Gene expression analysis of *MMP12* and *S100A4* of grafted bone using allograft and xenograft in mice

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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 2019
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การศึกษาลักษณะการแสดงออกของยีนเอ็มเอ็มพี12 และเอส100เอ4 ของกระดูกปลูกถ่ายชนิดเอกพันธุ์และวิวิธพันธุ์ในหนู

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Thesis Title	Gene expression analysis of *MMP12* and *S100A4* of grafted bone using allograft and xenograft in mice
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Field of Study	Prosthodontics
Thesis Advisor	Assistant Professor JAIJAM SUWANWELA, Ph.D.

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ทิพาพร จุไรรัตน์พร : การศึกษาลักษณะการแสดงออกของยีนเอ็มเอ็มพี12 และเอส100เอ4 ของกระดูกปลูกถ่ายชนิดเอกพันธุ์และวิวิธพันธุ์ในหนู. (Gene expression analysis of *MMP12* and *S100A4* of grafted bone using allograft and xenograft in mice) อ.ที่ปรึกษาหลัก : ผศ. ทพญ. ดร.ใจแจ่ม สุวรรณเวลา

การศึกษานี้มีวัตถุประสงค์เพื่อประเมินยีนที่น่าสนใจ ซึ่งบ่งชี้การทำงานของเซลล์สร้างกระดูก และเซลล์สลายกระดูก ภายหลังการปลูกถ่ายกระดูกด้วยวัสดุปลูกถ่ายเอกพันธุ์ชนิดที่ผ่านการละลายของแร่ธาตุภายใต้สภาวะแช่แข็งหรือดีเอฟดีบี (DFDBA) และวัสดุปลูกถ่ายวิวิธพันธุ์ที่ได้จากวัวที่ผ่านการสลายโปรตีนหรือดีบีบีเอ็ม (DBBM) เปรียบเทียบกับการหายของกระดูกตามธรรมชาติ

วิธีการทดลอง หนู C57BL / 6MLac เพศชาย 9 ตัว จะถูกสร้างช่องว่างบนกระดูกแคลวาเรียมในส่วนกระดูกพารัยทอลทั้งสองข้าง และถูกแบ่งออกเป็น 3 กลุ่ม โดยกลุ่มที่ 1 กลุ่มควบคุมคือไม่มีการปลูกกระดูกในช่องว่าง กลุ่มที่ 2 ทำการปลูกกระดูกด้วย DFDBA และกลุ่ม 3 ทำการปลูกกระดูกด้วย DBBM การแสดงระดับของเอ็มอาร์เอ็นเอของยีนเอ็มเอ็มพี12 (*MMP12*) และเอส100เอ4 (*S100A4*) จะถูกวิเคราะห์โดย ใช้ปฏิกิริยาลูกโซ่โพลิเมอร์แบบเรียลไทม์เชิงปริมาณในเดือนที่ 1 และเดือนที่ 3

ผลการศึกษาแสดงให้เห็นว่าในเดือน 3 ทั้งในกลุ่ม DFDBA และกลุ่ม DBBM มีการแสดงออกของยีน *S100A4* สูงกว่า *MMP12* อย่างมีนัยสำคัญ นอกจากนี้การแสดงออกของ *MMP12* และ *S100A4* ในกลุ่ม DFDBA และกลุ่ม DBBM สูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ และยังมีการแสดงออกของยีน *MMP12* ในกลุ่ม DFDBA มีการลดลงอย่างมีนัยสำคัญเดือน 3 เมื่อเทียบกับเดือนที่ 1

สรุปผลศึกษาได้ว่า DFDBA และ DBBM ส่งเสริมการปรับปรุงกระดูกในเดือน 3 นอกจากนี้ DFDBA มีคุณสมบัติที่จะช่วยให้การสร้างกระดูกดีกว่า DBBM

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5975814132 : MAJOR PROSTHODONTICS
KEYWORD: Real-time PCR, DFDBA, DBBM, MMP12, S100A4

Tipaporn Jurairutporn : Gene expression analysis of *MMP12* and *S100A4* of grafted bone using allograft and xenograft in mice. Advisor: Asst. Prof. JAIJAM SUWANWELA, Ph.D.

Objectives: This study aimed to evaluate the genes of interest that could explain osteoblast and osteoclast activities in bone remodeling after bone grafting with demineralized freeze-dried bone allograft) (DFDBA) and demineralized bovine bone mineral (DBBM) to compare with normal bone healing.

Methods: Calvarium defects were created on both side of the parietal bone in nine male C57BL/6MLac mice. Defects were divided into 3 groups; group 1: defect without grafting as a control group, group 2: defect grafted with DFDBA, and group 3: defect grafted with DBBM. The mRNA expression levels of *MMP12* and *S100A4* were analyzed using real-time PCR in 1month and 3 months.

Results: In month 3, the expression of *S100A4* gene was significantly increased, compared with *MMP12* gene in both DFDBA and DBBM groups. Moreover, the expression of *MMP12* and *S100A4* genes was significantly increased in both DFDBA and DBBM compared to the control group. In DFDBA group, the expression of *MMP12* gene was significantly decreased in month 3 compared to month 1.

Conclusion: The DFDBA and DBBM promoted bone remodeling in month 3. In addition, DFDBA has properties to help bone formation better than DBBM.

Field of Study:	Prosthodontics	Student's Signature ....
Academic Year:	2019	Advisor's Signature ....

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ACKNOWLEDGEMENTS

The authors would like to express their sincerest gratitude to my advisor, Assistant Professor Dr Jaijam Suwanwela, who has supported me throughout my study. I also would like to thank Dr. Thanyaporn Kangwannaronkul for kind suggestion and help in the sample collection. Finally, I would like to thank the service from the Biomaterial testing center of Chulalongkorn University. This research was supported by the faculty of dentistry, Chulalongkorn University.

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LIST OF ABBREVIATIONS

MMP12	*Matrix metalloprotein12*
S100A4	*S100 Calcium Binding Protein A4*
DFDBA	Demineralized freeze-dried bone allograft
DBBM	Demineralized bovine bone mineral
RUNX2	*Runt related transcription factor 2*
M-CSF	Macrophage-colony-stimulating factor
BMPs	Bone morphogenic proteins
MSCs	Mesenchymal stem cells
IL-6	Interleukin 6
RANKL	*Receptor Activator of Nuclear Factor kappa-B ligand*
TNF	Tumor necrosis factor
TGF	Transforming growth factor
PDGF	Platelet-derived growth factor
IGF-I	Insulin-like growth factor I
IGF- II	Insulin-like growth factor II
MCP-1	Monocyte chemoattractant protein 1
CSF-1	Colony stimulating factor 1
OPG	Osteoprotegerin
MMPs	*Matrix metalloproteinases*
TGF- 3	Transforming Growth Factor

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PDGF	Platelet-derived growth factor
FGF	Fibroblast growth factor
FDBA	Freeze-dried bone allograft

CHAPTER I INTRODUCTION

Dental implant success requires both quantity and quality of alveolar bone [1-3]. They are considered as the key factors for primary implant stability and an influence of the load-bearing capacity of the implant [4].

Alveolar bone resorption in patient is more rapid in the first 3-6 months after tooth extraction which 52% of resorption of bone volume occurs in the first 3 months and 72% of resorption of bone volume reduces within 1 year after extraction [3, 5]. Bone grafting is used at the bone defect to repair and augment the area to provide the structural and mechanical support for dental implants [5].

