GENE EXPRESSION OF *Bcl2a1* AND *Cxcl9* IN SOFT TISSUE COVERING BONE GRAFTING MATERIALS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Prosthodontics
Department of Prosthodontics
Faculty of Dentistry
Chulalongkorn University
Academic Year 2018

การแสดงออกของยีน *Bcl2a1* และ *Cxcl9* ในเนื้อเยื่ออ่อน ที่ปกคลุมอยู่บนกระดูกปลูกถ่าย

น.ส.บวรลักษณ์ ทวีเดช

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาทันตกรรมประดิษฐ์ ภาควิชาทันตกรรมประดิษฐ์
คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2561

Thesis Title	GENE EXPRESSION OF *Bcl2a1* AND *Cxcl9* IN SOFT TISSUE COVERING BONE GRAFTING MATERIALS
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Field of Study	Prosthodontics
Thesis Advisor	Assistant Professor Jaijam Suwanwela, Ph.D.

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การศึกษานี้มีวัตถุประสงค์เพื่อประเมินการแสดงออกของยีน *Bcl2a1* และ *Cxcl9* ในเนื้อเยื่ออ่อนที่ปกคลุมกระดูกที่ได้รับการปลูกถ่ายด้วยกระดูกเอกพันธุ์สกัดแร่ธาตุแบบระเหิดและกระดูกวัวสกัดโปรตีนเปรียบเทียบกับเนื้อเยื่อที่ปกคลุมกระดูกที่ไม่ได้รับการปลูกถ่าย สัตว์ทดลองจะได้รับการสุ่มเข้ากลุ่มทดลองสามกลุ่ม แต่ละตัวจะถูกกรอกระดูกเพื่อเตรียมพื้นที่สำหรับปลูกถ่ายกระดูกบนกระดูกข้างกระหม่อมทั้งสองฝั่ง โดยกลุ่มควบคุมจะไม่ได้รับการปลูกถ่ายกระดูก หลังจากปลูกถ่ายกระดูกเป็นเวลา 1 เดือนและ 3 เดือนแล้ว สัตว์ที่อยู่ในกลุ่มทดลองของแต่ละช่วงเวลาจะถูกทำให้เสียชีวิต เนื้อเยื่ออ่อนที่ปกคลุมอยู่บนกระดูกปลูกถ่ายจะถูกเก็บนำมาสกัดอาร์เอ็นเอเพื่อวิเคราะห์ผลการแสดงออกของยีนด้วยวิธีเพิ่มปริมาณสารพันธุกรรมชนิดปฏิกิริยาจริงอัตโนมัติ ผลการวิเคราะห์ทางสถิติพบว่าที่เวลา 1 เดือน เนื้อเยื่ออ่อนจากกลุ่มที่ได้รับการปลูกถ่ายด้วยกระดูกเอกพันธุ์สกัดแร่ธาตุแบบระเหิดและกระดูกวัวสกัดโปรตีนเพิ่มการแสดงออกของยีน *Bcl2a1* มากกว่ากลุ่มควบคุมที่ระดับนัยสำคัญทางสถิติน้อยกว่า 0.05 นอกจากนี้ยีน *Cxcl9* ในเนื้อเยื่อกลุ่มกระดูกเอกพันธุ์สกัดแร่ธาตุแบบระเหิดยังแสดงออกมากกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติที่ระดับนัยสำคัญทางสถิติน้อยกว่า 0.05 เช่นกัน อย่างไรก็ตามเมื่อเปรียบเทียบผลระหว่างกลุ่มทดลองที่เวลา 3 เดือน และเปรียบเทียบผลภายในกลุ่มทดลองระหว่างทั้งสองช่วงเวลา พบว่ายีนทั้งสองชนิดแสดงออกแตกต่างกันอย่างไม่มีนัยสำคัญทางสถิติ ที่ระดับนัยสำคัญทางสถิติมากกว่า 0.05

สาขาวิชา ทันตกรรมประดิษฐ์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2561 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5975822132 : MAJOR PROSTHODONTICS

KEYWORD: GENE EXPRESSION SOFT TISSUE GRAFTING MATERIALS

Borwonluk Taveedach : GENE EXPRESSION OF *Bcl2a1* AND *Cxcl9* IN SOFT TISSUE COVERING BONE GRAFTING MATERIALS . Advisor: Asst. Prof. Jaijam Suwanwela, Ph.D.

This study was to assess the expression of *Bcl2a1* and *Cxcl9* genes in soft tissues covering bone grafted with demineralized freeze-dried bone allograft (DFDBA) and deproteinized bovine bone mineral (DBBM) in comparison with ones covering without grafting. The animals were randomly separated into three groups of treatment. Calvarial defect models were created on parietal bones. Bare defect models without bone graft as a control group. After one and three months, animals were sacrificed. Soft tissues covering the defected area were collected by punch technique. RNA was isolated and proceeded to Real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR). Statistical analysis indicated that in comparison to control group at one month, DFDBA and DBBM upregulated *Bcl2a1* gene in soft tissue covering bone graft at $P < 0.05$. Allograft also upregulated *Cxcl9* gene ($P < 0.05$). However, the comparison among groups at three months and comparison within group between one and three months shown the expression of both genes but without significant difference ($P > 0.05$).

Field of Study: Prosthodontics Student's Signature .....

Academic Year: 2018 Advisor's Signature .....

ACKNOWLEDGEMENTS

I would first like to thank my thesis advisor Assistant Professor Dr.Jaijam Suwanwela for her great inspiration and guidance. Without her encouragements, this thesis could not have been successfully conducted.

I would like to thank Dr.Thanyaporn Kangwannarongkul for her advice and tissue samples providing.

I would also like to thank Professor Dr.Thanaphum Osathanon for his advice and support throughout my research. I also Thank Mr.Noppadol Sa-Ard-Iam and the staffs of Molecular Biology Research Center, Faculty of Dentistry, Chulalongkorn University, for their counseling service throughout my research.

I would also like to acknowledge Assistant Professor Dr.Keskanya Subbalekha , Assistant Professor Dr.Philaiporn Vivatbutsiri, and Dr.Pisaisit Chaijareenont. I am gratefully indebted to their valuable comments on this thesis.

Finally, I must express my very profound gratitude to my family for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and drafting this thesis. This accomplishment would not have been possible without them. Thank you.

Borwonluk Taveedach

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CHARPTER I

INTRODUCTION

Background and rationale

Dental implants are widely used and are one of several treatment options that can be used to replace missing teeth. Nowadays, it still challenging to create the preferable outcome in an esthetic zone in order to achieve emergence profile. As buccal bone resorbs, It is a corresponding recession of gingival as well [1, 2]. It hardly to gain bone volume in any case without bone augmentation. Therefore, alveolar bone augmentation had been recommended to perform with implant placement [3-5]. A bone graft is a material or tissue used to repair bone defect or improve bone volume. There are four types of bone grafts including, autografts, allografts, xenografts, and alloplasts. There are various type of bone augmentation techniques performing in clinical practices [6]. In many situations, a barrier membrane may not be used, and bone graft alone can be more effective [7]. Bone graft is a material or tissue used to repair bone defect or improve bone volume. Osteogenesis, osteoinduction, and osteoconduction are the three principles of grafting materials [8]. These are used to decide which materials properly for an individual defect. Allograft and xenograft had been normally used in our clinical practices. The first one is demineralized freeze-dried bone allograft (DFDBA) and the other one is deproteinized bovine bone mineral (DBBM). DFDBA is produced from the same species of human which be receiving from bone bank. It is widely used in the case of ridge preservation [4]. DBBM is produced from a bovine bone under a low heat condition [9]. Due to the osteoconduction properties, DBBM mainly used in an insufficient bone defect. We had been founding inconvenient situations while augmented bone in an aesthetic area. Sometimes barrier membrane was hard to manipulated and some defect needs only bone graft to be performed. From these reasons, we questioned that the process of healing or properties of grafting materials might affect a soft tissue covering on bone grafted area. Bone graft as a subtype of

dental materials and may play a role as foreign body to promotes chronic inflammation and soft tissue destruction [10].

