mm and 15 mm in diameters at each study period of 2, 6, 8 and 12 weeks and there was also no difference of bone formation between 2 and 4 or between 8 and 12 weeks.[22] However, the actual diameter of the created defect in Sohn's study was 10 mm because the diameter of trephine was the outer diameter.

In rabbits, the bone metabolism is approximately three times faster than in humans.[23] Therefore, a healing period of 2 weeks was chosen for evaluating the early phase of the healing response, such as the stability of the materials or the host reactions. And a healing period of 8 weeks was selected since it is appropriate for evaluating the late phase of the healing response, such as bone incorporation, resorption of materials, bone remodeling, or the amount of bone regeneration.[22] From histological analysis, at 8 weeks the defect were not completely bridged in unfilled defects which confirmed the critical-size nature (Figure 5F).

Alloplastic bone substitute materials should secure the space necessary for bone in-growth. BCP1 and BCP2 groups showed larger augmented areas than the unfilled defect group which showed collapsed tissue profiles particularly at the central portion. These suggested that porous BCP has favorable space maintaining capacities at both 2 and 8 weeks. This result was consistence with the study from Lim et al that used BCP with HA/TCP ratio of 70/30 (Osteon^{TM}, Genoss. Co. Ltd., Suwon, Korea) and found effective space maintenance throughout 8 weeks of healing period.[24] If the space collapses, the blood clot cannot be stabilized and epithelial down growth cannot be prevented, consequently impairing new bone formation.[25]

Although micro-CT imaging data revealed that autogenous bone chip group had the highest bone regeneration than other groups significantly at both time frames ($p<0.05$), autogenous bone chip voxels and new mineralized bone voxels could not be discriminated while experimental groups represented only new bone formed voxels.

At 8 weeks of healing periods newly formed bone in BCP1 and BCP2 groups were 20.70±2.76 and 20.72±3.97 respectively. These results were slightly better than the previous study, used BCP with HA/TCP ratio of 60/40 (MBCP; Biomatlante, Nantes, France) filled in 8-mm circular defects which new bone formation was only 16.8±4.9.[26] BCP1 and BCP2 group also has higher bone regeneration than the unfilled defect groups however the difference was not statistically significant.

Moreover, from 2 to 8 weeks, increasing of BVF for BCP (10.8±2.00) and BCP2 (10.15±3.12) were higher than autogenous bone chip (5.10±0.99) and empty defects (4.17±3.29), proving the osteoconductivity of BCP. Furthermore, the newly formed bone was in close contact with the BCP1 and BCP2 particles, suggesting that these materials have osteoconductive properties.

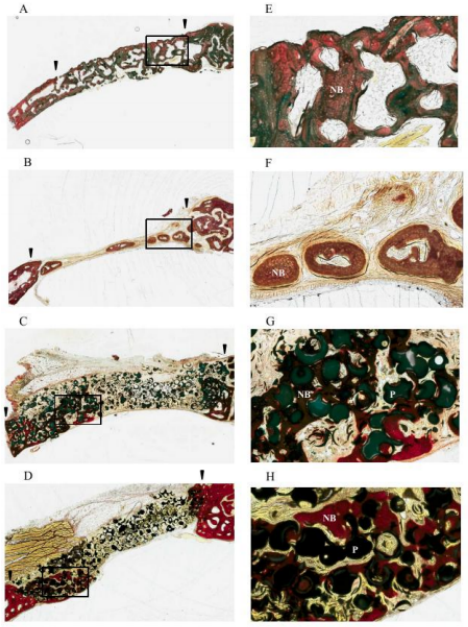

Figure 7- Histology examination at 8 weeks of healing periods: Autogenous bone chip (A, E), Unfilled defect (B, F), BCP1 (C, G) and BCP2 (D, H). Arrowhead = defect margin; GB = grafted bone; NB = newly formed bone; OB = original bone; P = BCP particle. Goldner's Masson trichrome, original magnification (A, B, C, D) and Goldner's Masson trichrome, original magnification x 5 (E, F, G, H)