The gold standard for osseous regeneration is using an autograft, a bone taken from patients' body. This is due to the fact that its properties are osteogenic, osteoinductive and osteoconductive [6, 7]. However, its low availability and donor site morbidity necessitate the development of alternative products, such as allografts and xenografts [5, 8]. Demineralized freeze-dried bone allograft (DFDBA) is a commonly used material because it has shown good clinical results for many years. The most likely reason why DFDBA provides an osteoconductive and osteoinductive effect is because of the presence of bone morphogenic proteins (BMPs)[6, 9, 10]. These BMPs promote bone formation [6]. However, the material also has disadvantages such as the possible transmission of diseases [6]. For this reason, another graft material such as deproteinized bovine bone mineral (DBBM) has been promoted. This grafting material

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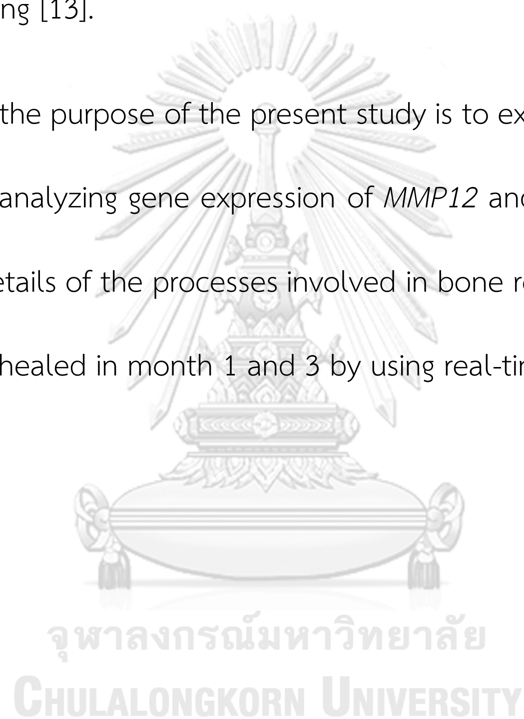
serves as the scaffold and the ingrowth of neovascular tissue and infiltration of osteogenic precursor cells in the grafted site [11, 12].

Data from one of our previous studies which we used two types of bone grafts mentioned above in mouse calvaria defects showed a significant activation of osteoblast gene expression and a slow resorption of graft material compared with natural bone grafting [13].

Therefore, the purpose of the present study is to extend the data described in the late study by analyzing gene expression of *MMP12* and *S100A4* in an attempt to explain in more details of the processes involved in bone remodeling after the defect in mice had been healed in month 1 and 3 by using real-time PCR.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CHAPTER II REVIEW OF LITERATURE

Implantology

Dental implants have been used since 1965 to replace natural root of the tooth and help in installing a dental prosthesis [3]. The conventional protocol for dental implantation was first proposed by Dr. Branemark, an orthopedic surgeon who was also a research professor in the study of implants and surrounding bone [3, 14]. It is consisted of 2 phases; the first phase is the placement of an implant in the surgical cavity, which requires 3-6 months for wound healing, depending on bone quality and the region of implantation, the second phase is a placement of the actual prosthesis [1]. Osseointegration is a microscopic phenomenon in which bone and the biomaterial implant become fused in order to replace load-bearing organs by restoring their form and function. This process is included with new bone growth, the integration of bone formation and stabilization of new bone, which depends on the mechanical connection of the implant surface and surrounding bone [3].

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Implant anchorage is divided into primary, secondary, and tertiary stability. Primary stability occurs immediately after the insertion of a dental implant [3], while secondary stability is obtained after osseointegration. Tertiary stability is the maintenance of osseointegration. Primary stability has an essential role in osseointegration and is dependent on bone quality and quantity, dimensions of implants, surgical technique, and loading condition. There are many factors that influence osseointegration [1, 3, 4, 14, 15].

Bone remodeling process

Bone is a supporting framework of the body. The structure of bone is constituted by inorganic and organic components. It is composed of support cells (osteoblast and osteocytes) and remodeling cells (osteoclasts) [16]. The fracture healing process has overlapping phases, including the inflammatory phase, reparative phase, and remodeling phase. The inflammatory phase consists of sub-stages, which are the stage of hematoma formation and stage of granulation tissue. While the reparative phase consists of stages of fibrocartilaginous callus and bony callus (proliferation of fibroblasts and collagen synthesis). The last phase, remodeling phase, which consists of bone-resorbing osteoclasts and bone-forming osteoblasts, is arranged within temporary anatomical structures, known as “basic multicellular units” (BMUs) [17].

The bone remodeling can be divided into the following six phases: quiescent, activation, resorption, reversal, formation, and mineralization.

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The quiescent phase is the phase when the bone is at rest. The following phase is the initial “activation” stage where the activation of mononuclear monocyte-macrophage osteoclast precursors starts. This leads to the differentiation, migration, and fusion of the large multinucleated osteoclasts. These cells are attached to the mineralized bone surface, and initiate resorption by the secretion of hydrogen ions and lysosomal enzymes along with cathepsin K to degrade the components of bone matrix,

including collagen, and low pH condition [18-21]. The next phase is the resorption phase, in which macrophages and foam cells release growth factors contained within the matrix, fundamentally transforming growth factor (TGF), platelet-derived growth factor (PDGF), and insulin-like growth factor I and II (IGF-I and II). After the parathyroid hormone (PTH) induced bone remodeling, osteoblasts produce the chemokine monocyte chemoattractant protein-1 (MCP-1) to induce osteoclastogenesis by using the osteoblast expression of osteoclastogenesis cytokines, colony-stimulating factor-1 (CSF-1) and RANKL. Moreover, there is also osteoprotegerin (OPG) which is modulated in response to PTH. The OPG expression is reduced, while CSF-1 and RANKL production is increased to promote osteoclast formation. RANKL will not only promote the proliferation of osteoclast precursors but also initiates resorption activity and prolongs the life of the mature cells. The mature osteoclasts dissolve the mineral matrix and decompose the osteoid matrix producing Howship's resorption lacunae. Before changing to formation phase, the bone remodeling will undergo reversal phase, the phase which bone resorption shifts to bone formation. The coupling signals connecting the termination of bone resorption to the beginning of bone formation are bone matrix-derived factors, such as transforming growth factor (TGF- 3), IGF-1, IGF-2, BMPs, platelet-derived growth factor (PDGF), and fibroblast growth factor including (FGF) [18-22]. Then, the formation phase occurs. The preosteoblasts synthesize a cementing substance, which the new tissue is attached. BMPs is responsible for the differentiation and the completed and differentiated osteoblasts synthesize the osteoid matrix. This

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is followed by the last phase, which is the mineralization phase. This process consists of a slow and gradual maturation of the mineral component which influences the mechanical strength of bone tissue [23].

Bone augmentation

It is understood that alveolar bone dissolves rapidly at 25% in the width of the crestal bone during the first year after the extraction, which may reach 40% in 1-3 years [24, 25]. As a result, the position of the labial plate changes from its beginning location. The preservation or recontouring of the labial appearance of the alveolar process is one of the keys to optimum implant aesthetics and long-term results [24].