An organism's phenotype is determined by its genotype, which is the set of genes the organism carries, as well as by environmental influences upon these genes. The etiology of chronic inflammation is largely unknown. Following acute inflammation, chronic inflammation is identified by the presence of mononuclear cells including lymphocytes and plasma cells [11]. Previous studies in mouse reported mRNA of *Bcl2a1* was produced during lymphocyte development [12], and lymphocyte and macrophage activation [13]. Cxcl9 is the chemokine (C-X-C motif) ligand 9 induced by IFN-**γ**. Cxcl9 mainly functions as the inducer of T-helper 1 cell [14]. Cxcl9 is secreted by various immune cell types including T lymphocytes, NK cells, dendritic cells, and macrophages, etc. [15]. This chemokine is associated with many T-cell-mediated conditions for example organ rejection [16], skin inflammatory such as contact hypersensitivity [17]. We have hypothesized *Bcl2a1* and Cxcl9 may upregulation in a tissue covering bone grafted area.

Mammalian calvarium has been used as a study model of a tissue-related biomaterial research, because of its morphology and approximately 85% of genome that resembles human [18, 19]. Mouse's calvarium is also recommended as a model to examine the tissue response to bone substitution materials.

In 2018, a previous study of Kangwannarongkul et al. founded DBBM and DFDBA promoted osteoblast-related genes expressed in the grafted area [20]. However, gene expression of inflammatory-related genes in soft tissue covering bone grafted area have never been assessed before. This study aimed to evaluate the expression of *Bcl2a1* and *Cxcl9* genes in soft tissue covering bone grafted with DFDBA and DBBM in a mouse model.

CHARPTER II
REVIEW OF LITERATURE

Dimensional ridge changes after tooth extraction and implant placement

Quantity and quality of alveolar bone are key factors to provide better stability of an implant and soft tissue. Treatment planning should be concerned about the volume and type of existing alveolar bone level of crestal bone, gingival biotype, the zenith of gingiva, biological width, a proximal contact area of adjacent teeth, duration of implant placement, and the relation of implant fixture to implant abutment to have an ultimate esthetic result [21].

After tooth extraction, about two – third of vertical and horizontal alveolar bone changes in a few months [22]. The resorption pattern is asymmetrical, a resorption rate of buccal bone is higher than lingual and palatal bone. The center of alveolar ridge changes lingually in 12 months after tooth extraction. Moreover, buccolingual width of ridge decreases to 50% [1]. Recent studies in human founded the vertical height of alveolar ridge could reduce about 0-2 mm. after implant had placed for 6 months [23-25]. There are two overlapping phases of buccal and lingual wall resorption. The first period, bundle bone resorbs, woven bone is then substituted. The main composition of buccal plate is bundle bone, while lingual plate has both bundle and lamellar bone. Bundle bone receives blood supply from periodontal ligament. A tooth extraction causes a periodontal ligament tearing resulting in rapid bundle bone resorption and lamellar bone is not replaced. Second phase, the external wall of both buccal and lingual alveolar bone resorb.[23] A traumatic injury during any surgical operations also stimulates the inflammation and inflammatory process is possibly one significant cause of resorption.

Bone augmentation

Tooth extraction has an effect on alveolar ridge deformities. Traumatic sequences and pathologic condition provide insufficient of bone volume for implant placement. In 2009, Chiapasco et al. concluded bone augmentation in five methods:

1) osteoinduction through the use of appropriate growth factors; 2) osteoconduction, in which a grafting material serves as a scaffold for new bone formation; 3) distraction osteogenesis, by which a fracture is surgically induced and the two bone fragments are then slowly pulled apart, with spontaneous bone regeneration between the two fragments; 4) guided bone regeneration (GBR), which use barrier membrane with or without bone graft; and 5) revascularized bone grafts, where a vital bone segment is transferred to its recipient site with its vascular pedicle such as fibular free flap used for mandible reconstruction [26, 27].

Osteoconduction by the grafting material and GBR, are the widely used method in an esthetic area. GBR can be used before and simultaneous implant placement to improve alveolar ridge morphology [28]. Bioresorbable and non-resorbable membranes are two types of the barrier membrane that used in GBR. A non-resorbable type needs a second operation for removal which causes discomfort to a patient. Moreover, with non-resorbable membrane, alveolar bone regeneration will be interfered and crestal bone will be resorbed due to flap operation. Thus, the bioresorbable collagen membrane with bone grafting is preferred. However, using a barrier membrane in an esthetic area is difficult to manipulate. In the case of thin biotype, it is prone to dehisce. Failures of GBR relating to a membrane exposure have been reported up to 50%. The exposure leads to infection and causes partial or total loss of the new bone formation [29-31]. In one randomized clinical trial (RCT), a horizontal bone augmentation using DBBM with a resorbable or non-resorbable membrane was evaluated. The result showed both types of membrane undergo high rates of membrane exposures of 64 and 71%, respectively. The same study reviewed clinical outcomes between autogenous bone and DBBM groups. The use of DBBM with or without membrane showed a mean defect was fulfilled higher than the autogenous bone group. Furthermore, implant survival rates of 93-100% have also been reported [32]. The turnover rate of soft tissue is higher than bone. Therefore, bone grafts are used alone when their interface has low resorption rates. In the other hand, the combination of bone graft and membrane should be used at the high resorption rate area [33]. If grafting materials have both supporting and modulate healing ability, it will be beneficial for the patients [34, 35].

The clinical outcome of ridge augmentation using autogenous bone or bone substitute material has shown no statistically significant difference between both types of grafts [36]. However, the evidence-based implant study analyzed the grafting material used for bone augmentation technique showed no scientific evidence providing clear guidelines of the best of bone grafting material due to lacking of the specific bone substitute comparison and the different sizes of defect [37].

Bone grafting materials

Bone graft is used as a filler and scaffold to facilitate bone regeneration to a grafted site. There are various bone graft classifications based on bone material groups:

Autogenous bone graft

Autogenous bone grafts or autograft obtained from the same individual receiving graft. Most compositions are inorganic materials and about 90 – 95% is hydroxyl apatite crystals. Only autograft has osteogenesis activity because of existence of osteocyte, osteoblast, osteoclast, and bone - lining cell. Their key role is induction of osteogenic cells to form a new bone. One key advantage of this graft is less tendency of graft rejection. Despite autograft as a gold standard for bone grafting [38].

Allograft

Allograft is derived from the same species, but different genotypes. There are many products available including: 1) Fresh frozen bone or FFB; 2) Freeze-dried bone allograft or FDBA; and 3) Demineralized freeze-dried bone allograft or DFDBA

Due to the higher risk of immunologic rejection and disease transmission than FDBA and DFDBA, FFB is not popularly used. Both FDBA and DFDBA have osteoinductive property. However, only DFDBA has bone morphogenic proteins (BMPs) which induces osteogenesis. The histological outcomes of alveolar ridge preservation using FDBA and DFDBA shown that no significant difference between both groups. DFDBA group has been reported a higher vital bone formation than FDBA group. Mean percentage of residual graft

particles in DFDBA group also had a significantly lower than FDBA group [4]. Demineralization of DFDBA aims to increase capabilities to adsorb BMPs molecules but causes DFDBA unstable to act as a scaffold. Therefore, grafting in some recipient site using DFDBA in combination with some biomaterials has been recommended. Recently, recombinant human bone morphogenetic protein 2 (rhBMP-2) has been suggested to fill in the extraction socket due to their osteoinductive effect [39].

Xenograft

Xenograft is derived from animal tissue. Organic substances are eliminated for reducing the cause of immunologic rejection and disease transmission to human. A deproteinized bovine bone or DBBM is shown to be highly compatible to oral hard tissue in human with osteoconductive ability [40]. It is produced from bovine cancellous bone which has a size of inner macropores resemble to natural cancellous bone [41]. Manufacturers have produced various DBBM products such as Bio-oss®, OsteoGraft®, Endobon®, and Cerabone®. However, the entire structures of materials have no differences. In vitro and in vivo study have founded multinucleated-giant cell acts as an osteoclast-like cell to resorb DBMMs and causes micropores on the surface of the graft. However, the study in human has shown some particles of DBMMs remain more than 10 years after DBMMs has been grafted [42].

Alloplast

Alloplast or alloplastic bone substitution is a synthetically derived graft material. Hydroxyapatite is mostly used because of its osteoinductivity and biocompatibility to the human body, but it has a slow resorption rate. However, this problem can be solved by combining with tricalcium phosphate [43].

Immune response to grafting materials

Following the implantation of biomaterials in tissue, inflammatory reactions consist of acute inflammation, chronic inflammation, and granulation tissue formation were expressed. The intensity and times are varied upon properties of biomaterials. Such as mechanical properties, porosity, surface chemistry, and degradability of materials (**Figure 1**) [44].

