Effect of Bone Marrow Concentrate on New Bone Formation in Rabbit Calvarial Defect

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ชื่อวิทยานิพนธ์ ผลของไขกระดูกเข้มข้นต่อการสร้างกระดูกใหม่ในรอยวิการกะโหลกศีรษะกระต่าย

ผู้เขียน นางสาวกนกพร สันตะวาลิ้ม

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บทคัดย่อ

วัตถุประสงค์ เพื่อศึกษาผลของไขกระดูกเข้มข้นต่อการสร้างกระดูกใหม่ในรอยวิการกะโหลกศีรษะกระต่าย

วัสดุและอุปกรณ์ ทำการศึกษาโดยสร้างรอยวิการกะโหลกศีรษะในสัตว์ทดลองจำนวน 15 ตัว ตัวละ 2 รอย แบ่งกลุ่มการศึกษาออกเป็น 4 กลุ่มตามชนิดของวัสดุทดแทนกระดูกที่ใช้ในการรักษารอยวิการ ได้แก่ กลุ่มที่ 1 ใช้กระดูกของกระต่ายเองเป็นวัสดุทดแทนกระดูก (AB), กลุ่มที่ 2 ใช้สารทดแทนกระดูกสังเคราะห์ชนิดเบต้าไตรแคลเซียมฟอสเฟต (β-TCP), กลุ่มที่ 3 ใช้สารทดแทนกระดูกสังเคราะห์ชนิดเบต้าไตรแคลเซียมฟอสเฟตร่วมกับไขกระดูกเข้มข้นของกระต่าย (β-TCP+BMAC), และกลุ่มที่ 4 รอยวิการที่ไม่ใส่สารทดแทนกระดูกเลย ทำการเปรียบเทียบผลการศึกษาจากลักษณะทางคลินิก ภาพถ่ายรังสีไมโครซีที และภาพถ่ายจุลพยาธิวิทยาที่ระยะเวลา 2, 4 และ 8 สัปดาห์ภายหลังการผ่าตัด

ผลการศึกษา วัสดุทดแทนกระดูกที่ใช้ในทุกกลุ่มศึกษามีความเข้ากันได้กับเนื้อเยื่อร่างกายเป็นอย่างดี ผลการศึกษาจากลักษณะทางคลินิกพบว่าในทุกกลุ่มศึกษามีการหายของแผลที่ดี ไม่พบปฏิกิริยาการอักเสบหรือติดเชื้อของแผล จากผลการศึกษาภาพถ่ายรังสีไมโครซีทีและภาพถ่ายจุลพยาธิวิทยา พบว่าในกลุ่ม AB มีการสร้างกระดูกใหม่มากที่สุด และมากกว่ากลุ่มศึกษาอื่นอย่างมีนัยสำคัญทางสถิติ กลุ่ม β-TCP+BMAC มีการสร้างกระดูกมากกว่ากลุ่มที่ใช้ β-TCP เพียงอย่างเดียว แต่ไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ แต่ปริมาณการสร้างกระดูกใหม่ในทั้งสองกลุ่มนี้มีปริมาณมากกว่ารอยวิการที่ไม่ใส่สารทดแทนกระดูกเลย นอกจากนี้ยังพบว่าปริมาณของวัสดุทดแทนกระดูกชนิดเบต้าไตรแคลเซียมฟอสเฟตมีปริมาณลดลดเมื่อเวลาผ่านไปมากขึ้น ในขณะเดียวกันพบว่ามีปริมาณกระดูกใหม่เพิ่มขึ้นตามระยะเวลาที่ผ่านไป

สรุปผลการศึกษา การใช้ไขกระดูกเข้มข้นร่วมกับสารทดแทนกระดูกชนิดเบต้าไตรแคลเซียมฟอสเฟต เป็นอีกหนึ่งทางเลือกของสารทดแทนกระดูกในการรักษารอยวิการของกะโหลกศีรษะกระต่าย

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Abstract

Objective: The aim of the present study was to compare new bone regeneration in rabbit calvarial defects treated by autogenous bone, beta-tricalcium phosphate alone (β-TCP), in combination of beta-tricalcium phosphate and bone marrow aspirate concentrate (β-TCP+BMAC), and empty defect.

Materials and methods: Two critical size defects were created on the calvarium of 15 male white New Zealand rabbits. The clinical evaluation and Micro-computed tomography (micro-CT) was used for evaluation of new bone regeneration at 2, 4 and 8 weeks after surgery respectively.

Results: The experimental models showed good clinical biocompatibility to all groups of the materials. The highest bone formation was achieved with autogenous bone treatment, and it was higher significantly than other groups. Bone formation in β-TCP and β-TCP+BMAC groups were not significantly different, but higher than empty defect. The quantity of residual graft materials were reduced gradually and new bone formation were increase with time in both groups.

Conclusion: This study showed that beta-tricalcium phosphate added with bone marrow aspirate concentrate could be one of the alternative material of choice for new bone formation in rabbit calvarial defect.

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Kanokporn Santavalimp

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Chapter 1

Introduction

Bony defects which are caused by accident, pathology, infection or congenital abnormally results in loss of functions and esthetics and need for reconstruction. Autogenous bone grafting is served as gold standard for bone grafting and reconstruction procedures because of its ideal bone regenerative properties; osteogenesis, osteoinduction, and osteoconduction. Donor sites for autogenous bone grafting were harvested from various sites include calvarium, mandible, rib, tibia and ilium[1]. Most commonly donor site for alveolar bone grafting is the iliac crests[2-4].

However, autogenous bone grafting procedures still have its adverse effect espeacially at the donor sites[1]. Donor site complications in autogenous bone graft harvesting procedures can lead to paresthesia, hypersensitivity, and infection[4-7]. In order to avoid these disadvantage of donor site complication, tissue engineering strategies may be used[1, 7].

Nowadays, bone marrow-derived mesenchymal stem cells (BMSCs) are one of human adult stem cells which commonly used as cellular component in research about tissue engineering procedures in humans [8-10].

In oral and maxillofacial surgery, there are many studies, both *in vitro* and *in vivo*, which report effectiveness of tissue engineering by using BMSCs in bone grafting procedures[2, 7].

Beta-tricalcium phosphate (β-TCP) is one of synthetic biomaterials that have been use for many years. β-TCP shows great osteoconductive potential which leads to good bone ingrowth. Many studies report effectiveness of β-TCP as grafting materials for bone repair, augmentation and substitution[11]. For example, Zhang D. et al, reported that β-TCP combined with osteogenically induced bone marrow stromal cells could be used as bone substitution material in alveolar cleft defect in a canine models in beagle dogs, and allow for subsequent orthodontic tooth movement[12].

There are experimental evidences report the action of BMSCs from concentrated bone marrow aspiration serve as “readily available” cell source, which is widely used in orthopaedics.

According to cell-based tissue engineering concept, using of BMSCs with β-TCP scaffolds may enhance new bone regeneration in rabbit calvarial defects and can be used as an alternative for bone grafting to autogenous bone graft.

Literature Review

Autogenous bone grafting

Autogenous cancellous bone is ideal and serve as gold standard for bone grafting procedures because of its living cells supply osteogenic property without inducing immunological response [2, 6, 11, 13].

The main disadvantage of the use of the autogenous bone grafts is complication at donor sites, such as, postoperative pain, limits function which leads to a prolonged stay in hospital and waste a lot of money[1,2,6,14,15]. Moreover, autogenous bone harvesting procedures can result in paresthesia, hypersensitivity, and infection. However, tissue engineering principle might be used to solve these problems[7].

Tissue engineering in bone grafting

Tissue engineering principle is scientific approach that combined biologic and engineering principles consist of three basic components; cells, scaffolds and signaling molecule. Aim of these principles is to construct or regeneration a new tissue for replace and restore function of diseased or damaged tissues and integrally to surrounding host tissue[3].

There are several studies about using tissue engineering strategies for oral and maxillofacial bone reconstruction [7], for example,

- Using platelet-rich plasma (PRP) combined with autogenous bone graft in alveolar cleft defect
- The use of barrier membranes and fibrin glue in bone grafting procedures
- Combination of autogenous bone graft with calcium phosphate scaffolds as grafting materials.
- Using signaling molecules, such as, bone morphogenetic protein-2 (BMP-2)

- Combination of mesenchymal stem cells (MSCs) and grafting materials for better osteogenesis property of grafting materials [7].

Table 1 Overview of studies about tissue engineering in bone grafting in alveolar cleft defect.

Note: ReCT = non- randomized retrospectively controlled trial

RCT = randomized controlled trial

PCT = non-randomized prospectively controlled trial

ICBG = iliac crest bone graft

PRP = platelet rich plasma

BMP-2 = Bone morphogenic protein-2

MSC = Mesenchymal stem cell

Author [reference]	Study type	Case/ Control	Control group	Study group	Conclusion
Duskova[16]	ReCT	36/9	ICBG	Two type of collagen membrane: Bio-Gide or Hypo-Sorb membranes combined with ICBG	Bone formation and healing does not statistically significant between 2 type of materials and suggest to use in severe cases due to high cost of treatment
Lee[17]	ReCT	30/30	ICBG	PRP & ICBG	No significant difference in bone resorption rate between groups.
Luaces-Rey[18]	ReCT	10/10	ICBG	PRP & ICBG	No significant difference in bone formation between groups.
Oyama[19]	ReCT	7/5	ICBG	PRP & ICBG	Higher bone volume in the PRP group.
Clavijo-Alvarez[20]	ReCT	15/20	ICBG	Using acellular dermal matrix for lining at oral or nasal mucosa and ICBG for bone substitute material	No significant difference in bone formation and does not improve mucosal healing

Table 1 (Continued)

Author [reference]	Study Type	Case/ Control	Control group	Study group	Conclusion
Peled[21]	RCT	10/5	ICBG	- Resorbable or non-resorbable membranes with ICBG	Guided bone regeneration by using resorbable membrane with ICBG showed a significantly greater fill of the defect in radiological data.
Segura-Castillo[22]	RCT	13/14	ICBG	Fibrin glue & ICBG	Adding fibrin glue to autologous bone graft can reduce bone resorption in alveolar cleft defect.
Weijs[23]	ReCT	18/29	Chin bone	β-TCP & chin bone	No significant difference in alveolar bone height in occlusal radiographs and can be used as substitute material in large defect.
Thuaksuban [24]	RCT	14/13	ICBG	Bovine hydroxyapatite & ICBG	No significant difference in bone density detected by densitometer and bone height in occlusal radiographs.
Alonso[25]	RCT	8/8	ICBG	Collagen sponge with BMP-2	No significant difference in bone volume between 2 groups. Significantly less bone height but less postoperative pain in study group.
Herford[26)]	ReCT	10/2	ICBG	Collagen sponge with BMP-2	No significant difference in bone volume and can be used as alternative choice of grafting material in alveolar cleft defect.
Dickinson[27]	RCT	9/12	ICBG	Collagen sponge with BMP-2	Significantly higher bone volume and less postoperative pain in study group.
Gimbel[15]	PCT	21/48	ICBG	Collagen sponge loaded with MSC	Less postoperative pain in study group. Data on bone formation were not published
Benlidayi[28]	ReCT	11/12	ICBG	Bovine hydroxyapatite	No significant difference in bone height and density in long term study

Cell-based tissue engineering

Nowadays application of mesenchymal stem cells are the main roles in tissue engineering and regenerative medicine because of their mutipotent capacity which is potential to differentiate into multiple cell lines (such as osteogenic, chondrogenic, adipogenic and myogenic lines.

