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เพื่อยกพื้นโพรงอากาศแมกซิลล่าด้วยกระดูกปลูกถ่ายวิวิธพันธุ์

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ปีการศึกษา 2558

GENE EXPRESSION OF ALVEOLAR BONE WITH MAXILLARY SINUS FLOOR AUGMENTATION USING XENOGRAFT

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Prosthodontics
Department of Prosthodontics
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ดลยา พวงชัยพฤกษ์ : การแสดงออกของยีนของกระดูกขากรรไกรที่ได้รับการผ่าตัดเพื่อยกพื้นโพรงอากาศแมกซิลล่าด้วยกระดูกปลูกถ่ายวิวิธพันธุ์ (GENE EXPRESSION OF ALVEOLAR BONE WITH MAXILLARY SINUS FLOOR AUGMENTATION USING XENOGRAFT) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ทพญ. ดร. ใจแจ่ม สุวรรณเวลา, 45 หน้า.

ในปัจจุบันการใส่รากฟันเทียมเข้ามามีบทบาทเพิ่มมากขึ้น เมื่อเกิดการสูญเสียฟันหลังบน อย่างไรก็ตามหากมีปริมาณกระดูกที่จะรองรับรากฟันเทียมนั้นไม่เพียงพอ หรือเกิดการยื่นย้อยของโพรงอากาศแม็กซิลลาหลังการสูญเสียฟันหลังบน ส่งผลกระทบทั้งทางด้านคุณภาพและปริมาณของกระดูก อันเป็นปัจจัยสำคัญของความสำเร็จในการใส่รากฟันเทียม จะทำให้ยากที่รากฟันเทียมจะเกิดการคงอยู่ที่ดีได้ ดังนั้นการผ่าตัดยกพื้นโพรงอากาศแม็กซิลลาจึงเข้ามามีบทบาทเพื่อเพิ่มความสูงของกระดูกเบ้าฟันในบริเวณสันเหงือกว่างตำแหน่งฟันหลังในขากรรไกรบนโดยการใส่วัสดุปลูกถ่ายชนิดต่างๆ ถึงแม้มีการศึกษามากมายเกี่ยวกับความสามารถในการเข้ากันได้กับร่างกายมนุษย์ของกระดูกปลูกถ่ายวิวิธพันธุ์ที่ได้จากวัว แต่การศึกษาในระดับยีนที่เกี่ยวข้องกับการหายของบาดแผลในกระดูกยังมีไม่เพียงพอ ดังนั้นวัตถุประสงค์ของงานวิจัยนี้คือเพื่อเปรียบเทียบการแสดงออกของยีนที่เป็นตัวแทนการแสดงออกถึงการหายของบาดแผลในกระดูก ทั้งการทำหน้าที่ของเซลล์สร้างกระดูก สลายกระดูกและไซโตไคน์ที่กระตุ้นให้เกิดการอักเสบได้แก่ RUNX2, RANKL, OPG, MMP-9, TRAP, IL-1β และ RANKL/OPG ระหว่างกระดูกขากรรไกรบนที่ได้รับการปลูกถ่ายกระดูกเพื่อยกพื้นโพรงอากาศแม็กซิลลาผ่านช่องด้านข้างด้วยกระดูกปลูกถ่ายวิวิธพันธุ์ กับกระดูกขากรรไกรบนที่มีการหายของแผลแบบปกติ โดยทำการเก็บชิ้นกระดูกจากทั้งสองกลุ่ม กลุ่มละ 7 ชิ้น ที่มีระยะเวลาการหายของแผลหลังผ่าตัดปลูกถ่ายหรือถอนฟันไม่ต่ำกว่า 6เดือน มาทำการตรวจสอบการแสดงออกของยีนเป้าหมายโดยวิธีการเรียลไทม์ อาร์ที -พีซีอาร์ ผลการวิเคราะห์พบว่าการแสดงออกของยีนเป้าหมายไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ (Independent t-test, $p>0.05$) จากผลจึงสรุปได้ว่ากระดูกปลูกถ่ายวิวิธพันธุ์ที่ได้จากวัวไม่มีผลต่อการแสดงออกของยีนที่เป็นตัวแทนการแสดงออกถึงการหายของบาดแผลในกระดูก

ภาควิชา ทันตกรรมประดิษฐ์ ลายมือชื่อนิสิต .....