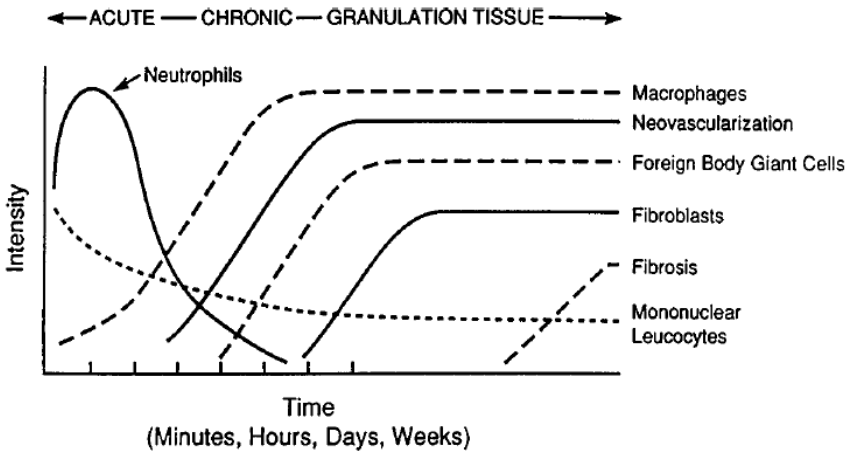

Figure 1 The temporal variation of inflammatory response to implanted biomaterial [44]

Within the first two weeks, acute and chronic inflammation occur after having biomaterials grafted. Monocytes, macrophages, and lymphocytes play a role in chronic inflammation follows acute inflammation. If biocompatible material was grafted, this process will be quickly resolved. Monocytes and macrophages still remain for interact at the interface of grafted material. However, chronic inflammation has been inspected to describe the foreign body reaction as well. It can be detected and present throughout a lifetime of implant material by the expression of Lymphocytes, adherent macrophages, and foreign body giant cells. [11, 45]. However, the clinical outcome acceptance however also depends on the host's tissue response with no infection [46, 47]. However, the ultimate success of various grafting materials is based on the host tissue response itself. Degidi et. al. 2006 studied the inflammatory infiltration and microvessel density in peri-implant soft

tissue of titanium and zirconium oxide healing caps in human. In titanium group, a greater number of vessels presented in the submucosa layer. The microvessels mostly found in an area of inflammatory cell infiltration [48].

Bcl2a1 (Mus musculus) is the gene symbol of B-cell lymphoma 2-related protein A1. The *BCL2A1* is the gene symbol of Homo Sapiens species also called Bcl-2 related gene expressed in fetal liver (Bfl-1). This gene mainly expressed in the hematopoietic system, especially in endothelial cells [49]. Tumor necrosis factor and NF-kB were identified as the regulator of *BCL2A1* expression [50, 51]. Simultaneously, *BCL2A1* transcription was reported to be induced in response to Lipopolysaccharides (LPS), Granulocyte-macrophage colony-stimulating factor (GM-CSF), and antigen receptor stimulation [52, 53]. The regulation of *BCL2A1* is shown in **Figure 2.** *BCL2A1* exerted a pro-survival function to prevent cell death. Previous studies in mouse reported mRNA of *Bcl2a1* was produced during lymphocyte development [12], and lymphocyte and macrophage activation. [13]. In 2010, study of gene expression profiles founded *BCL2A1* (Homo Sapiens) as the one of represented genes involving oral squamous cell carcinoma [54]. *BCL2A1* also expressed in synovial fluid of temporomandibular joint (TMJ) and associated with osteoarthritis[55].

Cxcl9 is the chemokine (C-X-C motif) ligand 9 induced by IFN-**γ** and also called monokine induced by IFN-**γ** (Mig). The *CXCL9* and MIG are the gene symbols of Homo Sapiens species. *CXCL9* is one of the three genes including CXCL(, CXCL10, and CXCL11 which induced by IFN-**γ** . These chemokines bind the CXCR3 receptor which is predominately expressed on activated CD4 and CD8 cells that are associated with a Th1 phenotype [56, 57]. These IFN-**γ**-inducible chemokines can be produced by a number of different cell types including hematopoietic cell types, e.g. macrophages and neutrophils and non-hematopoietic cell types, such as endothelial cells, and fibroblasts [16, 58-60] (**Figure 3**). This chemokine is associated with many T-cell-mediated conditions for example organ rejection [16], skin inflammatory such as contact hypersensitivity, [17], and oral lichen planus [61].

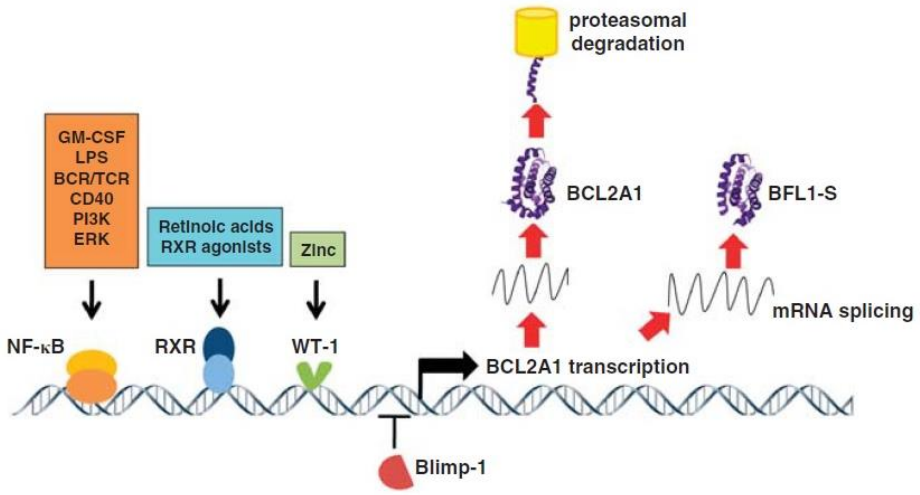

Figure 2 Regulation of *BCL2A1* [49]

An in vivo study of wound healing in maxillofacial region

Bone graft and dental implant have been developed to date by using researches of human and animals. Generally, the wound healing rate in the maxillofacial region in mammal is higher than human. The reason is a bigger size of a human skull and few muscle insertions causing less blood supply in human. Therefore, we have to create a smallest size of tissue defect that will not be able to heal throughout the lifetime of an animal, called 'Critical size defect (CSD)' [62]. The bony vault of the cranium of mammal has many similarities to the maxillofacial region in human due to morphological and embryological development. Mammalian calvaria develop from membranous precursors resemble to the membranous bone of the face. Anatomy of calvaria consists of two cortical plates and is inserted between plates with cancellous bone similar to mandible. Rats, rabbits and dogs have been recommended as calvarial wound models for studies of the biological reaction of cells after implantation [63]. However, mouse calvaria model is also used to study bone regeneration because mouse and human contain roughly the same number of genes with about 85% of gene identity [64, 65]. C57BL/6 is the most widely used inbred strain because these mice are easy to manipulate, well breeding.

This inbred strain had been used as a mouse model for investigation of the inflammatory response [66-69].

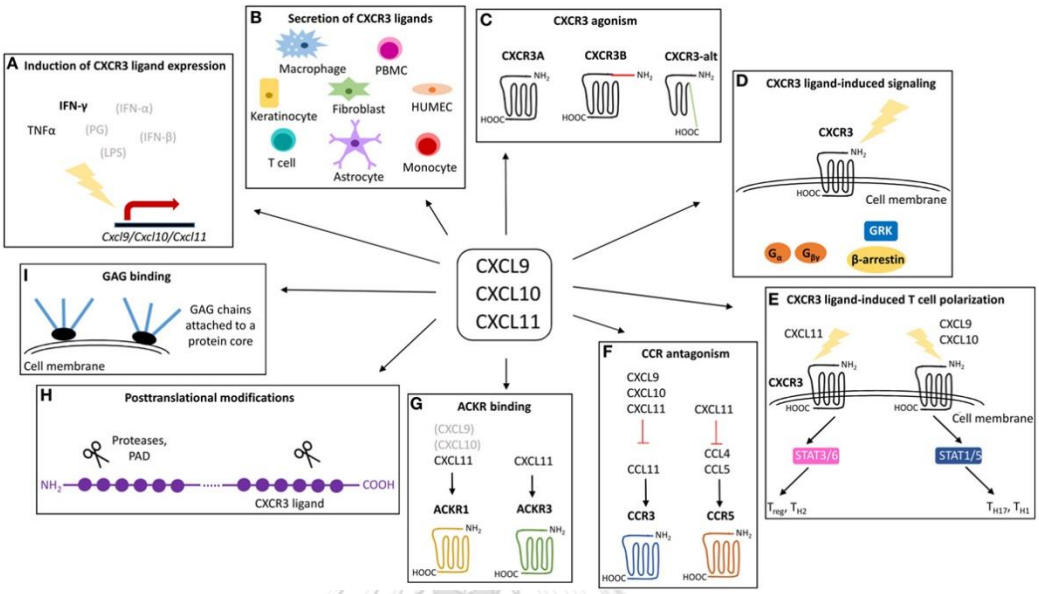

Figure 3 Mechanism and role of *Cxcl9* [70]

Real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR is a molecular study involving in mRNA transcription cycle measurement. RT-qPCR is a combination of three steps: 1) the reverse transcriptase (RT)-dependent conversion of RNA into cDNA, 2) the amplification of the cDNA using the PCR and 3) the detection and quantification of amplification products in real time [71]. Nevertheless, the quantity of detected RNA does not always direct variate to inflammatory cell levels. Moreover, researchers are not able to know which cells release cytokines. PCR is rather technically sensitive because it can detect cytokine producing cells only when that cell quantity is high enough [72].