The study of Mark Pittenger in 1999 showed that cells that were isolated from bone marrow aspirates can subsequently differentiated into three cell lines; osteogenic, adipogenic and chondrogenic[29]. Originally, the bone marrow is only one source of this cell population that contain multiple differentiation capacities. Later, many tissues can serve as these cell reservoir, such as, adipose tissue and muscle tissue.

In recent study, the concept of "stem cell niche" which is the perivascular space was explained. This principle determine that MSCs are localized in all place at vessels and perivascular space. From this principle, application for using MSCs can be indicated into three modes :

1. Culture expansion: cells selection from bone marrow, subsequent culture to increase number of cells in the laboratory, then transplant at the defect site;

2. Bone marrow concentration: bone marrow aspiration, then process for increase concentration and directly implanted into the defect site;

3. Using growth factors for systemic mobilization of stem cells and bone marrow precursors[30].

For example, Friedenstein. et al demonstrated the efficacy of the implantation of a culture expansion of undifferentiated precursor cells from the bone marrow in new bone formation[31, 32].

A recent clinical study has demonstrated new bone formation in critical bone defects in animal models by using porous hydroxyapatite (HA) ceramic scaffolds seeded with isolated and expanded BMSCs in culture.

Behnia H. et al was report a case using tissue-engineered bone in alveolar cleft defect, a combination of biphasic hydroxyapatite/tricalcium phosphate (HA/TCP) seeded with human derived mesenchymal stem cells and platelet growth factors. The results of the operation was uneventful and good bone incorporate with 51.3% fill of the bone defect at 3 months post-operation[34].

However, the culture expansion methods for using BMSCs are complex and costly procedures that limited to large research centers[29].

Another method for tissue engineering by using BMSCs as a cellular component is bone marrow aspirate concentration and directly implanted into the defect site[20].

Bone marrow aspirate concentrate (BMAC) is recently use in orthopaedics, both at the preclinical experimental level, and clinically. BMAC serves as the "readily available" cell source and directly placed or injected into the injury or defect sites or systemic administration. The BMAC represents a cell source with minimal manipulation and easy to use in clinical treatment and being categorized as "instant cell therapy"[30].

There are many studies using BMAC for tissue engineering. For example, systemic injection of autologous BMAC through the tail vain of mouse can improve callus formation at the defect site[35].

Vaz et al demonstrated that direct injection of the centrifuged osteogenic bone marrow aspirate into the defect can improve the healing of fibular osteotomies in rabbits models [36].

Dallari D. et al performed a study of bone regeneration in trabecular bone by creating critical size defects in the distal part of femurs of adult rabbits. They found that a combination of freeze-dried bone allografts, platelet-rich plasma, and autologous bone marrow stromal cells prepared by isopyknic centrifugation of bone marrow aspirate permitted an acceleration in bone healing and bone remodeling process[37.]

In orthopaedics, local injection of concentrated bone marrow aspirate from the iliac crest into necrotic lesion for the treatment of early-stage idiopathic osteonecrosis can reduce in pain and joint symptoms. This application of BMAC obtain promising results in long term follow up by reduce the incidence of fractural stage in early stages osteonecrosis of the femoral head, delay disease progression, and relieve symptoms[38].

Nowsadays, local injection of BMAC into fracture site of long bone is currently use for the treatment of long bone fractures in numerous centres. This method can improve bone heaing by accelerating callus formation and osteosynthesis. A retrospective study in 43 cases of open tibial fractures showed that local injection of BMAC is an alternative choice for management of long-bone delayed and nonunion which can reduce cost of treatment, promote good bone healing and low complication rate[39].

Not only in orthopaedics studies, in oral and maxillofacial surgery, there are also have many studies using BMAC with various bone substitute materials.

The study of Sununliganon L. et al, report that BMAC prepared by a single cycle of centrifugation is source of nucleated and progenitor cells. BMAC-derived MSCs still retain their multi-differentiation property *in vitro*, and can improve bone formation in maxillary sinus lift grafting when immediate implantation in rabbits[40].

A prospective randomized clinical trial in human show that BMAC seeded on BioOsss particles as grafting material in maxillary sinus lift procedure can induce new bone formation, results in sufficient bone volume for dental implant placement[41].

Previous study report various methods for reduce postoperative pain at donor site of alveolar cleft repair procedure, for example, autogenous bone harvest by minimally invasive technique by trocar with smaller access at donor site. But the best technique to reduce donor site morbidity in school-aged alveolar cleft repair was tissue engineering method by using BMAC from iliac crest seeded on resorbable collagen as a graft material[15].

From review literature, the application of BMSCs at the site of injury divided into two methods: (i) direct injection of cell suspension or (ii) by using scaffolds seeded with candidate cells through laboratory culture or direct absorption.

Despite the effectiveness of using culture expanded of MSCs, it still has limitation to use, because it is bound to the laboratory process of in vitro expansion, necessary to increase a number of cells to sufficient number and populate into the scaffold. Moreover, MSCs culture media usually contain foetal calf serum, which increase the risk of contamination or immunological reaction towards xenogeneic compounds[42]. This procedure certainly leads on to a greater manipulation of cells and limited into small number of research centers[30]. So the methods which using BMAC or bone marrow aspirate concentrate serves as "instant cell therapy" with scaffolds might be an alternative way for applying in clinical treatment.

Scaffolds

Currently, there are many types of materials use as scaffolds for tissue engineering include hydroxyapatite, collagen matrices and synthetic organic polymers[43]. However, there are some disadvantages with these materials in alveolar bone tissue engineering. Most synthetic degradable polymers, such as poly-lactic acid and poly-glycolic acid, are based on an ester backbone. These materials degrade when water is added to the ester linkage of the polymer backbone and their degradation products are acidic. When the degradation of this scaffold was occur, acidity of the host

tissue is increased and leads to increasing of inflammatory response, and further premature degradation of the grafting material[44, 45].

Beta-Tricalcium phosphates (β-TCPs) are bone-substitute materials that are marked out by their high biocompatibility, favourable resorption properties and osteoconductivity[46]. The application of β-TCP as a clinical scaffold in repairing bone defects was widespread in recent years. Many studies have reported the efficacy of β-TCP scaffolds which is good and proper for the attachment, proliferation, and differentiation of osteogenic cells, such as, osteoblasts and BMSCs[47].

Previously studies in animal models have shown that using β-TCP alone as grafting material results in severe resorption, but when adding with BMSCs via culture expanded method can promoted new bone formation and carry out favorable bone height in alveolar cleft defect. The overall effect of this grafting material consisting of β-TCP and BMSCs was equivalent to autogenous bone without obstruction the adjacent teeth to move into the newly formed bone in the grafted site[12, 47].

According to concepts of cell-based tissue engineering, using the advantage of bone marrow aspirate concentrate serves as "instant cell therapy" combination with β-TCP scaffolds which have high biocompatibility, favorable resorption properties and osteoconductivity, using of BMSCs with β-TCP scaffolds may enhance new bone regeneration in rabbit calvarial defects and can be used as an alternative option for bone grafting to autogenous bone graft.

The research problem

Can a BMSCs be prepared in chairside by simple centrifugation technique and use as instant cell therapy for enhance the new bone formation?

The purpose of the study

1. To prepared BMSCs from BMAC by simple centrifugation technique.
2. To investigate the effect of BMAC on new bone formation in rabbit cavalrial defects.

The objectives of the study

The aim of this study was to assess the effectiveness of bone marrow aspirate concentrate, obtained by means of simple centrifugation of bone marrow aspirates, by compare new

bone formation in rabbit calvarial defects treated by autogenous bone (AB), β-TCP alone (β-TCP), in combination of β-TCP and BMAC (β-TCP+BMAC), and empty defect.

Research question

Does using BMAC can enhance new bone formation in rabbit calvarial defect?

Hypothesis

The use of β-TCP added with BMAC could enhance new bone formation in rabbit calvarial defects.

Chapter 2

Materials and Methods

Research design

Randomized control trial

Experimental models

15 male New Zealand white rabbits aged between 5-7 months and weighting 3-4.5 kg were used in this study. All animals were housing and feeding according to standard animal care protocols of the Faculty of science, Prince of Songkla University. This study was submitted and approved by the animal experiment ethic committees of Prince of Songkla University.

Fig 1. New Zealand white rabbit

Group of study

The animals were divided into 4 groups according to types of grafting materials using in calvarial defects.

Group 1: Autogenous bone graft (AB)

Group 2: Beta-tricalcium phosphate added with bone marrow aspirate concentrate (β-TCP & BMAC)

Group 3: Beta-tricalcium phosphate alone (β-TCP)

Group 4: Empty defect

The animals were sacrificed with an overdose (1.2-1.3 ml) of pentobarbital sodium 200 mg/ml administered intravenously at two, four, and eight weeks after surgery.

Sampling technique

The samples were randomly divided by simple random allocation into four groups according to type of graft materials and were sacrifice in different three time points. The groups of study are shown in Table 2.

Table 2 Summary of the study groups

Sacifice period	**Type of grafting materials (number of defects)**			
	AB	**β-TCP & BMAC**	**β-TCP**	**Empty**
2 wks	3	3	3	1
4 wks	3	3	3	1
8 wks	3	3	3	1

Total - 30 defects (15 rabbits)

- 5 rabbits per each time point

Surgical procedures

All surgical procedures were performed under aseptic conditions by the same surgical team.