Bone substitutes degradation was assessed by comparing the material content for the two time points. In our observation periods, particle size of BCP1 and BCP2 were reduced. However, when comparing the 2 and the 8 week, the slight decrease in graft material content was not significant which probably due to a less TCP ratio. Theoretically, the TCP part will dissolve into Ca^{2+} and PO_4^{3-}ions, whereas HA will retain its form and structure and will not be resorbed.[27]

To this day, two ratios of HA/TCP (60/40 and 70/30) have been commercial and used for clinical application such as grafting material for maxillary sinus augmentation[28] and treating periodontal infrabony defects.[29]

The results of this study showed favorable outcomes of BCP with high ratios of HA/TCP in promotion of bone healing of the defects, which could be an effective material in various clinical conditions that space maintaining is important and slow degradation is a requirement such as atrophic alveolar ridge, large osseous defects, cystic cavity and sinus floor augmentation.

Conclusion

In conclusion, BCP1 (8:2) and BCP2 (9:1) presented good osteoconductive properties, biocompatibility with the living tissue and excellent space maintaining capacity with slow biodegradation rates and enhanced bone formation in animal model. BCP with high ratio of HA should be considered for further clinical trial study.

Acknowledgments

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Titles:

In vivo biocompatibility of porous BCP in two different ratios

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Objectives:

This study aimed to evaluate the effect of biphasic calcium phosphate with high HA/TCP ratios on bone formation in rabbit calvarial defects.

Methods:

Sixteen New Zealand white rabbits were randomly divided into 2 groups. Bilateral circular defects (10-mm diameter) were created on the calvarium in each animal. Group I (3 rabbits per each time frame) defects were grafted with autogenous bone chips or left empty. Group II (5 rabbits per each time frame) defects were grafted with BCP1 (HA:TCP, 8:2) or BCP2 (HA:TCP, 9:1). Micro computed tomography (micro-CT) and histological analysis were performed at 2 and 8 weeks after implantation.

Results:

Micro-CT analysis, at 2 and 8 weeks, autogenous bone chip group had a significantly greater bone volume fraction (29.48±9.84 and 34.58±8.85) than other groups ($p<0.05$). BCP1 (9.90±0.75 and 20.70±2.76) and BCP2 (10.57±0.85 and 20.72±3.97) showed higher percent of bone volume than unfilled defect groups (4.23±2.08 and 8.40±5.37), but no statistically significant difference was found. The material volume fraction of BCP1 (28.64±2.31 and 25.02±3.5) and BCP2 (29.92±4.39 and 24.71±3.91) at both time frames were not significant different and also no difference of each material between the 2 time frames was found.

Histological observation, autogenous bone group showed bridging defect with bone formed along the defect. The unfilled defect showed soft tissue healing defect. BCP1 and BCP2 showed more bone formation at 8 weeks than at 2 weeks.

Conclusions:

BCP at high ratios of HA showed osteoconductive properties and biocompatibility with the living tissue and could be considered for further clinical trial.

Keyword: Biphasic calcium phosphate, Bone substitutes, Calvarium, Micro-CT, Rabbits

Acknowledgments

This study was supported by grants from the National Metal and Materials Technology Center, Thailand Science Park, Pathumthani, Thailand (MT-B-53-BMD-07-183-I.), and Graduate School, Prince of Songkla University, Hatyai, Songkhla, Thailand. The authors would like to thank Asst. Prof. Korngrid Changkaew, Siriraj Hospital, Mahidol University, for facilitating in the scanning of histology sections.