Research questions

1. Are the gene expressions of *Bcl2a1* and *Cxcl9* in soft tissue covering bone grafted with DFDBA and DBBM different from the ones covering bone without grafting?

2. Are the gene expressions of *Bcl2a1*and *Cxcl9* in soft tissue covering bone grafted at one month different from three months?

Research Objectives

To study the gene expressions of *Bcl2a1* and *Cxcl9* in soft tissue covering bone grafted with DFDBA and DBBM in comparison with the ones covering bone without grafting.

Research Hypothesis

1. **$H_0$1**: The expression of *Bcl2a1* and *Cxcl9* in soft tissue covering bone grafted with DFDBA and DBBM are not significantly different from the ones covering bone without bone grafting

   H_A1: The expression of *Bcl2a1* and *Cxcl9* in soft tissue covering bone grafted with DFDBA and DBBM are significantly different from the ones covering bone without bone grafting
2. **$H_0$2**: The gene expressions of *Bcl2a1* and *Cxcl9* in soft tissue covering bone grafted at one month do not different from three months.

   H_A2: The gene expressions of *Bcl2a1* and *Cxcl9* in soft tissue covering bone grafted at one month different from three months.

Research Conceptual framework

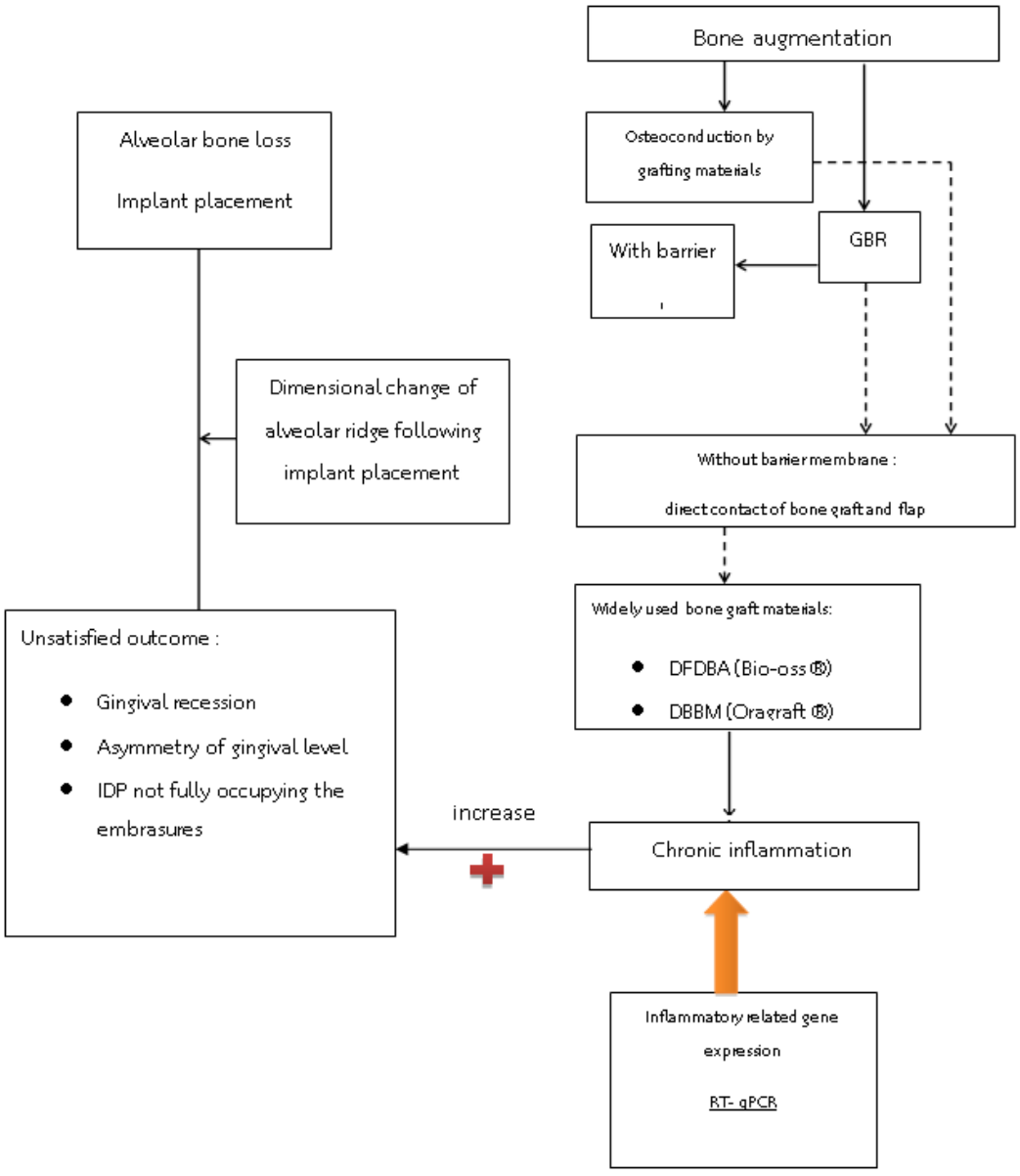

Figure 4 Research conceptual framework

CHAPTER III

RESEARCH METHODOLOGY

This research is the continuous study of Dr.Thanyaporn Kangwannarongkul, "GENE EXPRESSION AND MICRO-COMPUTED TOMOGRAPHY ANALYSIS OF GRAFTED BONE USING DEPROTEINIZED BOVINE BONE AND FREEZE-DRIED HUMAN BONE".

Animals

Male mice strain C57BL/6MLac age eight-weeks-old of previous study were used [20]. Animal use protocol No. 1832003 was approved by the Chulalongkorn University Animal Care and Use Committee (IACUC). Mice were housed in light and temperature-controlled facilities and given food and water ad libitum. Animals were separated into two groups: one month and three months. At each time point, defects were randomly assigned following three types of treatment: (1) bare defect without bone grafted as a control, or (2) defect grafted with DBBM (Bio-Oss®; GeistlichPharma AG, Wolhusen, Switzerland), or (3) defect grafted with DFDBA (OraGRAFT®; LifeNet, Virginia, USA) (**Figure 5**).

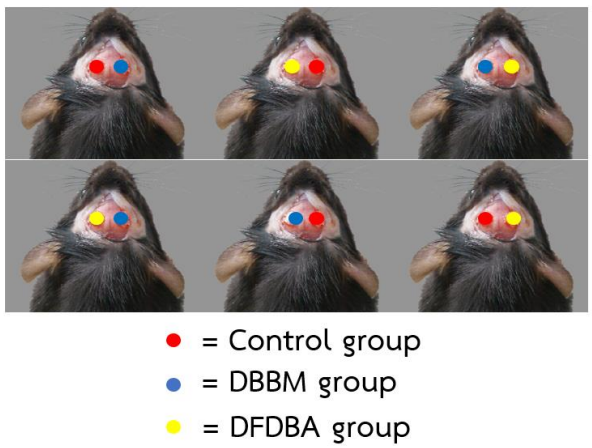

Figure 5 Randomization of treatment in each animal

(Courtesy to Dr. Thanyaporn Kangwannarongkul)

Surgical procedures

The surgical procedures were conducted in accordance with the IACUC of No. 1432001. Mice were sedated by mean of intraperitoneal injection. Pentobarbital (Nembutal®) was diluted with phosphate buffered saline (PBS) in the ratio of 1:10. Each animal was received at a concentration of 8 µL of dilution per weight (g). Hairs above the scalp were removed by blade and scalp was cleaned with alcohol and povidone iodine. To visualize the parietal bones, an incision of 1.5 mm length was made under 1% lidocaine with 1:10000 epinephrine following a midline through the skin and the periosteum of the calvarium. CSD size 3 mm in diameter were made both sides by mean of trephine bur with normal saline coolant. During this step, dura mater injury must be avoided. Ten milligrams of OraGRAFT® or Bio-Oss® were randomly grafted at defects of grafting groups. Cotton pellet soaking normal saline was used to packed bone graft particles. Scalp was sutured with nylon 3 – 0 with primary closure technique.