Anesthetic technique

All animals were placed on the surgical table in a prone position. Anesthesia was induced by 25 mg. kg Ketamine Hydrochloride and 5 mg/ kg diazepam intramuscularly into the gluteal region. Three minutes later, an intravenous catheter was placed into the marginal ear vein for intravenous anesthesia. 5 mg/ kg Thiopental was administered intravenously and then titrate at the rate of 2 mg/ kg every 15 minutes (maximum dose not more than 30 mg/ kg) until achieving consciousness. At the beginning of surgery, Penicillin G solution (50,000-100,000 U/Kg) was given as antibiotic prophylaxis for infection.

Preparation of the bone marrow aspirate concentrated (BMAC)

After adequate asepsis and antisepsis, bone marrow aspirate was prepared and process in sterile condition at 30 minutes before the beginning of surgical procedure. Start with bone marrow punch via 16G trocar with 16G needle into posterior iliac crest with gentle rotation movement until reaching into medullary cavity, then the trocar was withdraw. After that a 10 ml disposable plastic syringe soaked with a solution of heparin at 1:1000 was connected to 16G needle and 5 ml bone marrow was aspirated by firm traction of syringe plunger into plastic syringe. The 5 ml of bone marrow was transferred to a sterile graduated tube through 19G needle, then with a 22G needle, to disperse the cellular aggregation. This sample was then processed by centrifugation at 400xg at room temperature, for 10 minutes. After centrifugation, the material was separate into three layers; the supernant of plasma at the superior layer, the BMAC which perform as buffy-coat at intermediate layer, and the sedimented cells with higher density at the bottom. Then, the supernatant of the sample approximately 2 ml, was discarded with a sterile Pasteur pipette. 0.5 ml of buffy-coat was collected with Pasteur pipette which expected to be numerous number of hematopoietic precursors, mesenchymal and progenitor cells of the bone marrow stroma[36, 48]. A sample of 50 microliters from buffy coat was separated for in vitro study.

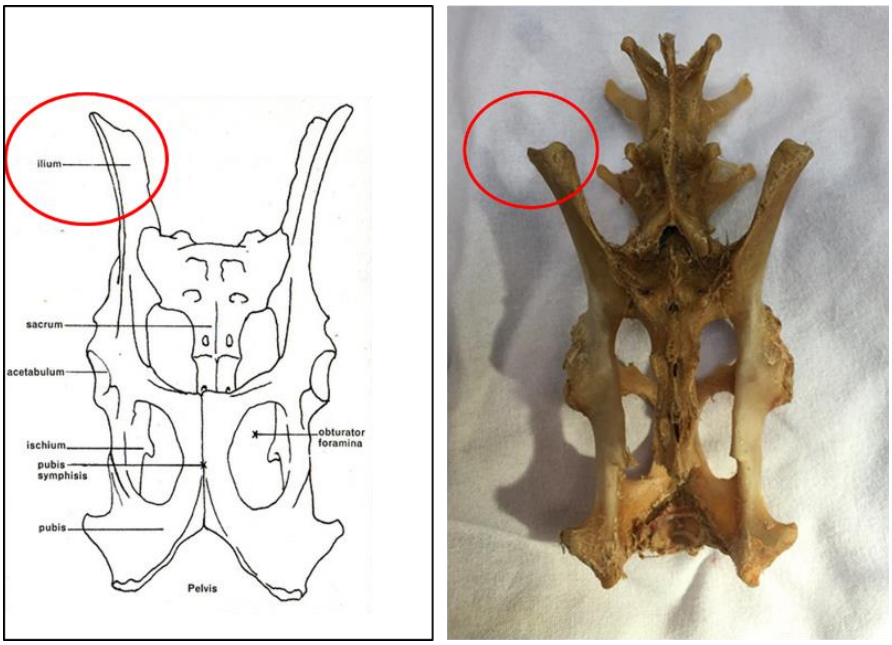

Fig 2. Schematic presentation of iliac crest in rabbit

Fig 3. Bone marrow was aspirate from iliac crest (A&B). After centrifugation at 400 g, room temperature for 10 minutes, it was separated into 3 layers (C). BMAC was be collected from the buffy-coat, which is form by the intermediate layer between discarded plasma and the sedimented cells at the bottom of the tube.

In vitro study

Following BMAC preparation, a sample of 50 microliters was culture in laboratory with culture medium consisting of Dullbecco's Modified Eagle Medium (DMEM, Gibco,USA) supplement with 10% fetal bovine serum (FBS, Gibco, USA) and antibiotics (penicillin/ streptomycin). After 3 days, non-adherent cells were discarded from culture plate by PBS washing and fresh medium changed. With medium change every 3 days, adherent cells cluster grew into colony-forming fibroblast-like cells in 10-14 days and were sub-cultured until the third passage, then the cells were used for testing MSCs properties .

Colony - forming unit fibroblast (CFU-F) assays for test self-renewal capacity was done by seeding hundred cells on to each well in six well plates, culture for about ten days. Then, stained with 0.1% toluidine blue and counted the colony that aggregates more than 50 cells or colony more than 2 mm in diameter.

Osteogenic deifferentiation potential was test by seeding 20,000 cells on to each well in six well plates, cultured with osteogenic medium consisting of α-MEM with 10% FBS, 100nmol/L dexamethasone, 50 μg/ml ascorbic acid, and 10 mmol/L β-glycerophosphate) and changed every 3 days. After 14 days, alizarin red was used to stain the calcified mass.

Operative phase

The implantation procedure in the rabbits were performed under general anesthesia as previously described. The animals was shaved and disinfected with 10% Povidone-Iodine at surgical field; over the cranium area between both ears of the rabbit. A mid- sagittal incision of 2-3 cm was made after local infiltration of 2% lidocaine hydrochloride with 1:100,000 epinephrine 1.8 ml at the skin area of the surgical field. Subperiosteal dissection was carried out and 2 bi-cortical defects 10 mm in diameter were created in the right and left parietal bone with a small round and fissures burs using micrometer according to wire template to ensure the same defect size in each animal. During surgery, 0.9% NaCl solution was use for coolant to avoid bone necrosis and rinsed out to remove abraded particles. A 1 mm deep circular mark was made with a small round bur at the defect edges and filled with preheated gutta-percha for later identification on the histological sections. The grafting material (AB, β-TCP&BMAC, β-TCP, or empty defect) was be randomly implied in created, and then the skin flap was be closed with 4/0 absorbable sutures. All rabbits were received antibiotic prophylaxis with 0.1 mL/kg cephalexine for 3 days. At the time points of 2, 4 and 8 weeks after the operation, the surgical wounds were clinically assessed. Healing and any

complications of the surgical sites were descriptively recorded. After that, the rabbits were sacrificed with an overdose of intravenous pentobarbital sodium. Each calvarium was removed in one piece. The gross specimens were examined before fixing in 10% formalin. After 48 hours, it was be cut along the mid sagittal suture into 2 pieces of specimens using a cutting-gridding machine (Exakt, Germany). Micro-computed tomography (μ-CT) analysis was be conducted in all specimens before processing them for histological analysis.

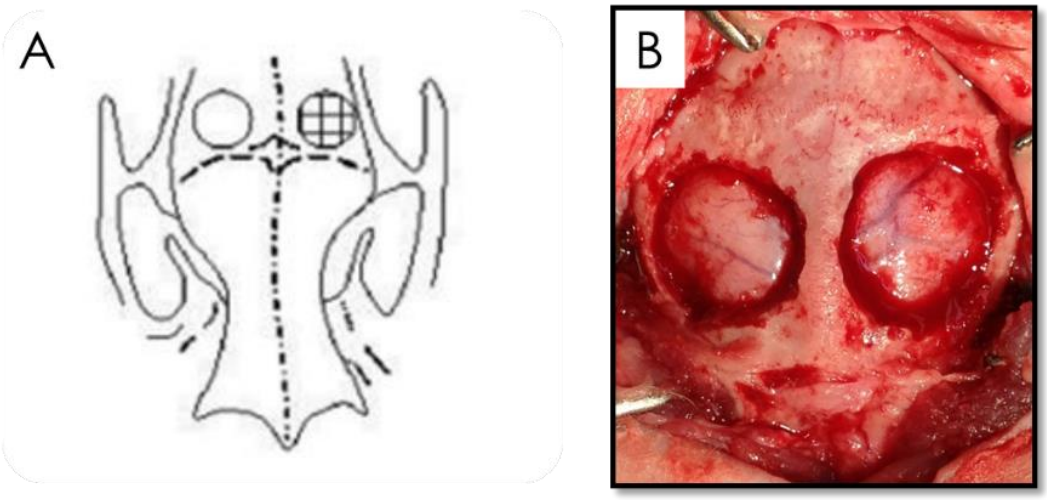

Fig 4. Schematic presentation of the critical size defects in rabbit calvaria (A). Two round defects 10 mm. imn diametor were created in the parietal bone on both sides of the calvarium (B).

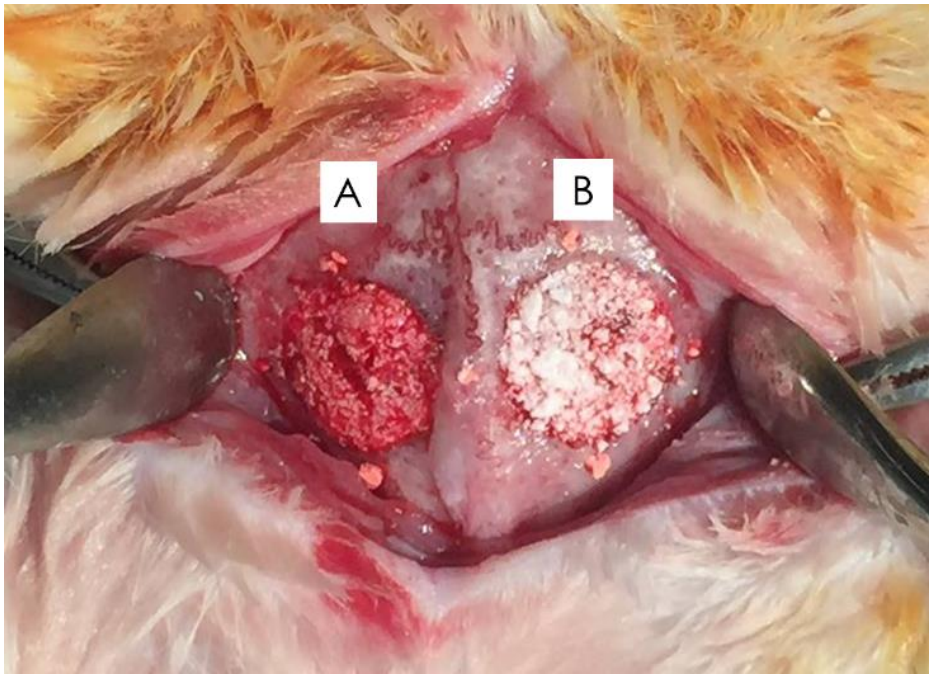

Fig 5. Grafting material (AB, β-TCP&BMAC, β-TCP, or empty defect) was be randomly implied in calvarial defects. This figure presents 2 type of grafting material which are β-TCP&BMAC (A) and β-TCP alone (B).