To satisfy the goals of implant dentistry, hard and soft tissues need to be presented with inadequate volumes and quality. Bone augmentation techniques may be applied to extraction socket, defect grafting, horizontal ridge augmentation, vertical ridge augmentation, and sinus augmentation [5, 26]. They are included with particulate grafting, membrane use, block grafting, and distraction osteogenesis, either alone or combination. To achieve this goal of therapy, it is desirable to provide a treatment that will aim at the preservation of the natural tissue contours as a preparation for the proposed implant prosthesis [25].

Bone graft material

Bone grafting is a surgical procedure for missing bone replacement with a material called a bone graft. Not only does this material replace missing bone but also

have been used for bone defect repair and alveolar ridge augmentation to provide structural and mechanical support for the placement of dental implants. Bone grafts are used as a filler and scaffold to facilitate bone formation and promote wound healing. This new bone growth strengthens the grafted area by forming a bridge between your existing bone and the graft. Over time the newly formed bone replaces much of the grafted material [5-7, 11, 27, 28].

An ideal bone graft substitute should be followings: biomechanically stable, able to degrade within an appropriate time frame, exhibit osteoconduction, osteogenesis and osteoinduction properties, and provide a favorable environment for invading blood vessel and bone forming cell [6, 28].

The biologic mechanisms that provide a rationale for bone graft healing process are osteogenesis, osteoinduction, and osteoconduction [6, 10].

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Osteogenesis: It is the process of new bone formation development using cells of bone graft from donor cells. Osteoprogenitor/osteogenic precursor cells within a graft material differentiate into osteoblasts, forming new bone structures that are connected to a recipient site. It is often found in bone autogenous bone graft [6, 10, 25, 28].

Osteoinduction: This material contains protein that is derived from a bone graft and is the growth factor for bone morphogenetic proteins (BMPs) [29]. Its role is organized into undifferentiated mesenchymal stem cells (MSCs) and osteoblast/osteoid. This type of treatment can be found in autografts and allografts [6, 10, 25, 28].

***Osteoconduction*:** While it grows on a surface, it uses graft material formation. Bone graft enhances the scaffold for bone formation to graft tissue in the growth of vascular tissue (neurovascular cell) into the graft site and mesenchymal cells (osteogenic precursor cell) [6, 10, 25, 28].

Bone graft material is divided into four types: autograft, allograft, xenograft, and alloplastic.

Autogenous bone graft

Autogenous bone graft is classified as the gold standard for osseous regeneration simply due to its reputation and the fact that it includes osteogenic, osteoinductive, osteoconductive properties, and its non-immunological response [6, 10, 30, 31]. Grafts are harvested from patients' extraoral (cranium, hip, tibia) and intra-orally (tuberosity, mandibular symphysis and retremium space). It requires two surgeries that would accumulate into higher morbidity cooperation. Operating time is increased by 8.6%, and it is more prone to complications leading to infection, with constant pain and loss of blood. An autogenous bone graft is indeed costly, which is the reason why other options for graft bone material are being utilized such as allograft and xenograft [6].

Allogenous bone graft

Allograft is derived from the human body that is harvested from another person, and it is freeze-dried to be preserved and sterilize the material. There are three

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types of bone allograft: fresh frozen bone allograft, freeze-dried bone allograft (FDBA), and demineralized freeze-dried bone allograft (DFDBA). FDBA and DFDBA are the materials commonly used because they have provided good clinical results for many years. FDBA provides a source of type I collagen, which is a major organic component of bones, while DFDBA contains demineralized inorganic substance. While both DFDBA and FDBA are osteoconductive, only DFDBA has been proven to be osteoinductive because demineralization by osteoclast is necessary in order to release BMPs from the mineralized matrix. However, demineralized bone does not produce the inorganic calcium that serves as scaffolding needed for bone regeneration [9, 28].

DFDBA shows more significant bone formation and lower amounts of graft material than FDBA after grafting process in human for 19 weeks (by Wood and Mealey) [32]. The osteoinductive potential of DFDBA is related to the amount of BMPs, which can be either active or inactive due to a number of functions. If it were inactive, the inductive properties would lose [29].

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Xenogenous bone graft

Xenograft is a graft material acquired from the bones of other species, i.e. bovine or pig, which is the deproteinized bone from inorganic bovine and has the familiar properties. Its compositions with biomechanical properties are similar to the properties of human bone. Human bone would extract protein, which avoids immunological reactions and at the same time gets rid of the substance during

osteoinduction. Xenograft is the graft material serving as a scaffolding, and it allows the ingrowth of neovascular and infiltration of osteogenic precursor cells go into the graft site [33, 34].

Demineralized bovine bone material (DBBM) is considered as a slow resorbing bone substitute. The inorganic component of DBBM is crystalline hydroxyapatite, and the organic components in DBBM are removed by efficient chemical and thermal processes [12]. The long-term clinical studies show a positive effect of DBBM in enhancing the lasting stability of the vertical augmentation [35]. In addition, *in vivo* experiments, the result shows that the new bone was formed and fused using DBBM particles after 2 weeks [33]. Bio-Oss® is low-resorbable DBBM. It has a compressive strength of 35 Mpa and 75-80% porosity. It promotes osteogenesis, increases angiogenesis, and represents a scaffold for bone formation [36]. The physical and chemical properties are very similar to human bone [37, 38].

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Alloplastic (synthetic) graft

Alloplastic grafts are derived from non-biological materials such as calcium sulfate, hydroxyapatite, bioactive glass, and hard-tissue replacement polymer. This type of graft is qualified as osteoconductive [6, 28, 34].

Selected genes involved in bone remodeling

MMP12

The matrix metalloproteinases play important roles in bone remodeling and repair, in which an imbalance or a nonfunctioning enzyme may lead to defection in bone healing (nonunion) [39]. *MMPs* play active roles in the migration of inflammatory cells, degradation and remodeling of extracellular matrix proteins, and in the angiogenesis process, described as being essential for bone healing [39]. Some reports have shown osteoclast requires *MMP* activity to move and to solubilize bone matrix. *MMP12* also promotes macrophage migration and angiogenesis, which is the important regulator of these repair processes [40]. Studies into *MMP12* in mice show *MMP12* expression of osteoclasts in calvaria and long lone, but *MMP12* does not appear to be critical for osteoclast recruitment or resorption in bone remodeling or grafting bone resorption after complete bone formation [41]. *MMP12* expression was higher in TRAP-positive multinucleated cells. *In vitro*, the generated TRAP-positive multinucleated cells possess the characteristics of osteoclasts [42].

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S1004A

S100A4 is a member of the calcium-binding S100 protein family, which is expressed by several normal cell types in various human, bovine, rat, and mouse tissues and found in, such as, smooth muscle, liver, bone marrow, smooth muscle cells of arteries, and osteoblastic cells [43]. A study in 1997 reported *S100A4* was expressed by several cell types, including mesenchymal cells surrounding the cartilage model of developing bones and those surrounding the tooth bud during mouse

embryonic development. *S100A4* may play a role in osteoblast differentiation *in vivo* [43]. *S100A4* mRNA is highly expressed by osteoblastic cells at the early stages of osteoblast differentiation before matrix mineralization and is absent when the tissue is fully mineralized [43]. *S100A4* expression decreases to undetectable levels in mature osteoblast and in osteocytes. In addition, *S100A4* is important for the regulation of bone formation by activating the NF-κB signaling pathway in osteoblasts [44].