At each time point, animals were sacrificed by cervical dislocation and mid-sagittal incision line was made. Center of soft tissue above grafted area had been located by measuring from lambdoid suture 4.5 mm length and sagittal suture 3 mm length and center of the grafted area were marked on scalp. Soft tissue size 4 mm in diameter was cut with biopsy punch. Each tissue was cut, one half was used for RT-qPCR analysis, while other half was used for further experiment. Tissue samples were labeled including control group, bare defect; DBBM, Bio-Oss®; and DFDBA, OraGRAFT®. Tissues were frozen in liquid nitrogen and stored in -80°C (**Figure 5**).

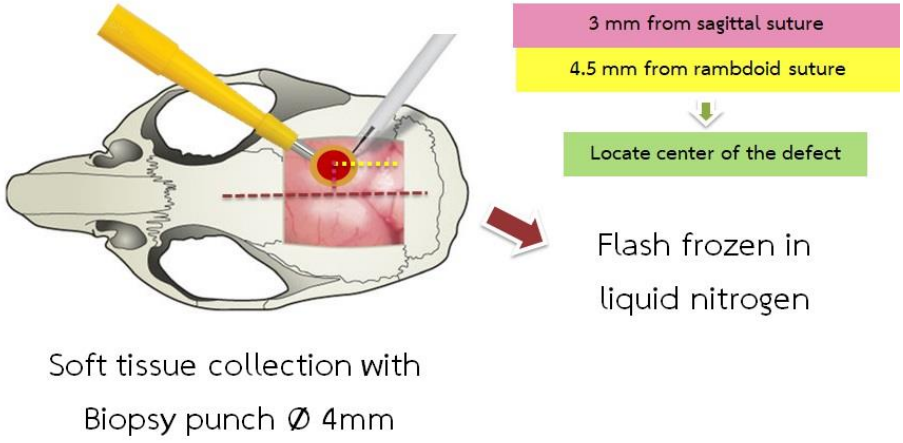

Figure 6 Soft tissue collection

Tissue homogenization and RNA isolation

Three samples per group and each time-point were analyzed in this experiment. All procedures were done under aseptic RNA handling techniques. RNA isolation was done with Rneasy Plus Minikit (QIAGEN N.V., Germany) following the manufacturer 's directions. Tissue homogenization and RNA isolation was followed these steps (**Figure 7**):

1. RLT Plus lysis buffer (350 µL) and β-Mercaptoethanol (3.5 µL) were prepared by adding into ceramic bead tube (bead size 2.8 mm in diameter). Next, frozen tissue sample was transferred into bead tube. Homogenization were done with PowerLyzer® Homoginizer (Mo Bio Laboratories, QIAGEN N.V., Germany) by Speed of 3,500 for 2 cycle x 45 s and 30 s of pause time. This step was done quickly, and tissue thawing must be avoided.
2. RNA isolation (**Figure 8**), gDNA was eliminated from homogenized tissue by centrifuging homogenized lysate into gDNA eliminator spin column. Centrifugation was performed for 30 s at ≥8000 x g (≥10,000 rpm). The spin column was discarded and the flow-through was saved.
3. Added 350 µL of 70% ethanol solution into flow-through solution of previous step and mixed well by pipetting. Mixed solution was transferred in to Rneasy spin column and centrifuged for 30 s at ≥8000 x g (≥10,000 rpm). The flow-through was discarded. The spin column and collection tube were saved.

4. The solution of previous step was added with 700 µL of Buffer RW 1 and centrifuged for 15 s, at ≥8000 x g (≥10,000 rpm) and flow-through was discarded.
5. Buffer RPE 500 µL in volume was added to spin column and centrifuged for 2 min, at ≥8000 x g (≥10,000 rpm) and the flow-through was discarded. This step was performed for two times.
6. Spin column of previous step was placed in new microtube. RNA was eluted from spin membrane by full speed centrifuging with 30 µL of Rnase- free water.
7. RNA quantification analyzed by spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Inc., USA). RNA quantity was determined by its UV absorbance at 260 nm. RNA quality was assessed using A260/A280 ratio. The ratio of A260/A280 about 1.8 to 2.1 was accepted to analyses RT-qPCR. RNA samples were kept in -80°C.

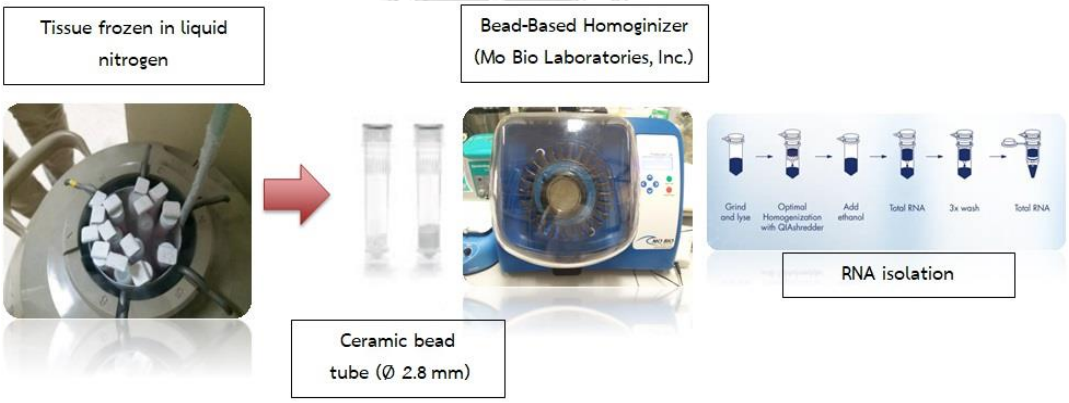

Figure 7 Tissue homogenization

RT-qPCR analysis

In this study, we chose two-step RT-qPCR to observe mRNA expression. The protocol also separated into two steps - step 1, reverse transcription protocol and step 2, Real-time polymerase chain reactions. Primer BLAST (https://ncbi.nlm.nih.gov/tools/primer-blast/) was used for designing the specific

primers of *Bcl2a1* and *Cxcl9*. Specificity of the primer sequences were evaluated by Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). 18s rRNA was used as a reference gene to normalize an mRNA expression. The primer sequences are shown in **Table 1**.

Step 1 Reverse transcription

RNA was reverse transcript to cDNA by the reverse transcription kit (Sensiscript, Qiagen, USA). The experiment was done following the protocol of RNA less than 50 ng that shown below.

1. The primer, 10x Buffer RT, dNTP Mix, and RNase-free water was thawed at room temperature except template RNA was thawed on ice on ice. All solution should be vortex, briefly centrifuge, and store on ice.
2. The master mix were prepared according to **Table 2**. Vortex and briefly centrifuge had been done and immediately stored on ice.
3. Template RNA was added for a final component of master mix with gently vortex and briefly centrifuge.
4. The reaction was incubated for 60 min at 37 °C.
5. The reverse-transcription reaction was store at -20°C for long-term storage.