สาขาวิชา ทันตกรรมประดิษฐ์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2558

5675807532 : MAJOR PROSTHODONTICS

KEYWORDS: MAXILLARY SINUS FLOOR AUGMENTATION / XENOGRAFT / GENE EXPRESSION / REAL TIME RT-PCR

DOLLAYA PUANGCHAIPRUK: GENE EXPRESSION OF ALVEOLAR BONE WITH MAXILLARY SINUS FLOOR AUGMENTATION USING XENOGRAFT. ADVISOR: JAIJAM SUWANWELA, Ph.D., 45 pp.

A lack of alveolar bone in the maxillary sinus area often causes difficulty in placing implants. Alveolar bone resorption and sinus pneumatization may occur after maxillary posterior tooth extraction. An adequate quality and quantity of bone is important for successful implant placement. Therefore, maxillary sinus floor augmentation (MSFA) using different types of grafts is performed when the amount of alveolar bone is insufficient. Histologic, and histomorphometric studies and systematic reviews have shown the clinical success of the use of anorganic bovine bone (ABB, Bio-Oss®) in maxillary sinus floor augmentation (MSFA). The molecular processes involved in bone healing are, however, still unclear. To explore gene expression associated with bone remodeling and inflammation, the mRNA expression levels of RUNX2, RANKL, OPG, MMP-9, TRAP, and IL-1β, as well as the ratio of RANKL/OPG were compared between alveolar bone of a group after MSFA with ABB and a maxillary posterior edentulous bone group. Seven bone samples from each group were collected at the time of implant placement, after 6 months of MSFA or tooth extraction. Real time RT-PCR was used to analyze gene expression. Real time RT-PCR revealed no statistically significant difference in gene expression level of RUNX2, RANKL, OPG, MMP-9, TRAP, and IL-1β, and in the ratio of RANKL/OPG. After a healing period of 6 months, ABB particles do not have an effect on the expression of genes associated with bone remodeling and inflammation.

Department: Prosthodontics Student's Signature .....

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

Academic Year: 2015

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CHAPTER I INTRODUCTION

A lack of alveolar bone in the maxillary sinus area often causes difficulty in placing implants. Alveolar bone resorption and sinus pneumatization may occur after maxillary posterior tooth extraction.[1] An adequate quality and quantity of bone is important for successful implant placement.[2] Therefore, maxillary sinus floor augmentation (MSFA) using different types of grafts is performed when the amount of alveolar bone is insufficient.[3,4] Because of certain limitations of autografts such as a limited amount of graft material available, bovine hydroxyapatite can be used as a substitute bone graft either with or without combining with autografts when used for MSFA.[5] Deproteinized sterilized cancellous bovine bone or anorganic bovine bone (Bio-Oss®, ABB) has been used as a substitute bone graft and was studied for over two decades. Most of these studies are histologic or histomorphometric studies, and in addition some systematic reviews have been published.[6-10] ABB has a slow resorption rate and grafted particles can remain in close contact with newly formed bone in the grafted site for years.[7] ABB particles do not interfere with the normal osseous healing process after MSFA and promote new bone formation in tooth extraction sockets in a similar degree as ungrafted sockets. On the other hand, Heberer et al. showed that bone formation in ungrafted human extraction sockets was higher than bone formation in sockets grafted with ABB.[8,11,12]

Bone remodeling consists of two main phases: the formation and the resorption phase. [13] In the bone formation phase, osteoblasts that are derived from mesenchymal stem cells initially deposit the bone organic phase, the osteoid layer. [14] They secrete next to type 1 collagen, which is the basic organic building block of bone, numerous non-collagenous proteins among which osteocalcin, bone sialoprotein and the enzyme and alkaline phosphatase. The latter enzyme plays an important role in mineralization of the osteoid layer. The gene necessary for osteoblast differentiation is Runt related