RESEARCH QUESTIONS

1. Are the gene expressions of *MMP12* and *S100A4* of bone grafted with DFDBA and DBBM different from the gene expressions of *MMP12* and *S100A4* from normal bone healing in 1 month and 3 months?

2. Are the gene expressions of *MMP12* and *S100A4* in bone grafted with DFDBA, DBBM, and normal bone healing at 3 months different from the expression of *MMP12* and *S100A4* in bone grafted with DFDBA, DBBM, and normal bone healing at 1 month?

RESEARCH OBJECTIVES

1. To study the differences in gene expressions of MMP12 and S100A4 of bone grafted with DFDBA and DBBM and the gene expressions of MMP12 and S100A4 from normal bone healing in 1 month and 3 months.

2. To study the differences in gene expressions of MMP12 and S100A4 in bone grafted with DFDBA, DBBM, and normal bone healing at 3 months and the differences in expression of MMP12 and S100A4 in bone grafted with DFDBA, DBBM, and normal bone healing at 1 month.

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HYPOTHESES

$H_0$1: The gene expressions of *MMP12* and *S100A4* of bone grafted with DFDBA and DBBM are not significantly different from the gene expressions of *MMP12* and *S100A4* from normal bone healing in 1 month and 3 months.

H_A1: The gene expressions of *MMP12* and *S100A4* of bone grafted with DFDBA and DBBM are significantly different from the gene expressions of *MMP12* and *S100A4* from normal bone healing in 1 month and 3 months.

$H_0$2: The gene expressions of *MMP12* and *S100A4* in bone grafted with DFDBA, DBBM, and normal bone healing at 3 months are not significantly different from the expression of *MMP12* and *S100A4* in bone grafted with DFDBA, DBBM, and normal bone healing at 1 month.

H_A2: The gene expressions of *MMP12* and *S100A4* in bone grafted with DFDBA, DBBM, and normal bone healing at 3 months are significantly different from the expression of *MMP12* and *S100A4* in bone grafted with DFDBA, DBBM, and normal bone healing at 1 month.

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EXPECTED BENEFIT

In clinical situation, the differences in healing patterns between natural bone and bone graft can be distinguished by the radiographic examination as bone grafting materials show more radiopacity than natural bone. As the effectiveness of bone grafting materials is still in controversy, this study aims to investigate the bone regeneration in animal model after placing deproteinized bovine bone and freeze-dried human bone compared with the ones from natural bone healing by using gene expression. The result can lead to more understanding in clinical use of grafting material and further research applications.

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CHAPTER III RESEARCH METHODOLOGY

Animal used for experiments

This research is the continuation of the previous study: Gene expression and micro-computed tomography analysis of grafted bone using deproteinized bovine bone and freeze-dried human bone by Thanyaporn Kangwannarongkul, DDS., MSc. Graduated student, Department of Prosthodontics, Faculty of dentistry, Chulalongkorn University [13].

Sixty-six 8-week male C57/BL6 mice were obtained from National Laboratory Animal Center of Salaya campus, Mahidol University (Bangkok, Thailand). The experiment was approved by Animal Care and Use Committee of Chulalongkorn University (ethical approval number 1432001). The mice were housed in light and temperature-controlled facilities and given food and water ad libitum. Nine mice were divided into 3 groups according to the type of graft: group 1, bare defect as control; group 2, demineralized freeze-dried human bone [OraGraft™; LifeNet, Virginia, USA]; and group 3, deproteinized bovine bone [Bio-Oss®; Geistlich Pharma AG, Wolhusen, Switzerland].

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Harvest and transplantation of bone graft (all the surgical procedures were done from previous study) Under general anesthesia, Nembutal® (Pentobarbital), were prepared with a phosphate buffered saline into a dilution of 1:10, and the concentration of 4 mg/kg [or 8 μL of dilution/wt (g)] was used. The anesthesia was injected into peritoneum layer, and the surgical field was prepared with alcohol and

povidone iodine after a mouse's hair was removed with blade. Afterwards, subcutaneous of skull was injected by 0.2 ml of 1% lidocaine with 1:100,000 epinephrine. A 1.5 mm midline incision was made on the skull, and the soft tissue and periosteum were elevated. Under constant saline irrigation, the 3-mm diameter circular defects and 0.2 mm in depth were created on both left and right sides of parietal bone by trephine burs. The cavity was created on each side of the parietal bone at 1.5 mm away from sagittal and 3 mm from lambdoid suture (Figure 1). Then, the defect was randomly filled with the bare defect control, OraGraft™ (LifeNet, Virginia, USA) and Bio-Oss® (Geistlich Pharma AG, Wolhusen, Switzerland). The bone graft was then inserted into skull cavity and stitched up with nylon 3-0.

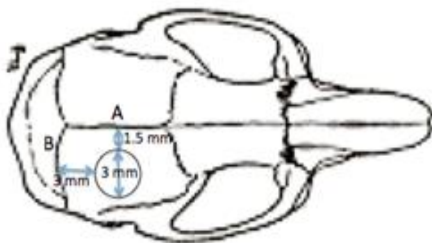

Figure 1 Location of where the defect was created A= sagittal suture; B = lambdoid suture

Under general anesthesia, each animal was utilized at 4 and 12 weeks after grafting. Bone samples that were adjacent to parietal and coronal suture were collected using a 5-mm. diameter trephine bur (Figure 2) and then stored in liquid nitrogen at -80°c.

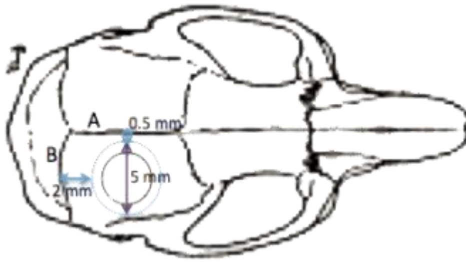

Figure 2 Location of collected sample bone

RNA preparation

RNA was prepared from the previous study. Briefly, bone samples that were adjacent to the defect were collected then lyzed and grounded. After that purified RNA was obtained by RNeasy mini spin column, and then the amount of extracted RNA was determined by a nanodrop machine (Nanodrop 2000, Thermo Fisher Scientific, Inc., USA). The nanodrop machine was used to detect the amount of nucleic acid and protein based on surface tension and light absorption principle.

Real-time polymerase chain reaction

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Complementary DNA (cDNA) was reverted from 50 ng of RNA by Sensiscript® reverse transcription (Qiagen, Qiagen, Inc., USA) system using methods provided by manufacturer. RNA sample was mixed with 10μM Oligo-dT primer, 5mM dNTP mix, 10x Buffer RT, 10 unit/μl RNase inhibitor and Sensiscript® reverse transcriptase and then incubated in 37°C for 60 minutes by thermocycler (Bio-Rad CFX96TM real time -PCR system; Bio-Rad laboratories, Inc., USA). The cDNA was analyzed for gene expression of *MMP12*, and *S100A4* by SYBR® FAST Universal kit (Kapa Biosystem, Inc., USA) with Bio-Rad CFX96TM real-time PCR system (Bio-Rad laboratories, Inc., USA). Rn18s was

used as an internal control. Primer and blast were used for designing primers (http://www.ncbi.nih.gov/tools/primer-blast/). Analysis of relative gene expression was performed using the $2^{-\Delta\Delta CT}$ method. The primers used for real-time PCR are shown in the table below (Table 1).