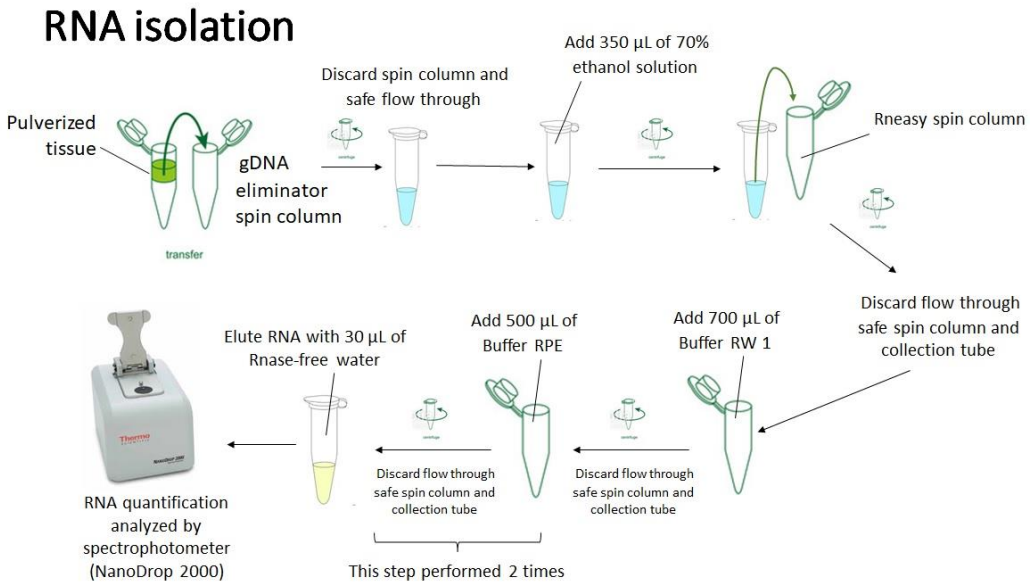

Figure 8 RNA isolation using Rneasy Plus Mini Kit (QIAGEN®)

Step 2 Real-time polymerase chain reaction (qPCR)

The FastStart SYBR Green Master (FastStart Essential DNA Green Master kit (Roche Diagnostics GmbH Mannheim, Germany) was used for performing the polymerase chain reaction. Bio – Rad CFX96TM RT-PCR system (Bio-Rad laboratories, INC., USA) was utilized to verify the ability of mRNA to observe gene expression. Real-time polymerase chain reactions had been perform and the protocol was shown below.

1. Solutions were thawed, mixed by pipetting up and down motion, and store on ice.

2. PCR mix 50 µl per reaction was prepared following the solution components in **Table 3.** PCR mix was mixed carefully by pipetting up and down. Vortex must be avoided.

3. qPCR reactions were run following the Bio – Rad CFX96TM RT-PCR system protocol in **Table 4**.

During the qPCR cycles, melt peak were plot and showed a single amplicon on the mRNA expression of interested genes (**Figure 9**).

Table 1 Sequences of gene specific primers used for RT-qPCR

Gene symbol	Primer	Primer sequences (5' to 3')
Bcl2a1	Forward	CTT CAG TAT GTG CTA CAG GTA CCC G
	Reverse	TGG AAA CTT GTT TGT AAG CAC GTA CAT
Cxcl9	Forward	CAC TTC GCT GCT ATC TAA TTG G
	Reverse	TAG GCA CTG TGG AAG ATT TAG G
18s rRNA	Forward	AGG GGA GAG CGG GTA AGA GA
	Reverse	GGA CAG GAC TAG GCG GAA CA

Table 2 Reverse transcription components of < 50 ng RNA

Component	Volume/reaction	Final concentration
Master mix		
10x Buffer RT	2.0 µl	1x
dNTP Mix (5 mM each dNTP)	2.0 µl	0.5 mM each dNTP
Oligo-dT primer (10µM)	2.0 µl	1 µl
RNase inhibitor (10 units/µl)	1.0 µl	10 units (per reaction)
Sensiscript Reverse Transcriptase	1.0 µl	
RNase-free water	Variable	-
Template RNA	Variable	< 50 ng (per reaction)
Total volume	**20 µl**	**-**

Table 3 Components of FastStart SYBR Green PCR mix

Component	Volume	Final concentration
FastStart SYBR Green Master	25 µl	1x
Forward primer (30 µM)	0.5 µl	300 nM
Reverse primer (30 µM)	0.5 µl	300 nM
Water PCR Grade	19 µl	
cDNA (up to 250 ng)	5 µl	
Total volume	**50 µl**	

Table 4 qPCR protocol of Bio – Rad CFX96TM RT-PCR system

Step	Temperature	Duration	Cycles
Enzyme activation	95 °C	3 min	Hold
Denature	95 °C	1 -3 s	40
Anneal/extend	60 °C	≥ 20 s + plate read	
Dissociation (melt curve)	60 - 95 °C	5 s + Plate read	Increment 0.5 °C until reach 95 °C

Data analysis

Quantitative PCR data analysis was done using qbase+ software, version 3.0 (Biogazelle, Zwijnaarde, Belgium – www.qbaseplus.com). Relarative gene expressions were calculated using $2^{-\Delta\Delta CT}$ method [73, 74]:

Where

$$\Delta\Delta CT = \Delta CT_{\text{Grafted group}} - \Delta CT_{\text{Control group}}$$

$$\Delta CT_{\text{Grafted group}} = CT_{\text{Gene of interest}} - CT_{\text{Internal control gene (18s rRNA)}}$$

$$\Delta CT_{\text{Control group}} = CT_{\text{Gene of interest}} - CT_{\text{Internal control gene (18s rRNA)}}$$

The relative gene expression data was evaluated the difference by one-way analysis of variance (ANOVA). Post hoc Tukey's Honestly test was used for multiple comparison between every two group. The difference between two time points, one and three months after bone grafted, was analyzed with independent t-test. All statistics were performed at the 95% significance level. *P* value less than 0.05 was indicated statistically significant difference.

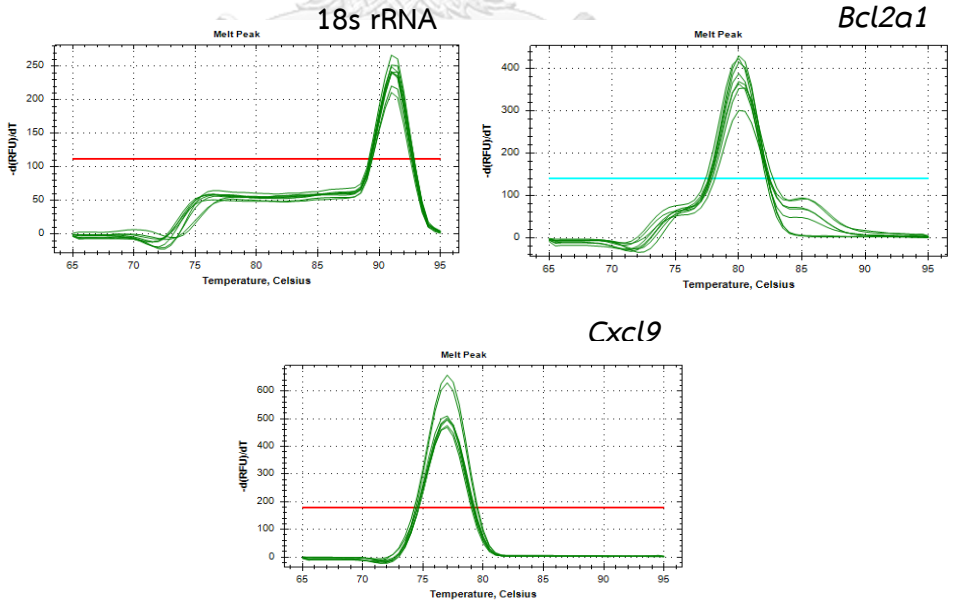

Figure 9 Melt curve of 18s rRNA, *Bcl2a1*, *Cxcl9* showed the dissociation characteristics of double-stranded DNA during heating.

CHAPTER IV

RESULTS

The comparative gene expression results are shown in **Figure 10** to **Figure 13**. At one month, the gene expression levels of *Bcl2a1* were statistically significant difference ($P < 0.05$). DFDBA and DBBM groups statistically significant upregulated *Bcl2a1* comparing with control group ($P < 0.05$). The expression levels of *Cxcl9* showed statistically significant difference between groups ($P < 0.05$). *Cxcl9* of DFDBA group statistically significant upregulated comparing with control group (**Figure 10**). At three months, the relative gene expression of *Bcl2a1* and *Cxcl9* showed the difference among groups without statistically significance ($P > 0.05$) (**Figure 11**). Analysis results of *Bcl2a1* and *Cxcl9* expression within group are shown in **Figure 12** and **Figure 13**, respectively. An independent t-test analysis indicated that there was no statistically significant difference ($P > 0.05$) on the relative gene expression between two time-points.