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In vivo biocompatibility of porous BCP in two different ratios

Pongsakorn Praserttham, Prisana Pripatnanont

Introduction

Bone grafting procedures are essential in the treatment of maxillofacial osseous defects.[1] One of the most popular groups of bone substitutes is biphasic calcium phosphate (BCP), the incorporation of slow resorbable HA and rapidly resorbable TCP. Basically, BCP is usually used as an osteoconductive matrix maintaining space for bone formation and its dissolution rate varies upon the dissolution of TCP. Higher HA contents lead to slow degradation rate, but ensure volume maintenance while TCP is gradually resorbed and replaced by newly formed bone.[2]

This study aimed to evaluate the effect of biphasic calcium phosphate with high HA/TCP (8:2 and 9:1) ratios on bone formation in rabbit calvarial defects.

Materials and Methods

The porous BCP with HA/TCP ratios of 8:2 and 9:1 were selected from 5:5 to 9:1 ratio which had been tested for the best results of *in vitro* biocompatibility. There are 80% porosity, well-interconnected pore structure and a pore size of 100 to 300 µm in both ratios of BCP.

The rabbits were randomly divided into 2 groups. After anesthetized and disinfected surgical field, two identical bicortical bone defects diameter of 10 mm were created on the rabbit calvarium using a trephine bur. In control groups (n=6), defects were filled with either minced autogenous bone chips 0.15 gm by weight plus 15µl of 0.9% normal saline solution or left empty. In experiment groups (n=10), defects were randomly filled with porous BCP1 (8:2) or BCP2 (9:1) at 0.10 gm by weight plus 15µl of 0.9% normal saline solution.

Figure 1- Bilateral calvarial defects filled with different materials. (A: Unfilled defect, B: Autogenous bone chip, C: BCP1 and D: BCP2)

The rabbits were sacrificed at 2 and 8 weeks after surgery. Each calvarial specimen was cut mid-coronally along the center of two circular defects into 2 halves. One half was used for micro-CT analysis to evaluate the percent of bone volume fraction (BVF, BV/TV) and material volume fraction (MVF, MV/TV).

The other half was for histological analysis. The specimens were processed according to undecalcified techniques and were stained with Goldner's Masson trichrome.

Study Group	Detail	Number of rabbits		
		2 weeks	8 weeks	Total
Control	A : Autogenous bone chip B : Unfilled defects	3	3	6
Experimental	C : BCP1 (8:2) D : BCP2 (9:1)	5	5	10
Total				16

Table 1- Groups of study

Statistical Analysis

Significant differences among groups were identified by One-way ANOVA with Scheffé's post-hoc test. Data was presented as means ± SD, and P-value less than 0.05 was considered statistically significant.

Results

All rabbits were recovered and healed uneventfully during the study period.

Micro-CT analysis, within the healing periods, only autogenous bone chip group had a significantly greater bone volume fraction, BVF, (29.48±9.84 and 34.58±8.85) than other groups ($p<0.05$). BCP1 (9.90±0.75 and 20.70±2.76) and BCP2 (10.57±0.85 and 20.72±3.97) showed higher percent of bone volume than unfilled defect groups (4.23±2.08 and 8.40±5.37), but no statistically significant difference was found. The material volume fraction of BCP1 (28.64±2.31 and 25.02±3.5) and BCP2 (29.92±4.39 and 24.71±3.91) at each time frames were not significant different. There was also no difference between the 2 time frames.

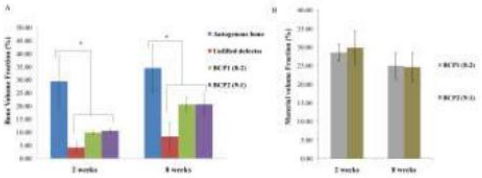

Figure 2- Micro-CT analysis, A: Bone volume fractions and B: Material volume fractions

Histological analysis, autogenous group; at 2 weeks, the defect was filled with autogenous bone chips. The defects were completely bridged and gained good continuity at 8 weeks, and the newly formed bone was well incorporated with the grafts. Unfilled defects group; at 2 weeks, the contour of the defects were collapsed, and the defects were filled with connective tissue. There were some bony islands at 8 weeks but the defect were not bridged and still lost of continuity.