Gene	Primer sequences	
MMP12	Forward	5`- GCT AGA AGC AAC TGG GCA AC-3`
	Reverse	5`- ACC GCT TCA TCC ATC TTG AC-3`
S100A4	Forward	5`-CCA CAA GTA CTC GGG CAA AG-3`
	Reverse	5`-GTC CCT GTT GCT GTC CAA GT-3`
Rn18s	Forward	5`-AGG GGA GAG CGG GTA AGA GA-3`
	Reverse	5`-GGA CAG GAC TAG GCG GAA CA-3`

Table 1 Primer design

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Statistical analysis

The data was analyzed using Student's t tests (SPSS version 16.0, SPSS Inc., Chicago, USA). The difference of the relative gene expression among groups was evaluated with one-way analysis of variance (ANOVA), followed by Post hoc Tukey's honestly Significance Difference with a significant level of 5%. The difference of the relative gene expression within each group was evaluated with independent t-test with a significant level of 5%.

CHAPTER IV RESULTS

To investigate the effects of bone grafted with DFDBA and DBBM on the mRNA expression of *MMP12* and *S100A4* of mouse calvariae defects, the expressions of genes in bone samples from DFDBA, DBBM and control groups after 1 month and 3 months were measured using real-time PCR.

At 1 month, the expression of *S100A4* gene was not different from *MMP12* gene in the DFDBA and DBBM (Figure 3).

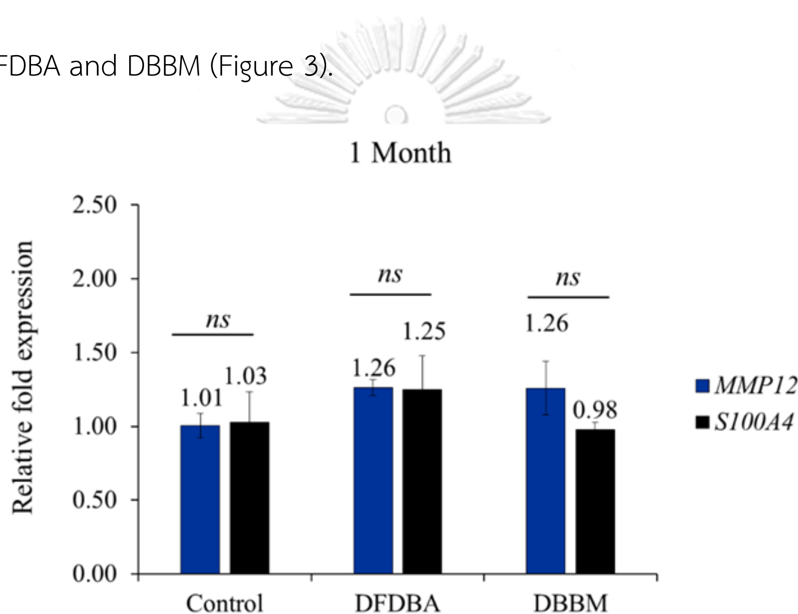

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Figure 3 The expressions between of *MMP12* and *S100A4* genes of the control groups, DFDBA and DBBM after 1 month. Data are represented as mean ± SEM (n=3). ns = not significant (P > 0.05), t-test.

In the control groups, there was 1.019 in the average value of the gene expression of *S100A4* of 1.03-fold and of *MMP12* of 1.01-fold, leading to the proportion of the gene expression of *S100A4* per *MMP12*. In the bone grafted with DFDBA, it presented 0.992 in the average value of the gene express of *S100A4* of 1.25-fold and of *MMP12* of 1.26-fold, leading to the proportion of the gene expression of *S100A4* per

MMP12. In the bone grafted with DBBM, it presented 0.778 in the average value of the gene expression of *S100A4* of 0.98-fold and of *MMP12* of 1.26-fold, leading to the proportion of the gene expression of *S100A4* per *MMP12*.

These results indicate the bone remodeling processes in these following: firstly, the activities of *S100A4*, associated osteoblast cells, contributes to bone formation; secondly, *MMP12*, associated osteoclast cells, contributes to bone resorption. Among three experiment groups, the proportion is close to 1, demonstrating that the trend in bone formation and bone resorption in the remodeling processes in 1-month period has no significant difference in each group.

In addition, no differences in gene expressions of *MMP12* and *S100A4* were found, in both DFDBA and DBBM compared to the control group on month 1 (Figure 4). Likewise, at 1 month, the expressions of *MMP12* and *S100A4* genes were not different in DBBM compared to DFDBA group (Figure 4). These results indicate that the activities of *MMP12*, associated osteoclast cells, and *S100A4*, associated osteoblast cells, were not different among experimental groups in month 1.

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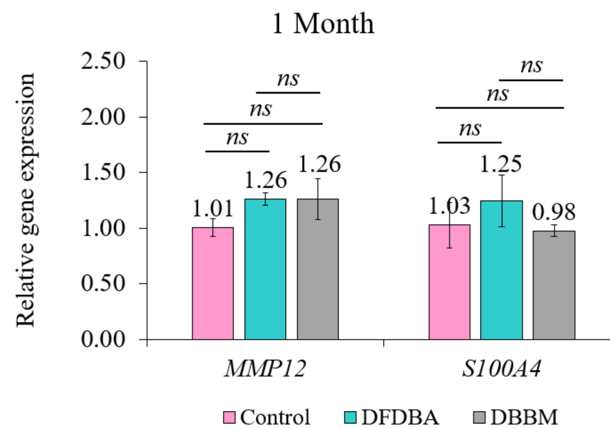

Figure 4 The effect of bone grafted with DFDBA and DBBM after 1 month on *MMP12* and *S100A4* gene expression. Data are represented as mean ± SEM (n=3). ns = not significant (P > 0.05), t-test.

At 3 months, the expression of *S100A4* gene was significantly increased, compared with to *MMP12* gene in both the DFDBA and DBBM groups (Figure 5).

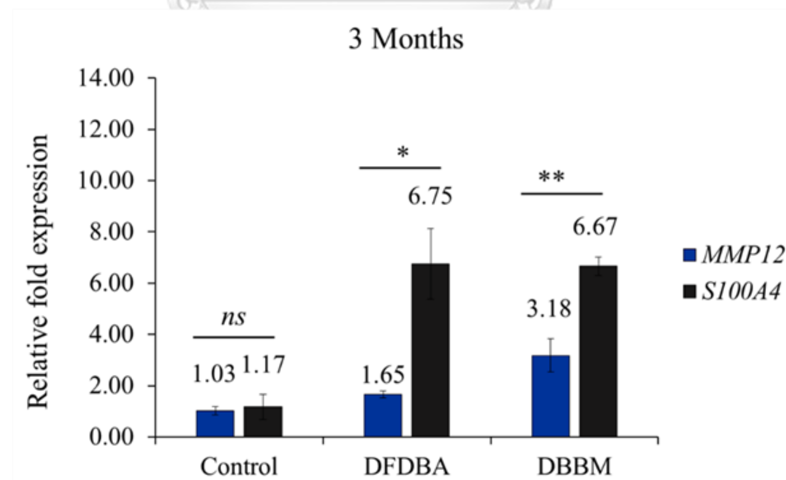

Figure 5 The expression between of *MMP12* and *S100A4* genes of the control groups, DFDBA and DBBM after 3 months. Data are represented as mean ± SEM (n=3). * p < 0.05 and ** p < 0.01 compared to the MMP12 group.