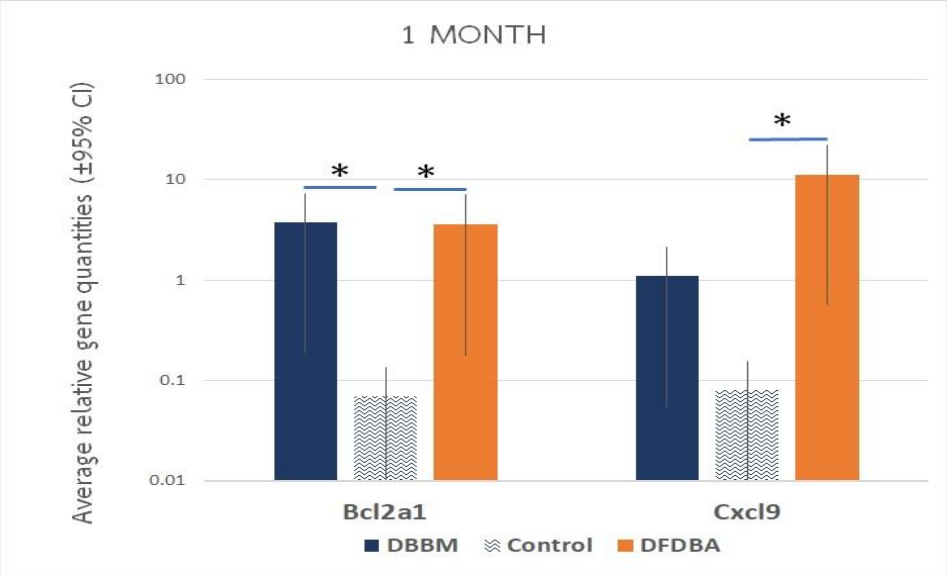

Figure 10 RT-qPCR of *Bcl2a1* and *Cxcl9* relative expression at 1 month Each sample was analyzed and 18s rRNA was used as the reference gene. Bars represent logE Mean ± 95% CI, *$P < 0.05$.

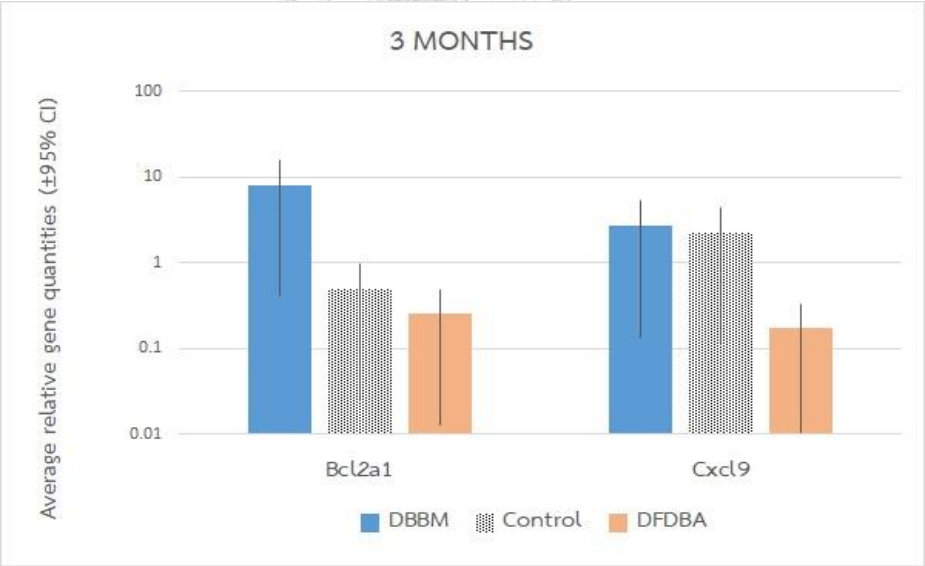

Figure 11 RT-qPCR of *Bcl2a1* and *Cxcl9* relative expression at 3 months Each sample was analyzed and 18s rRNA was used as the reference gene. Bars represent logE Mean ± 95% CI, *$P < 0.05$.

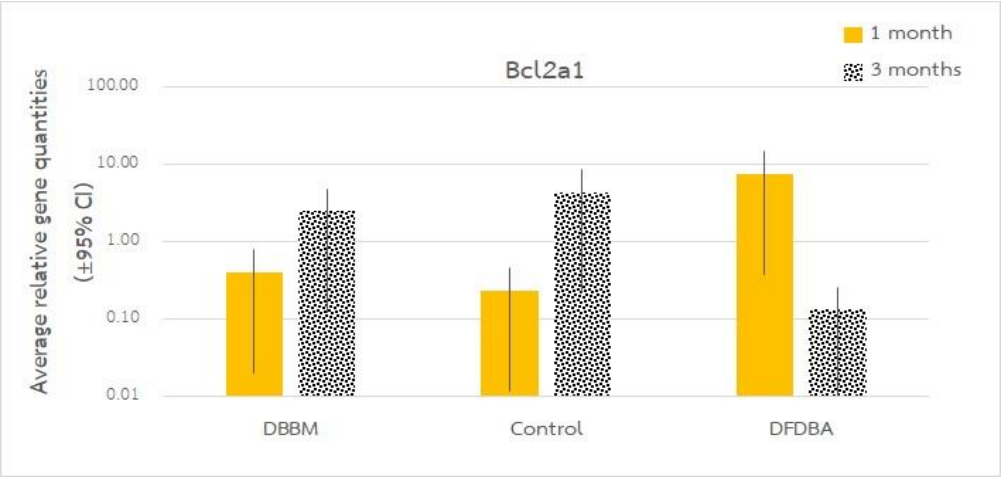

Figure 12 RT-qPCR of *Bcl2a1* relative expression between 1 and 3 months Each sample was analyzed and 18s rRNA was used as a reference gene. Bars represent logE Mean ± 95% CI, *P < 0.05.

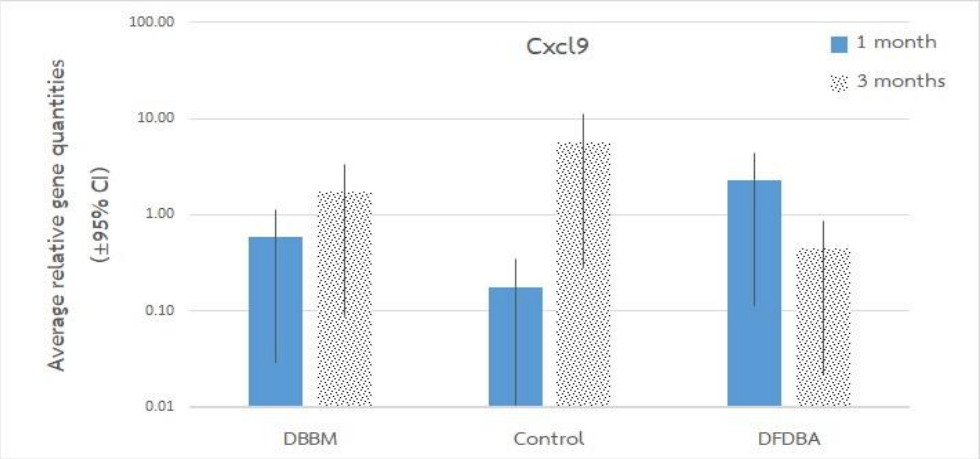

Figure 13 RT-qPCR of *Cxcl9* relative expression between 1 and 3 months Each sample was analyzed and 18s rRNA was used as a reference gene. Bars represent logE Mean ± 95% CI, *P < 0.05.

CHAPTER V
DISCUSSION

The aim of this study was to assess the expression of *Bcl2a1* and *Cxcl9* genes in soft tissues covering bone grafted with DFDBA and DBBM in comparison with the ones covering bone without grafting. The study used two types of commercial bone grafts and a bare defect as independent variable groups. A relative gene expression as a dependent variable group. Controlled variables consist of the methodology of animal sampling, surgical procedures, RNA purification technique, and quality of nucleic acid. Bio-Oss® is a bovine xenograft collected from different species except human. A deproteinization process was done to reduce an immunologic reaction of this graft. OraGRAFT® derived from the same species of human, but different genotypes. Although these bone grafts are biocompatible, the residual graft in the augmented area may still stimulate chronic inflammation.