Analysis

***μ*-CT analysis**

The specimens of each time point was be scanned using a *μ*-CT (*μ*CT 35, SCANCO Medical AG, Switzerland) in a direction parallel to the coronal aspect of the calvarium with a setting of 55 kVp, 72 μA and 4 W. The measuring parameter is the new bone volume fraction (VF) calculated by analysis software (*μ*CT 35 Version 4.1, SCANCO Medical AG, Switzerland) using the following formula.

$$\text{New bone VF (\%)} = \frac{[\text{New bone volume}]}{\text{Defect volume}} \times 100$$

Fig 6. Micro-CT 35, Scanco, Medica AG

Histological analysis

Following μ-CT analysis, **t**he specimens were decalcified by soaked in formic acid and subsequently changed every day for three weeks. After complete decalcification, the specimens were cut into 2 pieces, each piece containing either type of grafting material (AB, β-TCP&BMAC, β-TCP, or empty defect), then devided into 2 pieces at the center before dehydrating in graded series of alcohol with an automatic tissue processor (Leica TP 1020) and embedded in paraffin blocks. Each specimen was sectioned along a sagittal plane to the bone surface by using a diamond saw microtome in 5-µm-thickness of three serial sections of each specimen. Haemotoxylin and Eosin (H&E) was used for staining histological section and then descriptively analyzed by using a light microscope to evaluate new bone formation.

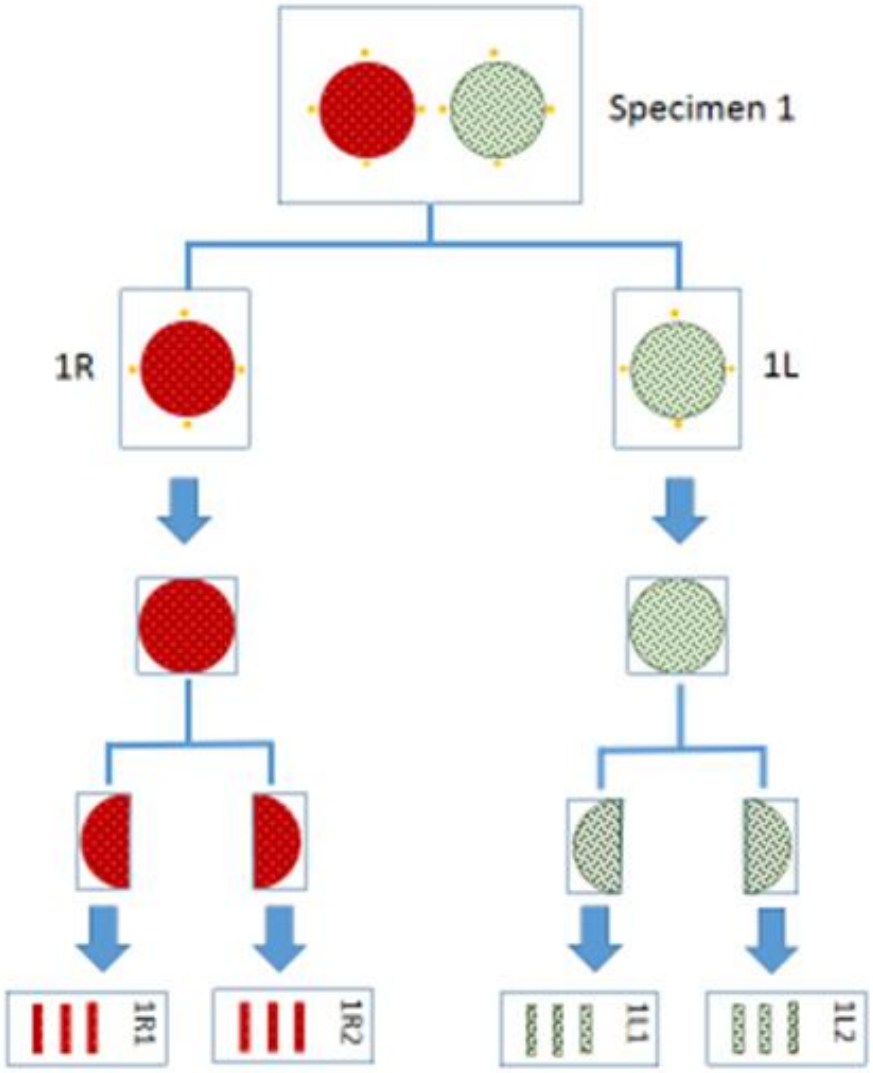

Fig 7. Slide- labeled diagram

Statistical analysis

The data was analyzed using the statistical analysis software (SPSS version 15.0, USA). A p-value < 0.05 was considered significant. The microscopic features of the scaffolds and the surrounding tissue was assessed descriptively. One-way Analysis of Variance (ANOVA) followed by Tukey HSD was applied to compare the differences of the measuring parameters among the healing intervals within each group. The paired *t*-test was applied to compare the differences of those parameters between the two groups for each time point.

Chapter 3

Results

Stemness and osteogenic capacity

After BMAC preparation and culture expanded in culture medium into third passage, the cultured cells can be perform self-renewal capacity and osteogenic deifferentiation potential.

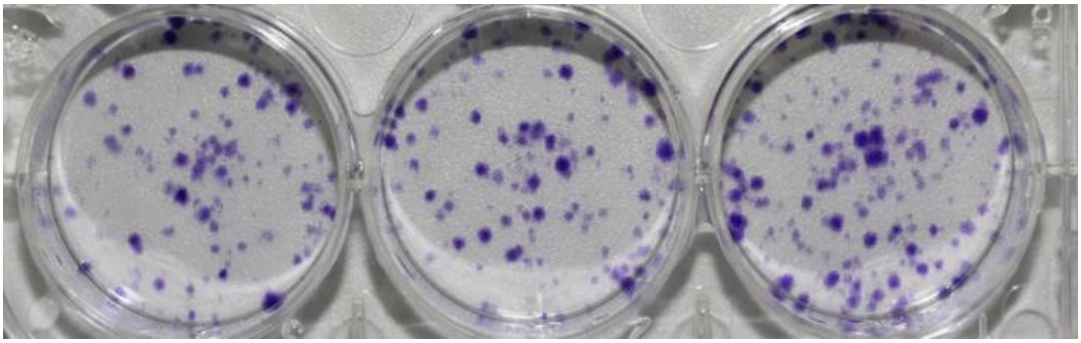

Fig 8. Colony - forming unit fibroblast (CFU-F) assays for test self-renewal capacity by seeding hundred cells on each well in six well plates, culture for about ten days. Then, stained with 0.1% toluidine blue and counted the colony that aggregates more than or colony more than 2 mm in diameter.

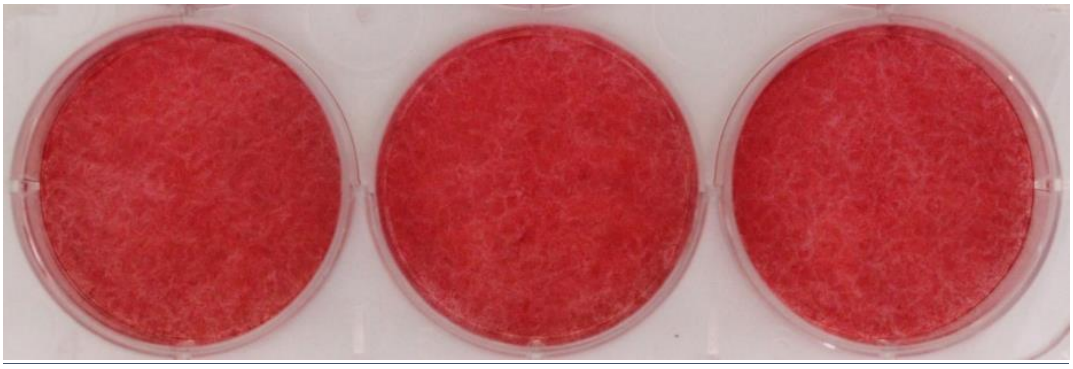

Fig 9. After cultured in osteogenic medium for 14 days, calcified mass was stained with alizarin red.

Clinical evaluation of the animals

All rabbits tolerated the operation well and had an uneventful recovery. Histopathologic features of graft-versus-host disease or immune rejection were not observed in any experimental groups.

Gross specimens

The gross specimens showed that healing of the implanted sites integrated well to the surrounding host bone. The surgical defects were covered with dense fibrous tissue without signs of foreign body reaction in all groups. In empty group revealed loss of contour, rubbery in consistency and fibrous like colour at defect site. In all group that treated with grafting material (AB, β- TCP&BMAC or β- TCP alone) showed normal contour, hard in consistency and not compressible. In groups that used β-TCP&BMAC or β-TCP revealed that margin of defects were easily identified by white colour of β-TCP and cannot detect any different in this both groups at every time point. In contrast with AB group, autogenous bone particles were observed surface and texture of regenerated tissues similary to native bone.