In the control groups, it showed 1.1359 in the average value of the gene expression of *S100A4* of 1.17-fold and of *MMP12* of 1.03-fold, leading to the proportion of the gene expression of *S100A4* per *MMP12*. In the bone grafted with DFDBA, it showed 4.971 in the average value of the gene expression of *S100A4* of 6.7500-fold and of *MMP12* of 1.6475-fold, leading to the proportion of the gene expression of *S100A4* per MMP12. In the bone grafted with DBBM, it showed 2.0951 in the average value of the gene expression of *S100A4* of 6.6667-fold and of *MMP12* of 3.1820-fold, leading to the proportion of the gene expression of *S100A4* per *MMP12*. These results indicate that the bone remodeling processes in the control groups illustrate the proportion which is close to 1, demonstrating that the trend in bone formation and bone resorption in the bone remodeling processes has no considerable difference. For the bone grafted with DBBM, there is a presence of bone formation affecting bone resorption in 4.971 and 2.0951, respectively.

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Moreover, the expressions of *MMP12* and *S100A4* genes were significantly increased in the in both the DFDBA and DBBM groups (Figure 6). When compared the expressions pattern of *MMP12* and *S100A4* genes in DBBM versus DFDBA group, it was found that DBBM had no difference in gene expressions of *MMP12* and *S100A4* in 3 months group compared to DFDBA group (Figure 6). These results showed that the activities of *MMP12*, associated osteoclast cells, and *S100A4*, associated osteoblast cells, in the both DFDBA and DBBM were significantly higher than the control group in

month 3. In addition, the activities of *S100A4* in DFDBD and DBBM were higher than the activities of *MMP12*.

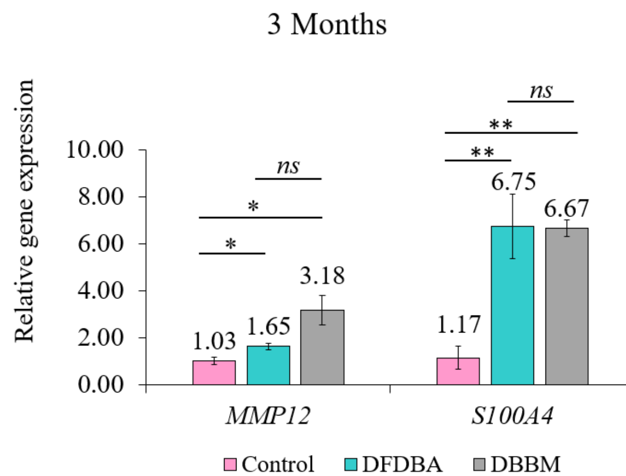

Figure 6 The effect of bone grafted with DFDBA and DBBM after 3 months on *MMP12* and *S100A4* gene expression. Data are represented as mean ± SEM (n=3). * $p < 0.05$ and ** $p < 0.01$ compared to the control group. ns = not significant ($P > 0.05$), t-test.

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To determine the difference in *MMP12* and *S100A4* gene expressions between 1 month and 3 months, the difference expression of genes at 1 month and 3 months was evaluated in each group. In the control group, the expressions of *MMP12* and *S100A4* genes were significantly decreased at 3 months, compared to 1 month (Figure 7A). In DFDBA group, the expression of *MMP12* was significantly decreased, while *S100A4* gene was no significantly increased, as compared to 1 month (Figure 7B). In DBBM group, *MMP12* and *S100A4* gene expressions in 3 months were not significantly different from 1 month (Figure 7C). These results revealed that the activities of *MMP12*

and *S100A4* in control group in month 3 were decreased from month 1. In addition, the activities of *MMP12* in DFDBA in month 3 were decreased from month 1.

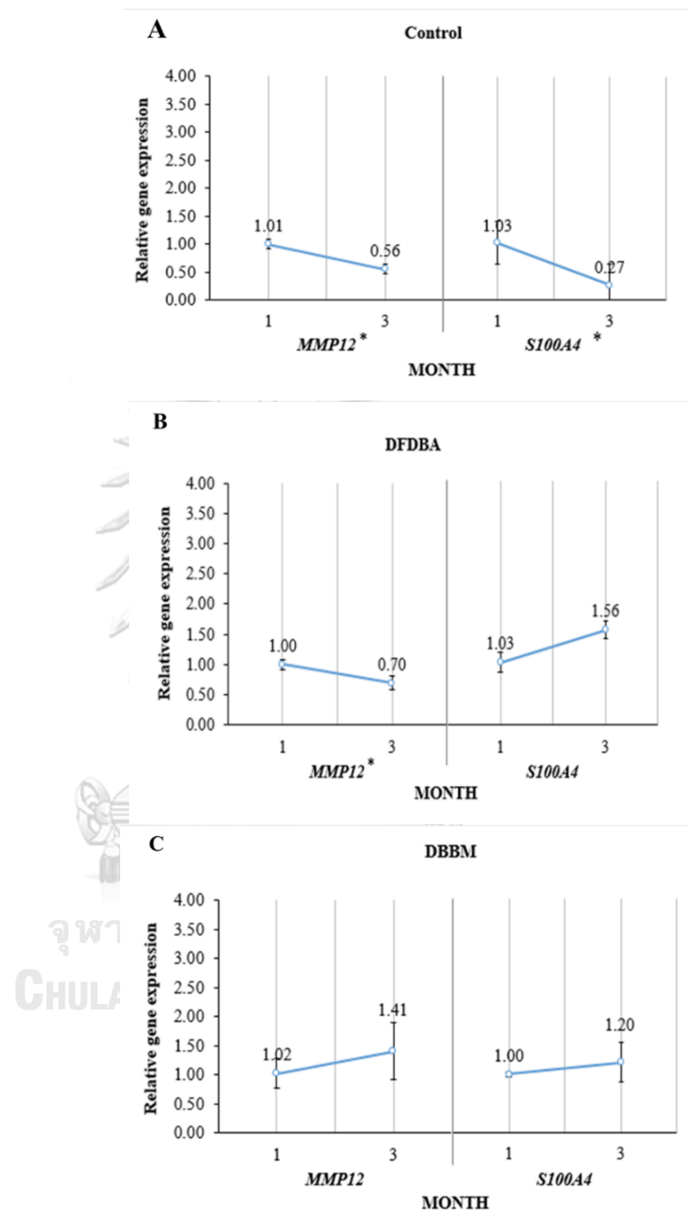

Figure 7 The expression of *MMP12* and *S100A4* gene between month 1 and month 3. Data are represented as mean ± SEM (n=3). * $p < 0.05$ compared to the month 1 group. (A; Control, B; DFDBA, C; DBBM)

CHAPTER V DISCUSSION

Our research was conducted by using DFDBA (OraGraft™) and DBBM (Bio-Oss®). DFDBA is synthesized from human bone and has osteoinduction and osteoconduction properties [10, 32, 34, 45]. DBBM (Bio-Oss®), is synthesized from bovine, which contains a quality of high osteoconduction and different bone grafts [46, 47]. Our research set an objective to find the results of applying those materials for grafting in the period of 1 month and 3 months to understand the involvement of bone resorption and bone formation.