transcription factor 2 (RUNX2).[15] Osteoblasts also produce cytokines, macrophage-colony-stimulating factor (M-CSF), Interleukin-6 (IL-6), and receptor activator of NF-kB ligand (RANKL). These cytokines control growth and differentiation of osteoclast precursor cells into mature osteoclasts.[16] In the bone resorption phase, osteoclasts play a crucial role. These cells are multinucleated and derived from a hematopoietic stem cell. Bone degradation is accomplished in the following sequence. First, osteoclasts attach to the bone matrix through a sealing zone. Next, osteoclasts become polarized and form a resorption area, the s-called ruffled border. Then, osteoclasts dissolve the mineral and degrade bone matrix in the resorption lacuna. Finally, degradation products are removed. Osteoclasts that attach to ABB particles and bone tissue have the same shape and size.[17] RANK is the ligand for RANKL, which is expressed on the membrane of preosteoclasts, connects with RANKL to provide a critical signal necessary for osteoclast differentiation.[18] Osteoclastogenesis-inhibitory factor (OCIF), also called osteoprotegerin (OPG), interferes with the cell-to-cell interaction between RANK and RANKL. Thus, OPG inhibits osteoclastogenesis.[19] A large amount of matrix metallopeptidase 9 (MMP-9), tartate-resistance acid phosphatase (TRAP), and cathepsin K (CTSK) are released by mature osteoclasts during the process of bone resorption.[20,21] Apart from the RANKL/RANK/OPG pathway, interleukin-1beta (IL-1β), which is an important inflammatory mediator, also can stimulate osteoclastic bone resorption.[22,23]

So far only a few genetic studies have been performed on the effect of Bio-Oss® on osteoblastic or osteoclastic gene expression. Moreover, most of these studies were in vitro studies.[24,25] In an in vivo study, no significant difference was found in the expression of genes associated with the osteogenic potential of mesenchymal cells by comparing non-augmented and ABB-augmented human extraction sockets after 6 weeks of healing time.[26] According to Caubet J et al., the expression of the bone-forming gene RUNX2 was significantly higher when using autologous maxillary bone

combined with Bio-Oss® than with BoneCeramic® after 4-5 months of MSFA.[27] They used 50% 0f Bio-Oss® or BoneCeramic® combined with autograft bone. The aim of this study was to explore the expression of specific genes that are associated with bone formation, bone resorption and inflammation (i.e. RUNX2, RANKL, OPG, MMP-9, TRAP, and IL-1β) after MSFA procedure using solely xenograft "ABB".

RESEARCH QUESTION

Are there any differences in the mRNA expression levels of RUNX2, RANKL, OPG, MMP-9, TRAP, and IL-1β, as well as the ratio of RANKL/OPG between alveolar bone of a group after MSFA with ABB and a maxillary posterior edentulous bone group (MPEB)?

RESEARCH OBJECTIVE

The objective of this study was to examine the differences between the mRNA expression levels of RUNX2, RANKL, OPG, MMP-9, TRAP, and IL-1β, as well as the ratio of RANKL/OPG of alveolar bone of a group after MSFA with ABB and a MPEB group.

HYPOTHESES

H_0: There are no differences in the expression level of RUNX2, RANKL, OPG, MMP-9, TRAP, and IL-1β, as well as the ratio of RANKL/OPG between alveolar bone of a group after MSFA with ABB and a MPEB group.

H_A: There are differences in in the expression level of RUNX2, RANKL, OPG, MMP-9, TRAP, and IL-1β, as well as the ratio of RANKL/OPG between alveolar bone of a group after MSFA with ABB and a MPEB group.

KEYWORD

Maxillary sinus floor augmentation

Xenograft

Gene expression

Real time RT-PCR

EXPECTED BENEFIT

This study is the first in vivo study that yields the gene expression associated with bone remodeling and inflammation between alveolar bone of a group after MSFA with ABB and a maxillary posterior edentulous bone group.

CHAPTER II REVIEW OF LITERATURE

Characteristics of maxillary sinus

The maxillary sinus is a pyramid shaped structure in the maxillary bone which has its deepest part at the first molar region.[28] The alveolar bone in the sinus area is often inadequate for implant placement because of sinus pneumatization and bone resorption after tooth extraction. There are factors that increase the chance of maxillary sinus pneumatization after maxillary teeth extraction, including superiorly curving of the sinus floor around surrounding teeth, protruding of tooth roots into the sinus, having extraction of several adjacent posterior teeth or extraction of a tooth with missing adjacent teeth,[1,29] and increasing positive intra-antral pressure.[30] MSFA should be performed to restore the atrophic edentulous maxilla before implant placement.[3,31]

Management of posterior maxilla with MSFA

Management of posterior maxilla with MSFA can be performed using two techniques. The introduction in 1994 to a new technique using a crestal approach by Summer.[32] Crestal Approach Technique is indicated when the residual crestal bone is greater than 5 mm. An osteotome is used to fracture the sinus floor after which the implant site is drilled below the sinus floor. The sinus membrane is elevated by inserting grafting material (Figure 1).