In experimental groups; there was indistinguishable between BCP1 and BCP2. At 2 weeks, dense BCP particles were found all over the defect. At 8 weeks, BCP particles were visually less than at 2 weeks; newly formed bone was projected from the defect edge, extended in a centripetal direction and corporated well with the BCP particles. The contours of the defects were well preserved by the grafting material.

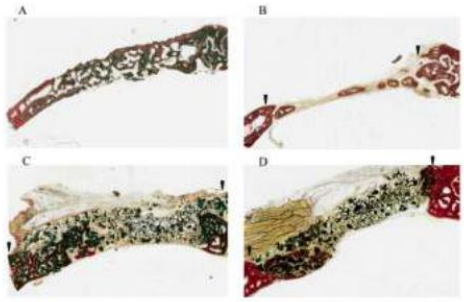

Figure 3- Histology examination at 8 weeks of healing periods: Autogenous bone chip (A), Unfilled defect (B), BCP1 (C) and BCP2 (D). Arrowhead = defect margin. Goldner's Masson trichrome, original magnification.

Discussion

In this study, the osteoconductive effect of newly developed biphasic calcium phosphate with high HA/TCP ratio was evaluated in rabbit calvarial defects.

Ideal bone substitute materials should secure the space necessary for bone in-growth. BCP1 and BCP2 groups showed normal and better contour of augmented areas than the unfilled defect group. These suggested that porous BCP has favorable space maintaining capacities throughout the healing periods.

Micro-CT imaging data revealed that autogenous bone chip group had the highest bone content than other groups significantly at both time frames ($p<0.05$), since the autogenous group included bone chips together with newly formed bone while experimental groups represented only new bone formed.

From 2 to 8 weeks, increasing of BVF for BCP (10.8±2.00) and BCP2 (10.15±3.12) were higher than autogenous bone chip (5.10±0.99) and empty defects (4.17±3.29), proving the osteoconductivity of BCP.

The results of this study showed favorable outcomes of BCP with high ratios of HA/TCP in promotion of bone healing. BCP could be effective in various conditions that space maintaining and slow degradation are required such as atrophic alveolar ridge, large osseous defects, cystic cavity and sinus floor augmentation.

Conclusion

In conclusion, BCP1 (8:2) and BCP2 (9:1) presented good osteoconductive properties, biocompatibility with the living tissue and excellent space maintaining capacity with slow biodegradation rates in animal model. BCP with high ratio of HA should be considered for further clinical trial study.

References

[1] Block MS, Jackson WC. Techniques for grafting the extraction site in preparation for dental implant placement. Atlas Oral Maxillofac Surg Clin North Am. 2006 Mar;14(1):1-25.

[2] LeGeros RZ, Lin S, Rohanizadeh R, Mijares D, LeGeros JP. Biphasic calcium phosphate bioceramics: preparation, properties and applications. J Mater Sci Mater Med. 2003 Mar;14(3):201-9.

In vivo biocompatibility of porous BCP in two different ratios

20

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Keyword: Biphasic calcium phosphate, Bone substitutes, Calvarium, Micro-CT, Rabbits

Abstract

Objectives: This study aimed to evaluate the effect of biphasic calcium phosphate with high HA/TCP ratios on bone formation in rabbit calvarial defects.

Methods: Sixteen New Zealand white rabbits were randomly divided into 2 groups. In each animal, bilateral circular defects (10-mm diameter) were created on the calvarium. Group I defects were grafted with autogenous bone chips or left empty. Group II defects were grafted with BCP1 (8:2) or BCP2 (9:1). Micro-CT and histological analysis were performed at 2 and 8 weeks after implantation.

Results: Micro-CT analysis, at 2 and 8 weeks, autogenous bone chip group had significantly ($p<0.05$) highest bone volume fraction (29.48±9.84 and 34.58±8.85). No statistically significant was observed for material volume fraction. Histological observation, autogenous bone group showed bridging defect while unfilled defects group showed connective tissue heal. BCP1 and BCP2 showed more bone formation at 8 weeks than at 2 weeks.