The first gene in the study was *MMP12*, which associated with osteoclast gene that generated bone resorption. *MMP12* expresses in osteoclast in calvariae and long bones, and its expression were higher in TRAP positive cells [41]. The second gene was *S100A4*, which associated osteoblast gene that generated bone formation. *S100A4* mRNA is highly expressed by osteoblastic cells at the stages of differentiation before matrix mineralization [43].

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From the result, it was found that there were changed that occurred in the control group without grafting from 1 to 3 months. In control groups at 3 months, the gene expressions of *MMP12* and *S100A4* showed significantly decreased in expression compared to 1 month. Interestingly, the gene expression of *S100A4* was more decreased than *MMP12* (Figure.7A). These results implied that bone remodeling activity

of normal bone healing had higher bone resorption activity than bone formation activity.

When the defect was grafted with either DFDBA or DBBM, it was found that there were no differences in the gene expressions of *MMP12* and *S100A4* from the control group in 1 month (Figure 3, 4). These results also indicated that there was no difference in bone remodeling activity between the healing with bone grafted materials and normal bone healing in 1 month. This was in accord with the previous study of Kangwannarongkul T. et al. (2018) that there were no statistically significant differences in the gene expressions of bone maker genes, *Runx2, Osx, OPN, OCN, and ALP,* observed among groups (control, DFDBA and Bio-Oss®) in 1 month.

From the result in 3 months, it was found that the bone grafted with the both DFDBA and DBBM had significantly higher gene expressions of *MMP12* and *S100A4* than the control group (Figure 6). This revealed that there was more bone remodeling activity in the bone grafted with both DFDBA and DBBM than the normal bone healing. This result was also similar to the previous study by Kangwannarongkul T et al (2018) that the expression of *Runx2* and *Osx* had significantly higher expression in the DFDBA and Bio-Oss® groups compared to the control group at 3 months [13]. This led to the conclusion that both bone graft materials promoted bone regeneration. Furthermore, James W. Turonis et al. (2006) studied the histological of bone grafted with a 2% DFDBA group as compared to all other groups. They found that bone filled in 3 months

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statistically significant increased in the 2% DFDBA group when compared to all other groups (ie, DFDBA at 1%, 2%, and 3% to 6% residual calcium levels and FDBA at 23% residual calcium) and the control group (no allograft) [48]. Besides, Kyung-Nam Moon et al. (2015) reported that the amount of new bone formation of DBBM (Bio-Oss®) and mineralized allogenic bone (Tutoplast®) compared to the control group in 3 months. It was demonstrated that the amount of new bone formation was significantly higher in the experimental groups (Bio-Oss®, Tutoplast®) than in the control group [49]. Taken together, the bone remodeling activity of bone grafted with both DFDBA and DBBM was higher than normal bone healing in 3 months.

In terms of the gene expressions of the *S100A4* and the *MMP12* in 3 months, it was found that in bone grafted with DFDBA, the proportion of the gene expression of *S100A4* per *MMP12* was 4.0971 (Figure 6). In the same way, in bone grafted with DBBM had the proportion of the gene expression of *S100A4* per *MMP12* was 2.0951 (Figure 6). It showed that the bone grafted with DFDBA had a higher proportion of *S100A4* per *MMP12* than the bone grafted with DBBM. This implied that bone graft with DFDBA had higher bone formation than the bone grafted with DBBM. This result was in accordance with a study of Samer Srouji et al. (2012) that compared the bone formation activity of bone grafted with bone graft materials included Bio-Oss®, OraGraft™, Bi-Ostetic™, and ProOsteon®. They found that the bone formation activity of bone grafted with OraGraft™ and ProOsteon® was higher than Bio-Oss®[50, 51]. Thus, the DFDBA (OraGraft™) could be help generate bone formation better than DBBM (Bio-Oss®).

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From the information above, we have calculated the relative gene expression value from the gene expression equation using the $2^{-\Delta\Delta CT}$ [52, 53]. This method is a convenient way to calculate relative gene expression levels between different samples. The relative gene expression is a fold change compared to the calibrator (untreated sample, time zero, etc.) [52, 53]. All the samples are compared to the calibrator, which means that if we compare the samples to different calibrators, it will get a different gene expression value [53]. For example, in our study, the relative gene expression of *MMP12* or *S100A4* at 3 months in figure 5 and 6, has shown different data from figure 7. This is because the calibrator in figure 5 and 6 is gene expression of *MMP12* or *S100A4* in the control group at 3 months, while the calibrator in the figure 7, which the relation fold change at month 3 is compared to month 1, is gene expression of *MMP12* or *S100A4* in other groups (control, DFDBA, DBBM) at 1 month.

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CHAPTER VI CONCLUSION

The results from this study show the gene expression level demonstrating a continuous bone remodeling after grafting bone with DFDBA and DBBM in mice. The bone remodeling process of transplantation of both DFDBA and DBBM was higher than normal bone healing in the control group at 3 months. In addition, DFDBA has properties to help bone formation better than DBBM.

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APPENDIX

Independent Samples Test of gene expression between *MMP12* and *S100A4* after 1 month (Figure 3)

DFDBA group

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
DFDBA	*MMP12*	3	1.2625	.09808	.05663
	S100A4	3	1.2467	.33292	.19221

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
DFDBA	Equal variances assumed	3.869	.121	.079	4	.941	.01586	.20038	-.54048	.57220
	Equal variances not assumed			.079	2.345	.943	.01586	.20038	-.73556	.76728

DBBM group

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
DBBM	*MMP12*	3	1.2597	.31670	.18285
	S100A4	3	.9767	.07572	.04372

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
DBBM	Equal variances assumed	4.483	.102	1.505	4	.207	.28300	.18800	-.23897	.80497
	Equal variances not assumed			1.505	2.228	.259	.28300	.18800	-.45156	1.01755

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Independent Samples Test of gene expression between bone grafts after 1 month (Figure 4)

MMP12

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
MMP12	Control	3	1.0069	.14286	.08248
	DFDBA	3	1.2625	.09808	.05663

Independent Samples Test

MMP12		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
MMP12	Equal variances assumed	.373	.574	-2.555	4	.063	-.25559	.10005	-.53336	.02218
	Equal variances not assumed			-2.555	3.543	.071	-.25559	.10005	-.54809	.03691

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
MMP12	Control	3	1.0069	.14286	.08248
	DBBM	3	1.2597	.31670	.18285

Independent Samples Test

MMP12		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference Lower	Upper
MMP12	Equal variances assumed	2.083	.222	-1.260	4	.276	-.25273	.20059	-.80965	.30420
	Equal variances not assumed			-1.260	2.782	.303	-.25273	.20059	-.92041	.41496

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
MMP12	DFDBA	3	1.2625	.09808	.05663
	DBBM	3	1.2597	.31670	.18285

Independent Samples Test

MMP12		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
MMP12	Equal variances assumed	3.582	.131	.015	4	.989	.00286	.19141	-.52859	.53431
	Equal variances not assumed			.015	2.380	.989	.00286	.19141	-.70667	.71240