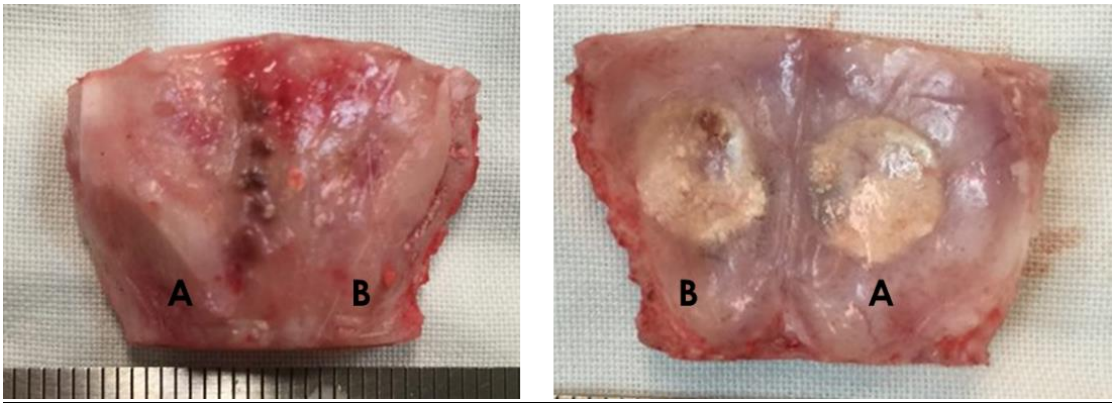

Fig 10 Gross photographs of rabbit calvarium at 4 weeks after implanted with β-TCP&BMAC (A) and β-TCP (B)

Micro-CT evaluation

To quantify the newly formed bone, the percentage of bone volume was calculated from micro-CT data. Figure 11 shows the percentage of new bone volume fraction. New bone volume fraction was highest in AB group, at 2 weeks after surgery new bone volume fraction was 41±5.97%, and then it rose to 50.95±3.44% at 4 weeks, and was reduced to 36.97±3.45% at 8 weeks. In β-TCP+BMAC group, new bone volume fraction was not different in any time point and less than AB group (at 2 weeks: 35.95±4.32%, at 4 weeks: 36.81±9.17%, at 8 weeks: 34.02±4.55%). In β-TCP group, new bone volume fraction was less than β-TCP+BMAC group but not statistically significant (at 2 weeks: 35.2389±2.41%, at 4 weeks: 31.26±5.33%, at 8 weeks: 29.76±3.46%). New bone volume fraction was lowest in empty group and statistically significant with other group in every time point. It was 4.81% at 2 weeks after surgery, and then raised to 13.81% at 4 weeks, then it reach to 15.15% at 8 weeks.

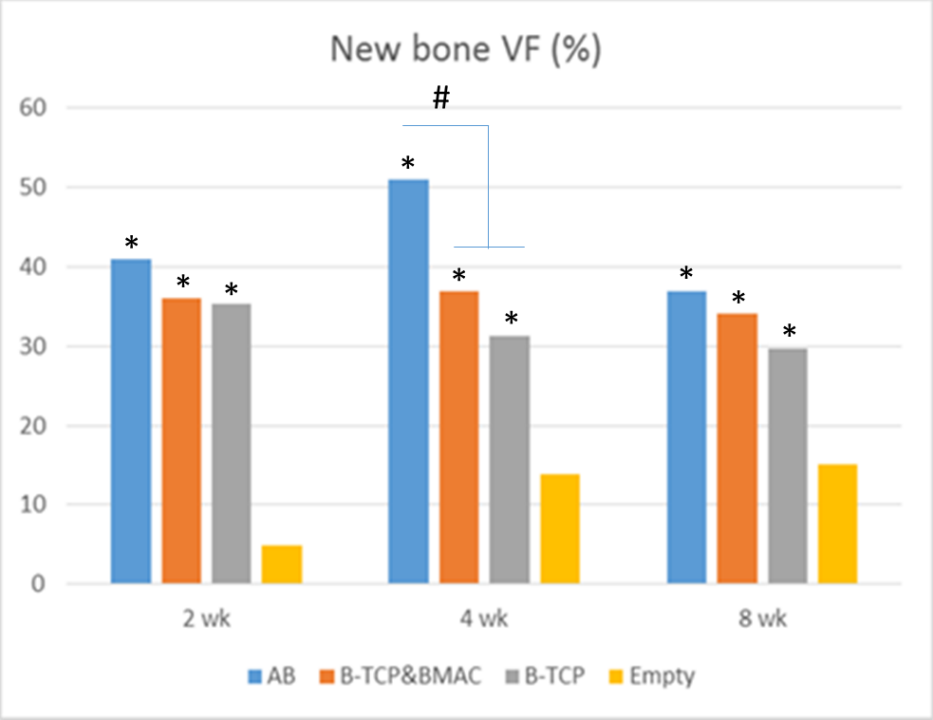

Fig 11 Relationship between new bone volume fractions in different time point.

* Statistically significant different when compared with empty group in each time point

\# Statistically significant when compared among group in same time point

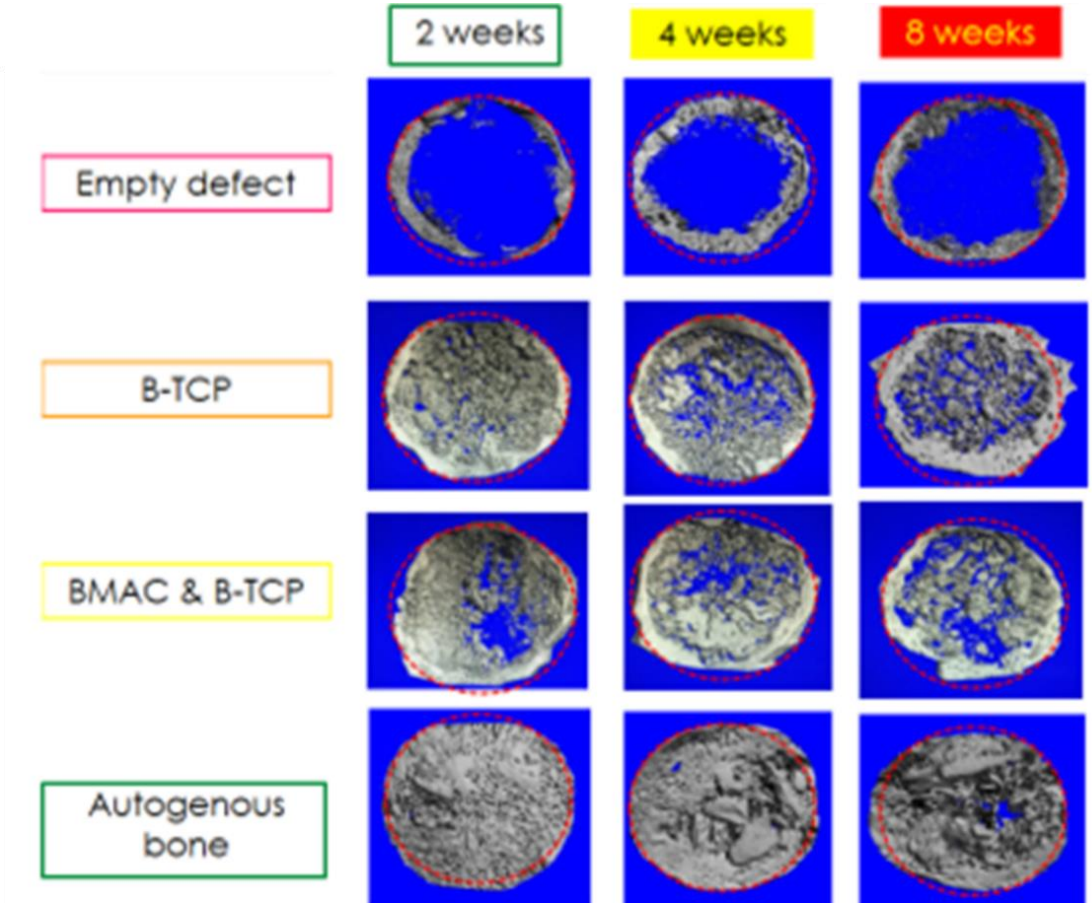

Fig 12. The 3-D reconstruction of the defects using μ-CT over the observation periods in axial cut.

Histologic evaluatiom

Histologic view of bone generated 2, 4 and 8 weeks after implantation, in the central area of bone defects. Two weeks after surgery, immature bone was observed in the fibrous connective tissue in AB group, and weak new bone formation occurred over the outer surfaces of β-TCP granules in central areas in β-TCP&BMAC groups, which was not found in β-TCP groups. After 4 weeks and 8 weeks, mature bone was further developed in AB and β-TCP&BMAC groups. In empty groups, only connective tissue was observed in central area at every time points.

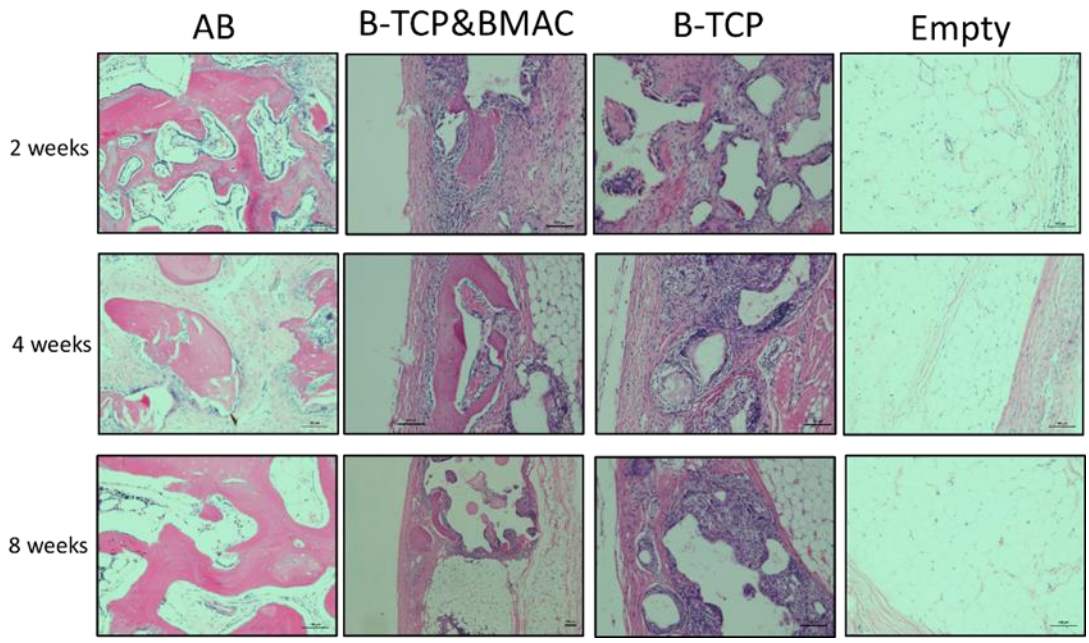

Fig 13. Histologic view of the bone generated at 2, 4 and 8 weeks after implantation, in the central area of bone defects. H&E stain. Scale bar = 100 μm.