Conclusions: BCP at high ratios of HA showed osteoconductive properties and biocompatibility with the living tissue and could be considered for further clinical trial.

Introduction

Bone grafting procedures are essential in the treatment of maxillofacial osseous defects.[1] One of the most popular groups of bone substitutes is biphasic calcium phosphate (BCP), the incorporation of slow resorbable HA and rapidly resorbable TCP. Basically, BCP is usually used as an osteoconductive matrix that maintaining space for new bone formation and its dissolution rate varies upon the dissolution of TCP. Higher HA contents lead to slow degradation rate, but ensure volume maintenance while TCP is gradually resorbed and replaced by newly formed bone.[2]

This study aimed to evaluate the effect of biphasic calcium phosphate with high HA/TCP (8:2 and 9:1) ratios on bone formation in rabbit calvarial defects.

Materials and Methods

Materials

The porous BCP with HA/TCP ratios of 8:2 and 9:1 were selected from 5:5 to 9:1 ratios which had been tested for the best results of *in vitro* biocompatibility. There are 80% porosity, well-interconnected pore structure and a pore size of 100 to 300 µm in both ratios of BCP.

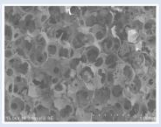


Fig.1- SEM of BCP granules. original magnification x 50

Experimental Design

The rabbits were randomly divided into 2 groups. After anesthetized and disinfected surgical field, two identical bicortical bone defects diameter of 10 mm were carefully created on the rabbit calvarium using a trephine bur with saline irrigation.

Study Group	Detail	Number of rabbits		
		2 weeks	8 weeks	Total
Control	A : Autogenous bone chip B : Unfilled defects	3	3	6
Experimental	C : BCP1 (8:2) D : BCP2 (9:1)	5	5	10
Total				16

Table.1- Groups of study

In control groups (n=6), defects were filled with autogenous bone chips 0.15 gm by weight plus 15µl of 0.9% normal saline solution, that were minced with a bone morselizer or left empty. In experiment groups (n=10), defects were randomly filled with porous BCP1 (8:2) or BCP2 (9:1) at 0.10 gm by weight plus 15µl of 0.9% normal saline solution.

Fig.2- Bilateral calvarial defects filled with different materials. (A: Unfilled defect, B: Autogenous bone chip, C: BCP1 and D: BCP2)

The rabbits were sacrificed at 2 and 8 weeks after surgery. Each calvarial specimen was trimmed and cut mid-coronally along the center of two circular defects into 2 halves. One half was used for micro-CT analysis to evaluate the percent of bone volume fraction (BVF, BV/TV) and material volume fraction (MVF, MV/TV). The other half was for histological analysis. The specimens were processed according to undecalcified techniques and two sections contained the central portion were selected and stained with Goldner's Masson trichrome.

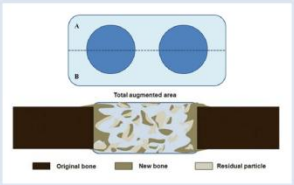


Fig.3- Specimen was cut into 2 pieces, A: For micro-CT analysis and B: For Histological analysis

Fig.4- Schematic drawing shows the ROI for Micro-CT analysis.

Statistical Analysis

Significant differences among groups were identified by One-way ANOVA with Scheffé's post-hoc test. Data was presented as means ± SD, and P-value less than 0.05 was considered statistically significant

Results

Animal

All rabbits were recovered and healed uneventfully during the study period.

Micro-CT analysis

Within the healing periods, only autogenous bone chip group had a significantly greater bone volume fraction, BVF, (29.48±9.84 and 34.58±8.85) than other groups ($p<0.05$). BCP1 (9.90±0.75 and 20.70±2.76) and BCP2 (10.57±0.85 and 20.72±3.97) showed higher percent of bone volume than unfilled defect groups (4.23±2.08 and 8.40±5.37), but no statistically significant difference was found. The material volume fraction of BCP1 (28.64±2.31 and 25.02±3.5) and BCP2 (29.92±4.39 and 24.71±3.91) at each time frames were not significant different. There was also no difference between the 2 time frames.