S100A4

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
S100A4	Control	3	1.0300	.29000	.16743
	DFDBA	3	1.2467	.33292	.19221

Independent Samples Test

S100A4		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
S100A4	Equal variances assumed	.159	.710	-.850	4	.443	-.21667	.25491	-.92440	.49107
	Equal variances not assumed			-.850	3.926	.444	-.21667	.25491	-.92968	.49635

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
S100A4	Control	3	1.0300	.29000	.16743
	DBBM	3	.9767	.07572	.04372

Independent Samples Test

S100A4		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
S100A4	Equal variances assumed	1.917	.238	.308	4	.773	.05333	.17304	-.42712	.53378
	Equal variances not assumed			.308	2.271	.784	.05333	.17304	-.61213	.71880

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
S100A4	DFDBA	3	1.2467	.33292	.19221
	DBBM	3	.9767	.07572	.04372

Independent Samples Test

S100A4		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
S100A4	Equal variances assumed	4.767	.094	1.370	4	.243	.27000	.19712	-.27729	.81729
	Equal variances not assumed			1.370	2.206	.293	.27000	.19712	-.50647	1.04647

Independent Samples Test of gene expression between *MMP12* and *S100A4* after 3 months (Figure 5)

DFDBA group

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
DFDBA	*MMP12*	3	1.6475	.23029	.13296
	S100A4	3	6.7500	1.94070	1.12046

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
DFDBA	Equal variances assumed	3.320	.143	-4.522	4	.011	-5.10251	1.12832	-8.23523	-1.96979
	Equal variances not assumed			-4.522	2.056	.043	-5.10251	1.12832	-9.83208	-.37294

DBBM group

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
DBBM	*MMP12*	3	3.1820	1.11122	.64156
	S100A4	3	6.6667	.50836	.29350

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
DBBM	Equal variances assumed	1.670	.266	-4.939	4	.008	-3.48468	.70551	-5.44349	-1.52587
	Equal variances not assumed			-4.939	2.802	.019	-3.48468	.70551	-5.82239	-1.14697

Independent Samples Test of gene expression between bone grafts after 3 months (Figure 6)

MMP12

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
MMP12	Control	3	1.0252	.26513	.15308
	DFDBA	3	1.6475	.23029	.13296

Independent Samples Test

MMP12		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference Lower	Upper
MMP12	Equal variances assumed	.137	.730	-3.069	4	.037	-.62233	.20276	-1.18527	-.05939
	Equal variances not assumed			-3.069	3.923	.038	-.62233	.20276	-1.18965	-.05501

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
MMP12	Control	3	1.0252	.26513	.15308
	DBBM	3	3.1820	1.11122	.64156

Independent Samples Test

MMP12		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference Lower	Upper
MMP12	Equal variances assumed	3.812	.123	-3.270	4	.031	-2.15683	.65957	-3.98809	-.32557
	Equal variances not assumed			-3.270	2.227	.071	-2.15683	.65957	-4.73480	.42114

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
MMP12	DFDBA	3	1.6475	.23029	.13296
	DBBM	3	3.1820	1.11122	.64156

Independent Samples Test

MMP12		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
MMP12	Equal variances assumed	4.229	.109	-2.342	4	.079	-1.53450	.65519	-3.35361	.28461
	Equal variances not assumed			-2.342	2.171	.134	-1.53450	.65519	-4.15062	1.08163

S100A4

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
S100A4	Control	3	1.1700	.70505	.40706
	DFDBA	3	6.7500	1.94070	1.12046

Independent Samples Test

S100A4		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
S100A4	Equal variances assumed	1.580	.277	-4.681	4	.009	-5.58000	1.19211	-8.88984	-2.27016
	Equal variances not assumed			-4.681	2.519	.027	-5.58000	1.19211	-9.81860	-1.34140

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
S100A4	Control	3	1.1700	.70505	.40706
	DBBM	3	6.6667	.50836	.29350

Independent Samples Test

S100A4		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
S100A4	Equal variances assumed	.120	.747	-10.953	4	.000	-5.49667	.50184	-6.89000	-4.10333
	Equal variances not assumed			-10.953	3.637	.001	-5.49667	.50184	-6.94659	-4.04675

Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
S100A4	DFDBA	3	6.7500	1.94070	1.12046
	DBBM	3	6.6667	.50836	.29350

Independent Samples Test

S100A4		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
S100A4	Equal variances assumed	2.129	.218	.072	4	.946	.08333	1.15826	-3.13253	3.29919
	Equal variances not assumed			.072	2.273	.948	.08333	1.15826	-4.36814	4.53480

Independent Samples Test of gene expression between 1 month and 3 months (Figure 7)

MMP12

Group Statistics

	Month	N	Mean	Std. Deviation	Std. Error Mean
MMP12	1	3	1.0067	.14259	.08233
	3	3	.5577	.14420	.08325

Independent Samples Test

Control		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
MMP12	Equal variances assumed	.016	.906	3.835	4	.019	.44900	.11708	.12393	.77407
	Equal variances not assumed			3.835	3.999	.019	.44900	.11708	.12391	.77409

Group Statistics

	Month	N	Mean	Std. Deviation	Std. Error Mean
MMP12	1	3	1.0020	.07795	.04500
	3	3	.7113	.09914	.05724

Independent Samples Test

DFDBA		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
MMP12	Equal variances assumed	.168	.703	3.992	4	.016	.29067	.07281	.08850	.49283
	Equal variances not assumed			3.992	3.789	.018	.29067	.07281	.08399	.49735

Group Statistics

	Month	N	Mean	Std. Deviation	Std. Error Mean
MMP12	1	3	1.0237	.25723	.14851
	3	3	1.4067	.49096	.28346

Independent Samples Test

DBBM		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference Lower	Upper
MMP12	Equal variances assumed	1.231	.329	-1.197	4	.297	-.38300	.32000	-1.27147	.50547
	Equal variances not assumed			-1.197	3.021	.317	-.38300	.32000	-1.39739	.63139

S100A4

Group Statistics

	Month	N	Mean	Std. Deviation	Std. Error Mean
S100A4	1	3	1.0281	.28970	.16726
	3	3	.2688	.16264	.09390

Independent Samples Test

Control		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
S100A4	Equal variances assumed	.620	.475	3.959	4	.017	.75932	.19181	.22676	1.29188
	Equal variances not assumed			3.959	3.147	.026	.75932	.19181	.16469	1.35395

Group Statistics

	Month	N	Mean	Std. Deviation	Std. Error Mean
S100A4	1	3	1.0262	.27233	.15723
	3	3	1.5613	.23905	.13801

Independent Samples Test

DFDBA		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference Lower	Upper
S100A4	Equal variances assumed	.050	.835	-2.558	4	.063	-.53509	.20921	-1.11595	.04577
	Equal variances not assumed			-2.558	3.934	.064	-.53509	.20921	-1.11982	.04964

Group Statistics

	Month	N	Mean	Std. Deviation	Std. Error Mean
S100A4	1	3	1.0019	.07483	.04321
	3	3	1.2036	.57375	.33126

Independent Samples Test

DBBM		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
S100A4	Equal variances assumed	9.660	.036	-.604	4	.579	-.20172	.33406	-1.12923	.72579
	Equal variances not assumed			-.604	2.068	.606	-.20172	.33406	-1.59470	1.19126

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