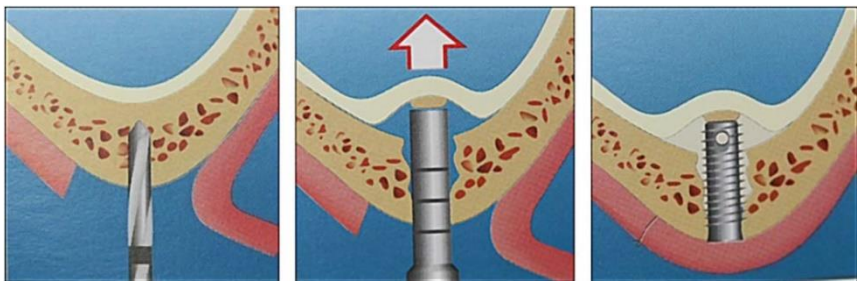


Figure 1 Crestal Approach Technique[32]

Alternatively, Lateral Window Technique may be used if the residual crestal bone height is less than 5 mm. It is preferred to delay implant placement by several months after the grafting phase.[33] Tatum was the first person to present the sinus floor elevation procedure with the Modified Caldwell-Luc approach in 1977.[34] The first article about this surgical technique was published in 1980 by Boyne and James.[35] Flap operation and window fenestration osteotomy at the maxilla is made to raise the sinus membrane. The space under the sinus membrane is then filled with different grafting materials (figure 2).

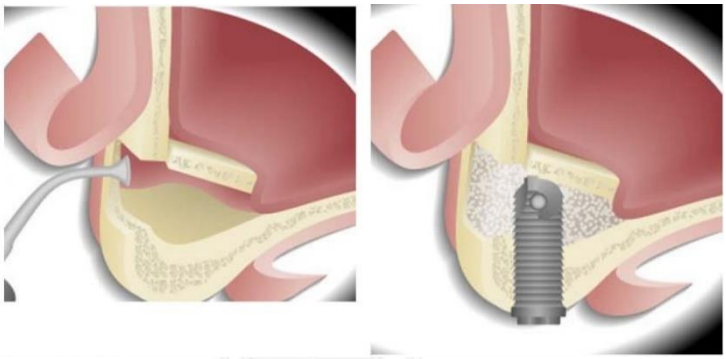


Figure 2 Lateral Window Technique[32]

Grafting materials

There are 4 groups of grafting materials.[35]

- Autograft/Autogenous bone graft
- Allograft/Allogenous bone graft
- Xenograft
- Alloplast

Autograft/Autogenous bone graft

This type of graft has been defined as the “gold standard”. It is defined as tissue transplanted from one site to another within the same individual. It provides a good source of cells, growth factors, and bone morphogenic proteins with no risk of antigenicity or cross infection. Mandible, iliac crest and tibia are the most common donor sites. The most important advantage of autograft is that it is the only graft that has osteogenesis, osteoconductive and osteoinductive abilities. Moreover, there is no risk of disease being transferred or risk of the bone graft being rejected by the body. However, there are a lot of disadvantages such as morbidity from the donor site, the need for hospitalization, longer healing time, surgical risk, and a limited amount of graft material available. Therefore, autogenous bone is often mixed with other grafting materials.

Allograft/Allogenous bone graft

Allografts are available in freeze-dried form from deceased human donors. They are tissue grafts between individuals of the same species but non-identical genetic composition. Both mineralized and demineralized forms have osteoconductive ability. The advantage is that no bone is being harvested from the patient. On the other hand, there are risks of immunogenic reaction and cross-infection. However, the grafts are processed and sterilized to eliminate the risk of cross-infection.

Xenograft

Xenografts are defined as tissue grafts between different species. They are usually derived from bovine bones and have osteoconductive ability with long-term resorption. The advantage is that no bone is being harvested from the patient, but there is a minimal risk of cross-infection from animals to humans.

Alloplast

Alloplasts are synthetically derived and do not originate from humans or animals. They are the mineral or inorganic components of bone such as β-tricalcium phosphate (β–TCP), coral hydroxyapatite and bioactive glasses. They have osteoconductive ability. There is no risk of disease transfer but there is a slight chance that the body will reject the graft.

Capability of grafting materials

The success of grafting material depends on its capability to make a suitable environment for the bone remodeling process. There are 3 capabilities.[36] Firstly, the grafting material contains a sufficient number of viable cells to form new bone. These cells must survive transplantation, proliferate, and differentiate into bone-producing cells is called "osteogenesis". [30] Secondly, the grafting materials serve as a scaffold to permit bone growth down into pores or on its surface and promote growth of new bone is called "osteoconduction". Finally, the grafting materials stimulate the immature cells to develop into pre-osteoblasts or bone-forming cell lineages is called "osteoinduction".