The RT-qPCR result showed the relative expression of *Bcl2a1* gene was significantly upregulated in DBBM group compared to control group ($P < 0.05$). DBBM group also upregulated *Cxcl9* gene compared to control group but statistical significance was not detected. DFDBA group significantly upregulated both genes compared to control group. From the result at one month, we found *Bcl2a1* and *Cxcl9* also upregulated in DBBM and DFDBA group compared to control group. However, at three months, *Bcl2a1* and *Cxcl9* genes were also expressed in each group. But the difference of expressions were not detected. According to the study of Anderson et al 2008, they found biomaterial surface properties play an important role in modulating the foreign body reaction in the first two to four weeks following implantation of biomaterial in the tissue [11]. In 2013, Brown and Badylak proposed that surface properties such as a particle size, porosity, and ions that can be released from the biomaterial are all biomaterial-specific factors influencing the immune response [75]. From this situation, properties of DFDBA and DBBM could regulate an inflammatory response in the soft tissues. The manufacturer reports a particle size of Bio-Oss® ranging from 250 to 1000 μm. But, Berberi et al 2014 found median particle

size of Bio-Oss® was only 1.32 μm [76]. In 2003, Laquerriere et al found hydroxyapatite with a small size (1 – 30 μm) could modulate inflammatory cells to produce the greatest amount of pro-inflammatory cytokines [77]. In accordance with the study of Laquerriere et al, bovine hydroxyapatite might play a role to stimulate inflammatory cells in our study as well. The main composition of DFDBA (OraGraft®) is collagen type I and particle size of DFDBA using in this study is 250 to 710 μm. In 2005, Honsawek et al analyzed the compositions of DFDBA (OraGraftTM, LifeNet®) using western blot analysis and found Bone morphogenic protein–4 (BMP-4) as one of the composition of this material [78]. In 1971, Urist et al published mesenchymal cells of muscle were induced by BMP-4 and differentiated into bone cells and bone marrow cells [79]. However, BMP-4 acts as an inflammatory factor by stimulating a specific adhesion molecule, ICAM-1 and Promote leucocyte extravasation in vivo [80, 81]. All of these previous studies, it may contribute to our finding that, at one month, *Bcl2a1* and *Cxcl9* of DBBM and DFDBA group were increased in an expression in comparison with control group.

At three months, *Bcl2a1* and *Cxcl9* genes were also expressed among groups but without significant difference (**Figure 11**). The relative expression of *Bcl2a1* and *Cxcl9* was found no statistically significant difference between two time-points. Interestingly, the expression of *Bcl2a1* and *Cxcl9* of DBBM and control group was increased at three months compared to one month. *Bcl2a1* demonstrated the trend of up-regulation like *Cxcl9*. In contrast, *Bcl2a1* of DFDBA group was decreased at three months compared to one month. *Cxcl9* also showed the trend of down-regulation similar to *Bcl2a1* (**Figure 12 and 13**). At 3 months, the data showed *Bcl2a1* and *Cxcl9* expressions of DFDBA and control group were higher than DBBM group. It might be due to the degradation rate of DBBM and DFDBA and resorption rate of normal bone healing. At the DFDBA grafted area and alveolar bone defect, there were begun to resorb within 2 - 4 months but DBBM in grafted area need more times about 15 – 30 months for degradation [82]. According to the previous study of Kangwannarongkul et al 2018, micro-computed images illustrated DFDBA group showed a high resorption rate of grafting particle at three months but DBBM group showed a slow resorption rate [20]. According to the result of Yang et al 2014, they

had grafted DFDBA (OraGraft®) in a space femur defect. After four weeks, the residual graft remained only 33% [83]. Chronic inflammation can result from the exposure to a low level of a particular irritant or foreign materials that cannot be eliminated by enzymatic breakdown or phagocytosis in the body [84]. So, by the time, when DFDBA had increased degradation, inflammation induced by chronic irritating also reduced as well. On the contrary, a study in human has shown some particles of DBBM remain more than 10 years after DBBM has been grafted [42]. The main organic composition of DFDBA is collagen type I. In 1978, PostLethwaite et al found degradation product of exogenous collagen type I-III can induced chemotaxis of human fibroblasts [85] to promote tissue structure and functionality [86]. It may be contribute to the results shown in **Figure 12** and **Figure 13** that the expression of *Bcl2a1* and *Cxcl9* of DFDBA group were decreased in comparison with control and DBBM groups at 3 months.

The limitation of this study is too small sample size, so the power of this study was decreased. The expression of *Bcl2a1* and *Cxcl9* might be due to others cells in tissue, not only from inflammatory cells in connective tissue layer [61]. However, *Bcl2a1* and *Cxcl9* may be used as the candidate genes in a further study.

CHAPTER VI
CONCLUSION

Within the limitation of this study, at one month, the expression of *Bcl2a1* was significant difference between DBBM and DFDBA comparing with control group. The expression of *Cxcl9* was also significant difference between DFDBA comparing with control group. The comparison among groups at three months and comparison within group between one and three months shown the expression of both genes but without significant difference. However, the further study should be performed with larger sample sizes, and immunohistochemistry need to be confirmed these results.

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APPENDIX

Table 5 RNA concentration and quality

Sample labeling indicate: SC = control group, SB = DBBM group, and SD = DFDBA group. A number in ten digit indicate month (1= one month and 3 = three months) and one digit = number of mice.

No.	Sample	RNA conc. (ng/µl)	A 260/280
1	SC17	17.8	1.9
2	SC13	12.6	2.16
3	SC11	25.4	2.2
4	SB11	28.5	2.17
5	SB16	38.6	1.85
6	SB13	22.7	2.08
7	SD18	41.6	1.99
8	SD17	40.2	2.27
9	SD12	141.2	2.14
10	SC37	11.6	2.02
11	SC33	12.9	1.86
12	SC36	31.1	2.09
13	SB33	20.4	2
14	SB39	16.6	2.15
15	SB34	30.3	1.9
16	SD34	15	2.19
17	SD31	17.8	1.9
18	SD39	6.6	2.08

Table 6 Average CT value of qPCR

Sample labeling indicate SC = control group, SB = DBBM group, and SD = DFDBA group. A number in ten digits indicate month (1= one month and 3 = three months) and one digit = number of mice. 18s rRNA as a reference gene.

No.	Sample	18s rRNA	*Bcl2a1*	*Cxcl9*
1	SC17	29.93	28.84	30.49
2	SC13	27.59	32.04	33.73
3	SC11	24.91	30.87	30.41
4	SB11	26.80	26.16	28.78
5	SB16	25.99	25.72	28.13
6	SB13	35.86	37.02	33.28
7	SD18	25.07	26.00	22.01
8	SD17	27.67	25.93	28.12
9	SD12	24.46	24.58	27.18
10	SC37	25.48	27.71	30.66
11	SC33	29.78	29.67	31.72
12	SC36	30.27	32.34	29.92
13	SB33	27.26	25.54	28.86
14	SB39	29.87	25.45	29.26
15	SB34	30.51	26.59	29.06
16	SD34	28.13	29.80	32.29
17	SD31	27.10	29.32	32.0
18	SD39	29.27	32.19	35.80

Table 7 qPCR results of *Bcl2a1* and *Cxcl9* relative expression at 1 month Each sample was analyzed and 18s rRNA was used as the reference gene.

Bcl2a1			
Group	Mean	95% value CI low	95% value CI high
DBBM	3.74	-1.479E-001	1.294E+000
Control	0.07	-4.007E+000	1.754E+000
Control	3.61	-4.569E+000	5.684E+000
Cxcl9			
Group	Mean	95% value CI low	95% value CI high
DBBM	1.09	-2.849E-001	3.613E-001
Control	0.08	-2.315E+000	1.328E-001
Control	11.29	2.274E-006	7.749E+000

Table 8 qPCR results of *Bcl2a1* and *Cxcl9* relative expression at 3 months Each sample was analyzed and 18s rRNA was used as the reference gene.

Bcl2a1			
Group	mean	95% value CI low	95% value CI high
DBBM	8.13	-4.244E+000	6.064E+000
Control	0.49	-4.478E+000	3.857E+000
DFDBA	0.25	-1.652E+000	4.533E-001
Cxcl9			
Group	mean	95% value CI low	95% value CI high
DBBM	2.72	-3.790E+000	4.660E+000
Control	2.21	-4.050E+000	4.737E+000
DFDBA	0.17	-2.204E+000	6.456E-001

Table 9 qPCR results of *Bcl2a1* and *Cxcl9* relative expression within group between 1 and 3 months

Each sample was analyzed and 18s rRNA was used as

Bcl2a1					
Group	Time (month)	Mean	95% value CI low	95% value CI high	*P*
DBBM	1	0.40	-1.114E+000	3.272E-001	0.30
	3	2.48	-4.761E+000	5.548E+000	
Control	1	0.23	-3.511E+000	2.241E+000	0.10
	3	4.32	-3.532E+000	4.803E+000	
DFDBA	1	2.26	-4.773E+000	5.480E+000	0.32
	3	0.44	-1.406E+000	6.994E-001	
Cxcl9					
Group	Time (month)	Mean	95% value CI low	95% value CI high	*P*
DBBM	1	0.58	-5.591E-001	8.711E-002	0.39
	3	1.72	-3.989E+000	4.461E+000	
Control	1	0.18	-1.979E+000	4.688E-001	0.12
	3	5.69	-3.638E+000	5.149E+000	
DFDBA	1	7.56	-5.818E+000	7.574E+000	0.17
	3	0.13	-2.303E+000	5.462E-001	

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