Empty groups

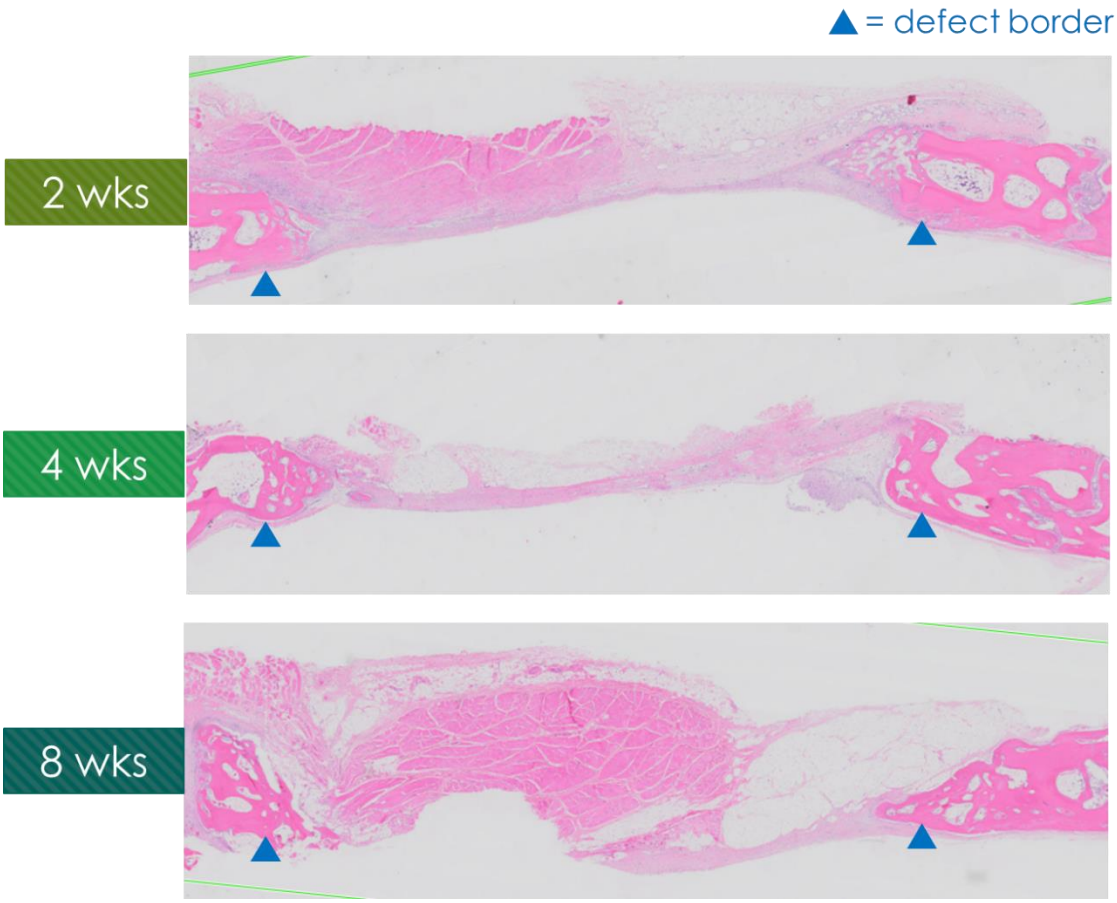

Fig 14. Histologic specimen of empty group showed little bone formation just only from margin of defects and only connective tissue was observed in central area at every time points.

AB groups

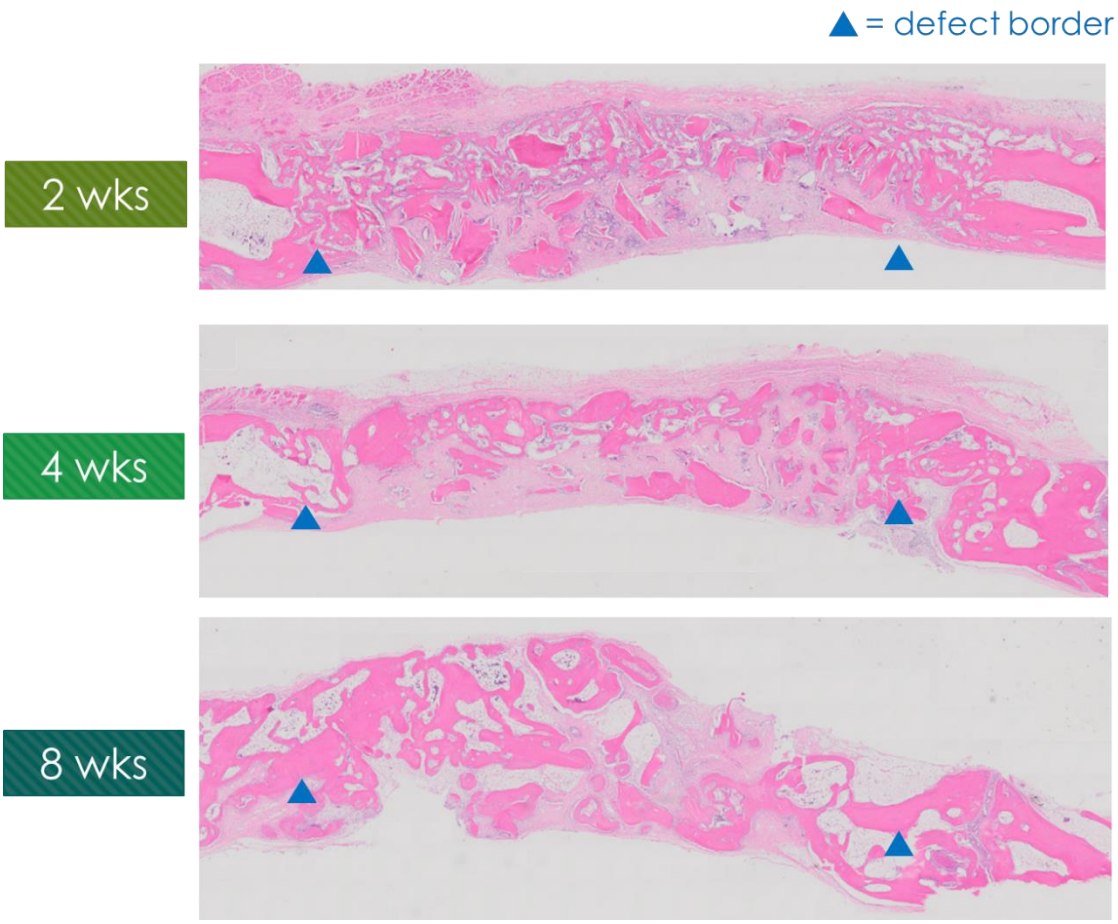

Fig 15 Histologic from AB group showed bone filled almost whole area and more maturation when time past.

β-TCP groups

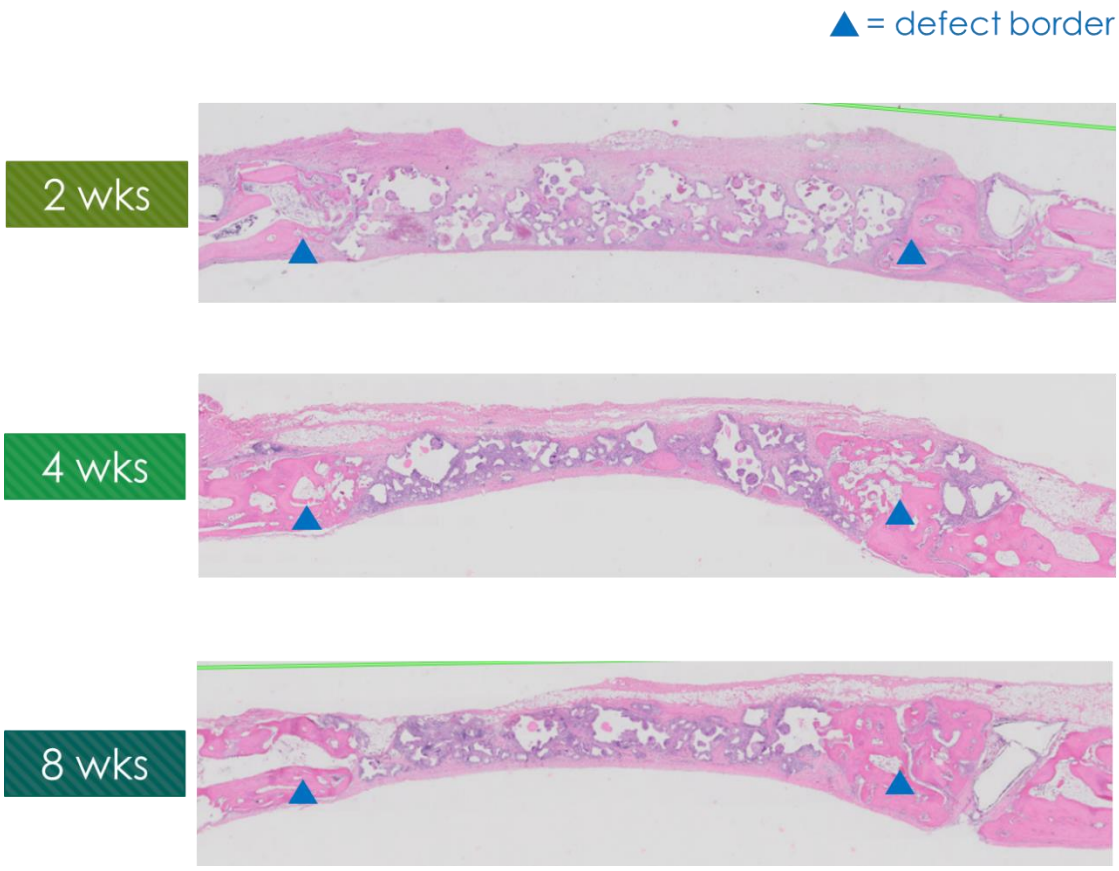

Fig 16. Histologic specimen from β-TCP groups showed new bone formation from margin of defects higher than empty groups.