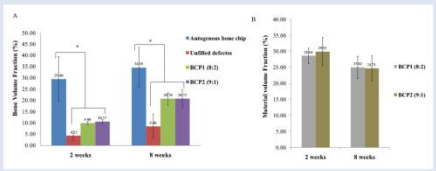
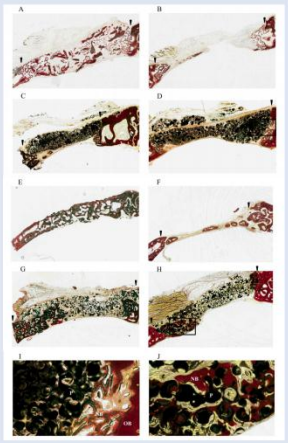


Fig.5 - Micro-CT analysis, A: Bone volume fractions and B: Material volume fractions

Histological analysis

Autogenous group; at 2 weeks, the defect was filled with autogenous bone chips. The connective tissue infiltrated from the periphery of the defect. The defects were completely bridged and gained good continuity at 8 weeks, and the newly formed bone was well incorporated with the grafts.



Unfilled defects group; at 2 weeks, the contour of the defects were collapsed, and the defects were filled with connective tissue. There were some bony islands at 8 weeks but the defect were not bridged and still lost of continuity.

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Fig.6- Histology examination at 2 weeks (A, B, C, D, I) and 8 weeks (E, F, G, H, J) of healing periods; Autogenous bone chip (A, E), Unfilled defect (B, F), BCP1 (C, G) and BCP2 (D, H, I, J). Arrowhead = defect margin; GB = grafted bone; NB = newly formed bone; OB = original bone; P = BCP particle. Goldner's Masson trichrome, original magnification (A-H) and Goldner's Masson trichrome, original magnification x 5 (I, J)

Discussions

A large number of bone grafting materials have been developed for rehabilitation of maxillofacial and periodontal defects. In this study, the osteoconductive effect of newly developed biphasic calcium phosphate with high HA/TCP ratio was evaluated in rabbit calvarial defects.

Ideal bone substitute materials should secure the space necessary for bone in-growth. BCP1 and BCP2 groups showed normal and better contour of augmented areas but the unfilled defect group showed collapsed tissue profiles. These suggested that porous BCP has favorable space maintaining capacities throughout the healing periods.

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References

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Ref. 01/54

หนังสือรับรอง

โครงการวิจัย เรื่อง **การศึกษาคุณสมบัติทางชีวภาพของวัสดุพรุนไบฟาสิกแคลเซียมฟอสเฟตในสัตว์ทดลอง**

หัวหน้าโครงการ **รศ.ทพญ.ปริศนา ปริพัฒนานนท์ คณะทันตแพทยศาสตร์**

ได้ผ่านการพิจารณาและเห็นชอบจาก คณะกรรมการจรรยาบรรณการใช้สัตว์ทดลอง มหาวิทยาลัยสงขลานครินทร์

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January 13, 2011

This is to certify that the research project entitled "In vitro biocompatibility of biphasic calcium phosphate" which was conducted by Assoc.Prof.Dr.Prisana Pripatananont, Faculty of Dentistry, Prince of Songkla University, has been approved by The Animal Ethic Committee, Prince of Songkla University.

K. Say

Kitja Sawangjaroen, Ph.D.
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Degree	Name of Institution	Year of Graduation
Doctor of Dental Surgery	Mahidol University	2007

List of Publication and Proceedings

Praserttham P, Pripatnanont P, Suttapreyasri S, Leepong N, Monmaturapoj N. In vivo biocompatibility of porous BCP in two difference ratios. ***The 11th Dental Faculty Consortium of Thailand Academic Meeting and Research Presentation (DFCT2013)*** May; 118-123.