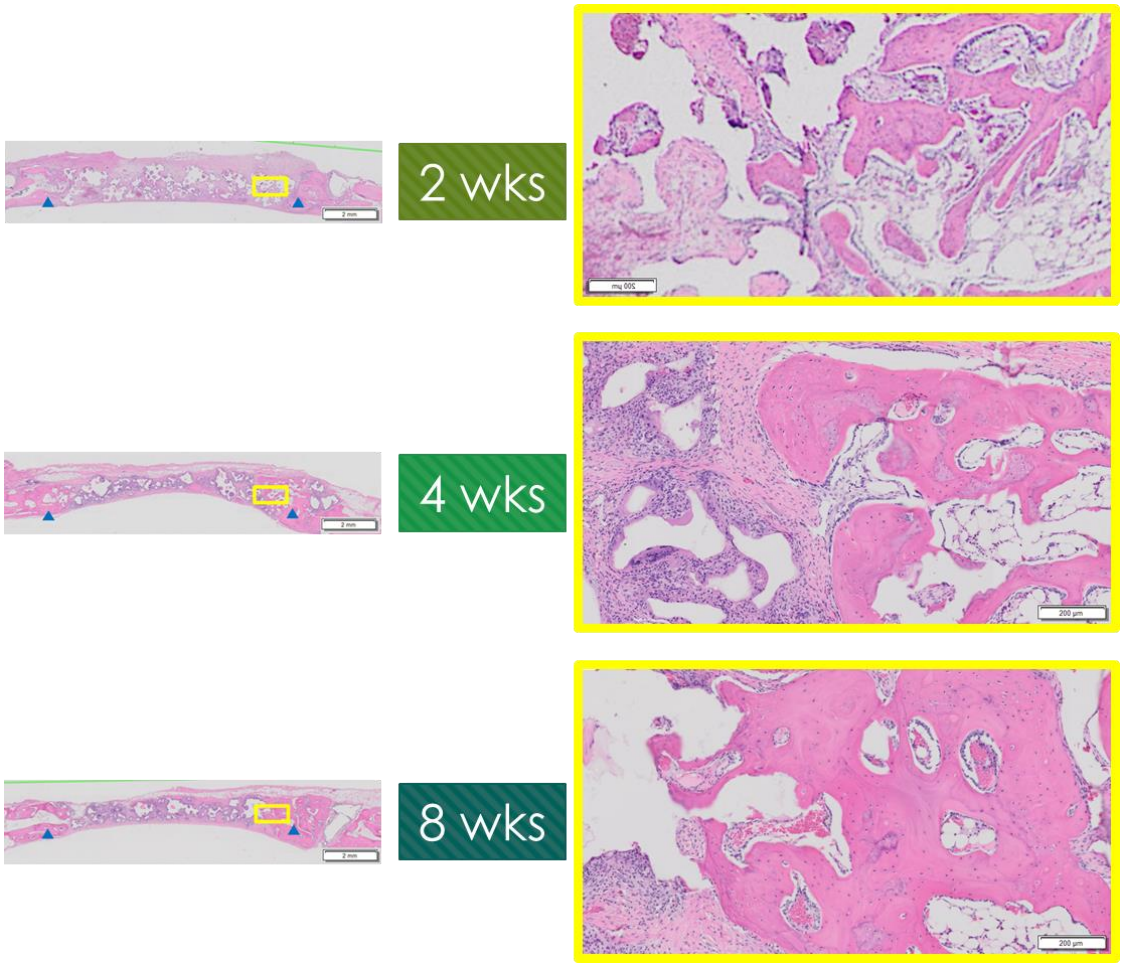

Fig 17. The picture amplified from margin of defects 0f histologic section of β- TCP group showed particles of scaffolds. At 2 weeks bone spicule was found at margin of defects. Woven bone was shown at 4 weeks. At 8 weeks, higher bone quantity and maturity bone formation was presented.

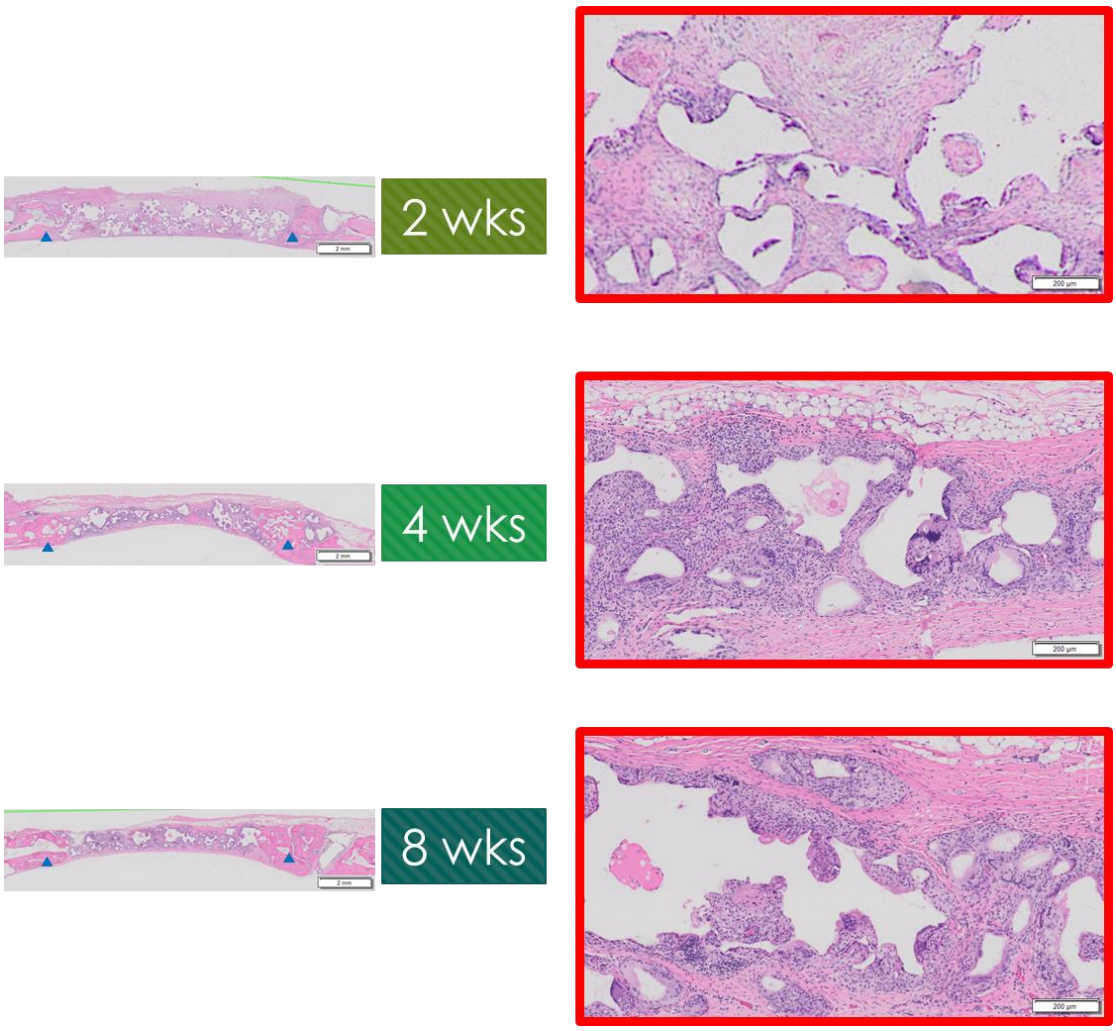

Fig 18. The picture amplified from center of defects 0f histologic section of β- TCP group showed particles of scaffolds, fibrous connective tissue, inflammatory cells and no bone formation.

β-TCP& BMAC groups

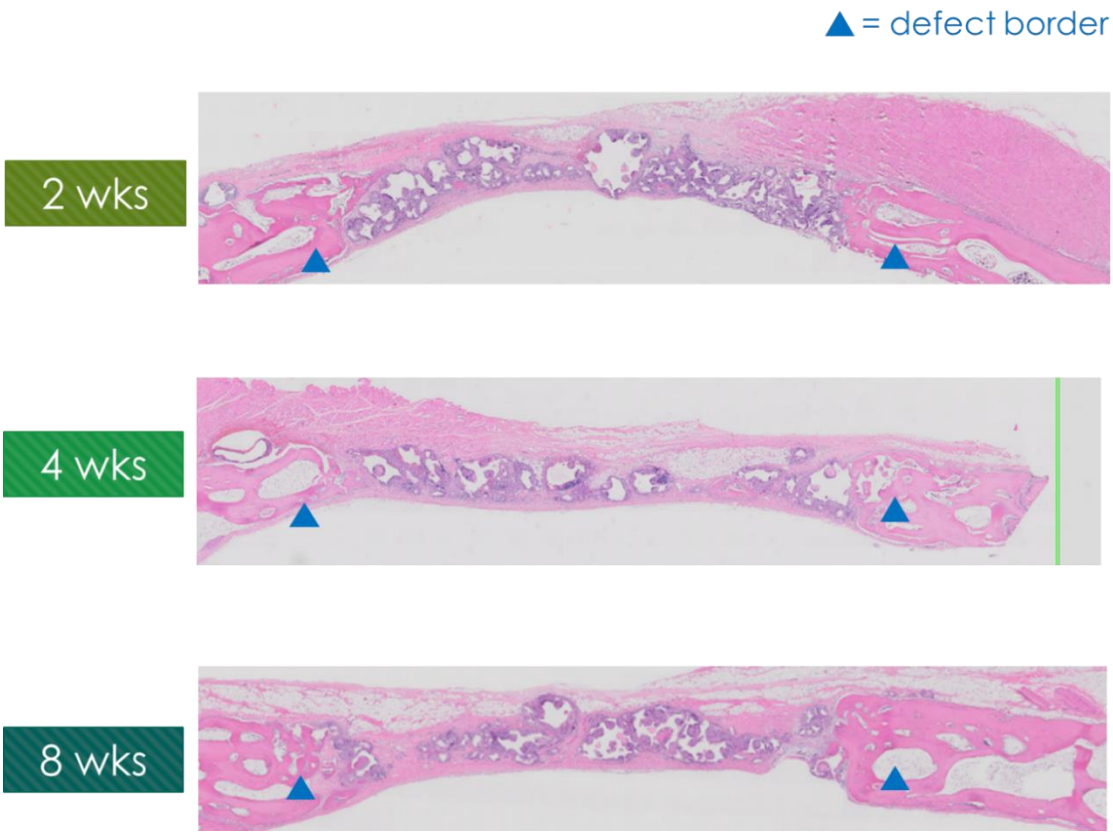

Fig 19. Histologic specimen from β-TCP&BMAC groups showed new bone formation from margin of defects higher than empty groups and β-TCP group.

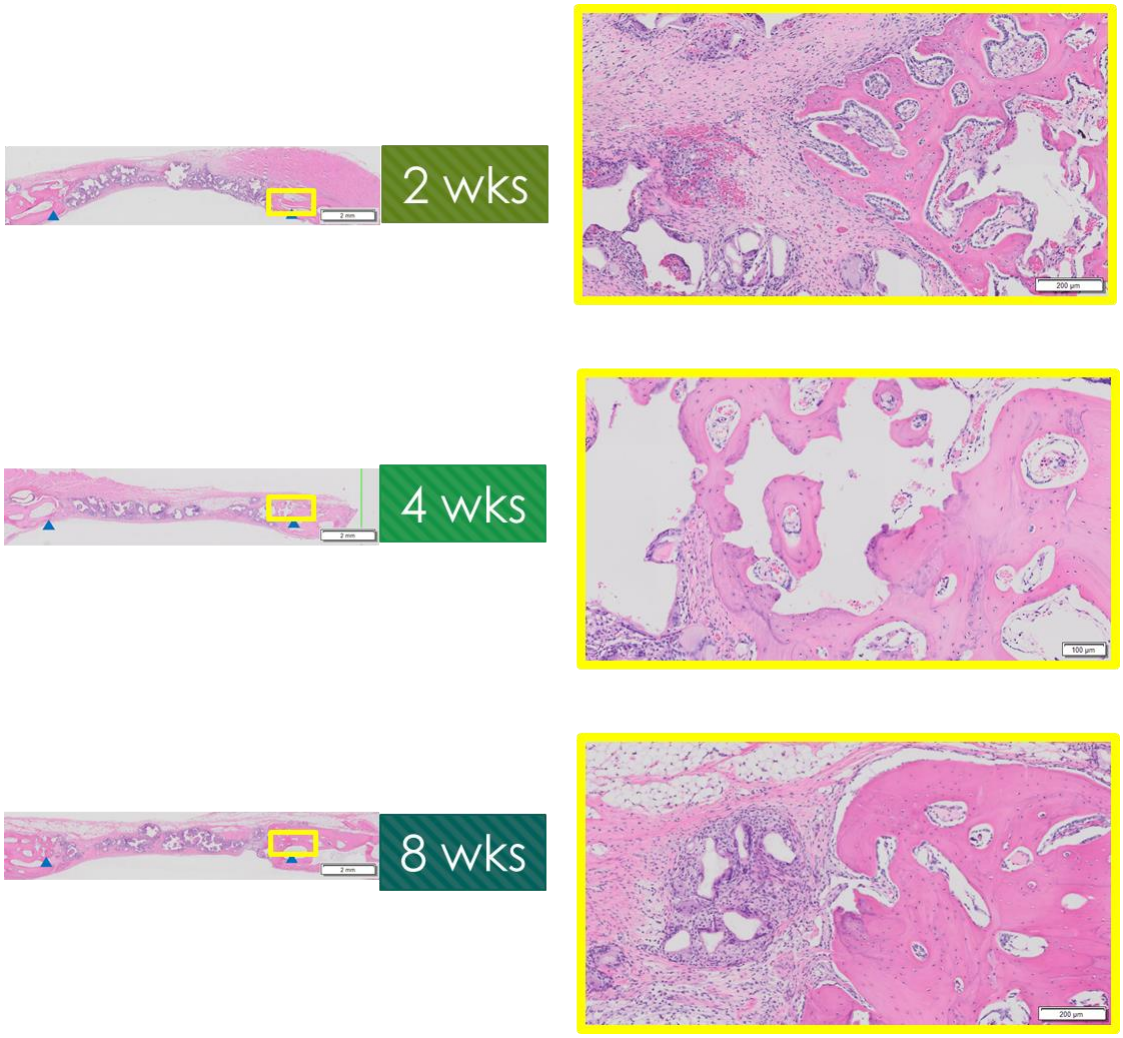

Fig 20. The picture amplified from margin of defects 0f histologic section of β- TCP&BMAC group showed particles of scaffolds. At 2 weeks woven bone was found at margin of defects. At 4 weeks and 8 weeks, higher bone quantity and more maturity bone was found when compared with β- TCP group.

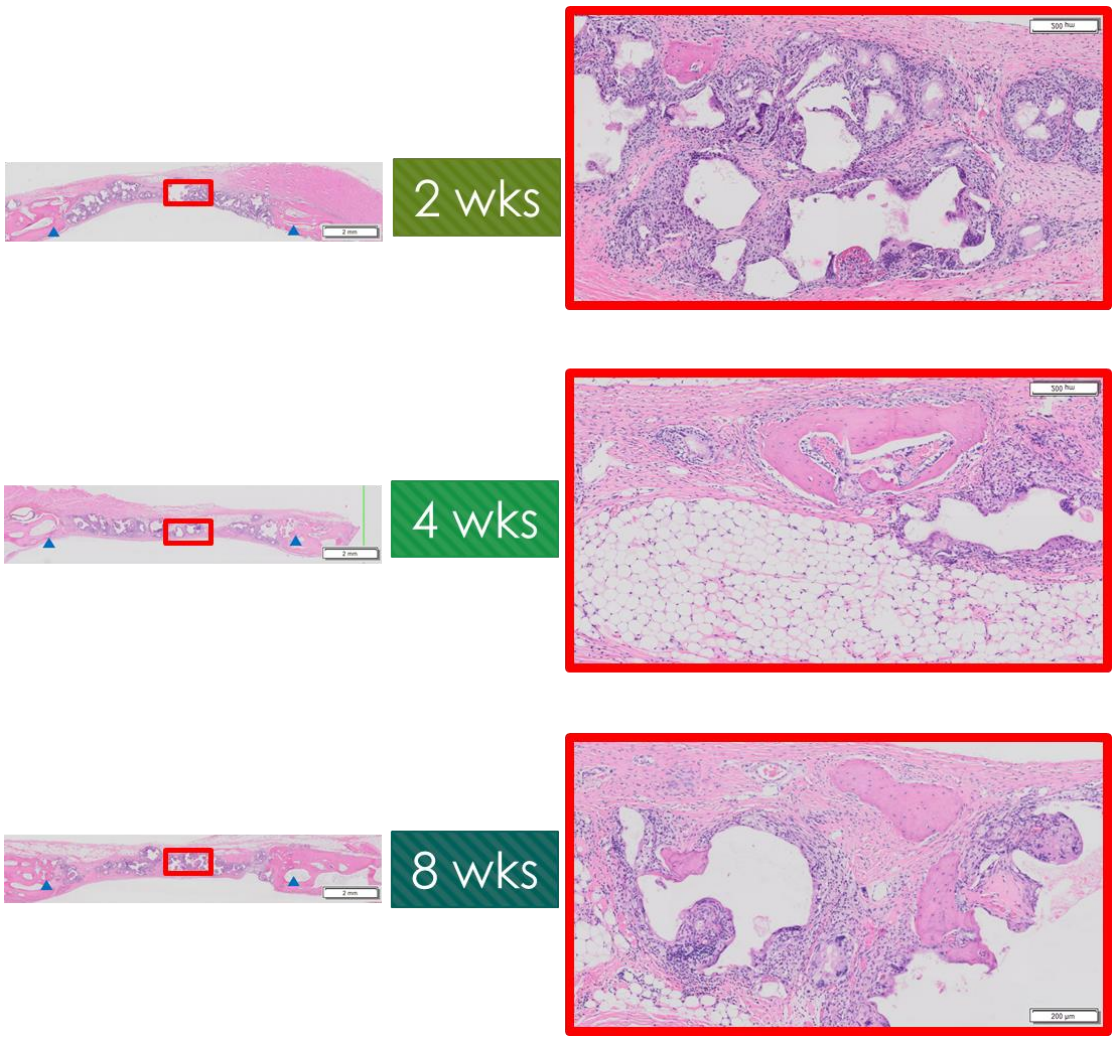

Fig 21. The picture amplified from center of defects 0f histologic section of β- TCP&BMAC group. At 2 weeks revealed inflammatory cells and some new bone formation. At 4 and 8 weeks, higher bone formation and more mature bone was found in center of defects. In contrast with specimens from β- TCP group which was not found any bone formation in center of defect.

Chapter 4

Discussion

In oral and maxillofacial surgery, there are many studies which report effectiveness of using tissue engineering principle by the transplantation of cultured of undifferentiated precursor cells, obtained from the bone marrow in bone grafting procedures, both *in vitro* and *in vivo* studies. However, cell culture methods are complex, limited to large research centers and costly procedure. To overcome this problems, the direct application of BMAC has been experimentally researched.

Even in healthy adults, the fresh bone marrow aspirate has moderate osteogenic capability. It is estimated that only one of every 50,000 nucleated cells of the fresh bone marrow is capable of differentiating itself to an osteoblast[36]. Due to these reason, the increasing of the concentration of mesenchymal progenitor cells could increase the number of nucleated cells and improve osteopotential property. In this study, 50 µl of BMAC that was separated for *in vitro* study can perform CFU-F which determine self-renewal capacity, one of stemness properties. Moreover, these cells are retained osteogenic capacity because after culture with osteogenic medium, these cells can produce mineralized matrix which can detected with alizarin red staining. So we can prove that the BMAC preparation technique by using simple centrifugation in this study, can caries out mesenchymal progenitor cells.

Beta-tricalcium phosphate (β-TCP) is one of synthetic biomaterials that have been in use for many years. β-TCP shows great osteoconductive potential which leads to good bone ingrowth. There are also many studies report effectiveness of β-TCP use as grafts for bone repair, augmentation, or substitution [11]. Same as this study, in experimental groups that contain β-TCP, new bone formation were occurred, without sign of infection or graft-versus-host reaction.

According to the micro-CT and histologic data, some of new bone formation can be detected from the defect side to the center site and increased by time in the empty group, but only connective tissue was presented in central area. The highest bone formation was observed in AB groups because of its ideal property in new bone regeneration, and it was higher significantly than empty groups in every time points. Newly bone formed in β-TCP+BMAC and β-TCP groups were not statistical significantly different, but higher than empty defect. New bone formation in the central area of defects can be found in autogenous bone treatment and also β-TCP&BMAC groups,

meanwhile β-TCP and empty groups cannot been found. Our result demonstrate that the use of BMAC can induced greater new bone volume compared with the group that treated with β -TCP alone because of improving osteogenesis and osteoinduction properties of BMAC.

Chapter 5

Conclusions

This study showed that beta-tricalcium phosphate added with bone marrow aspirate concentrate could be one of the alternative material of choice for new bone formation in rabbit calvarial defect. Methods for BMAC harvest should be developed to carry out higher quantity of bone marrow-derived mesenchymal stem cells, for enhance more bone formation.

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Appendix

Vitae

Name Miss Kanokporn Santavalimp

Student ID 5710820002

Educational Attainment

Degree	Name of Institution	Year of Graduation
Doctor of Dental Surgery	Mahidol University	2010
Higher Grad. Dip. in Clin. Sc. (Oral and Maxillofacial Surgery)	Mahidol University	2012

Work – Position and Address (If Possible)

Lecturer, Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Prince of Songkla University, Hat Yai Campus.

List of Publication and Proceeding (If Possible)

Santavalimp K, Nuntanaranont T, Kamolmatyakul S. "Effect of Bone Marrow Aspirate Concentrate on New Bone Formation in Rabbit Calvarial Defect" in The 40th National Graduate Research Conference 2016, during 20th- 21st October 2016 at Prince of Songkla University, International Convention Center, Prince of Songkla University, Hatyai Campus, Thailand.