Table 1 Capability of grafting materials [6]

Grafting Materials	osteogenesis	osteoinduction	osteoconduction
Autograft	+	+	+
Allograft	-	+/-	+
Xenograft	-	-	+
Alloplast	-	-	+

Applications of Bio-Oss®

Bio-Oss® has been studied and used as grafting material for maxillary sinus floor augmentation. It is a xenograft that consists of deproteinized, sterilized cancellous bovine bone. It has two particle sizes, large granule 1-2 mm and small granule 0.25-1 mm. Because of its slow resorption and osteoconductive ability, Bio-Oss® is suitable for a stable scaffold. Therefore, bone-forming cells at the peripheral of grafted site can penetrate and form new bone.[8,9,37,38] Histologically, Bio-Oss® showed newly formed lamellar bone in close contact with the graft particle.[7,38,39]

Bone

Bone is a specialized connective tissue. This tissue provides the organism with a rigid structure which, with muscle, facilitates stable locomotion. The bone matrix is predominantly a mixture of organic phase, which is type I collagen fibers and non-collagenous proteins. Inorganic phase consists of calcium and phosphorus in the form of hydroxyapatite crystals.[40] The cortex or compact bone is an external shell made of a dense layer of calcified matrix. The internal space is filled with trabecular bone. The spaces between these trabeculae are filled with hematopoietic bone marrow. The periosteal, endosteal and trabecular surfaces are lined with fibroblastic and osteogenic cells organized in layers.[41] Misch CE and Kircos LT classified the bone density into five groups (D1-5). D1 is primarily found in the anterior mandible, buccal shelf and midpalatal region while D2 is found in the anterior maxilla and the posterior mandible. D3, less dense, is found in the posterior maxilla and mandible. D4 is found primarily in the tuberosity region.[28] According to a dual energy x-ray absorptiometry, the posterior maxilla had the lowest bone mineral density.[42]

Bone remodeling

The bone remodeling has two main phases: bone resorption and new bone formation. Osteoblasts and osteoclasts play important roles in this process. First, osteoclasts remove the old bone, then osteoblasts form new bone replacing the tissue previously resorbed.[43] Osteoblast or bone-forming cell is derived from mesenchymal precursor cell. It is found in sheaths along the bone surface on the layer of bone matrix that it is producing.[44] It secretes type 1 collagen, which is the basic building block of bone, and osteocalcin and alkaline phosphatase, which are important non-collagenous proteins for mineral deposition. The essential gene necessary for osteoblast differentiation is Runt related transcription factor 2 (RUNX2).[15] Osteoblasts also produce cytokines, macrophage-colony-stimulating factor (M-CSF), interleukin-6 (IL-6), and receptor activator of NF-kB ligand (RANKL). These enzymes control growth and differentiation of osteoclast precursor cells into mature osteoclasts.[16] RANKL, expressed on the surface of osteoblasts, enhances the preosteoclasts differentiation into mature osteoclasts.[45] Toward the end of the matrix-secreting period, 15% of mature osteoblasts are entrapped in the new bone matrix and differentiate into osteocytes, whereas some cells remain on the bone surface, becoming flat lining cells. Osteocyte is related to the maintenance of bone structure by responding to mechanical stain and sending resorption or formation signals.[46]

The other main cell is osteoclast or bone resorption cell. It is a multinucleated cell derived from hematopoietic stem cell. RANK a counter part of RANKL, which is on preosteoclasts, connects with RANKL to provide a critical signal necessary for osteoclast differentiation.[18] To interrupts the cell-to-cell interaction between RANK and RANKL, osteoprotegerin (OPG) is released by osteoblast in order to bind RANKL. Thus, osteoclastogenesis is reduced. [19] OPG also directly inhibits mature osteoclasts. [47] Premature osteoclast is dose-dependent blocked by OPG.[48] Osteoclast dissolves crystalline hydroxyapatite and performs four steps to degrade organic bone matrix.

First, osteoclasts attach to bone matrix though the sealing zone, which is the specialized cell-extracellular matrix adhesion structure and the ruffled border. Then, osteoclasts degrade bone matrix in the resorption lacuna. Finally, degradation products are removed. Osteoclasts which attach to anorganic bovine bone particles and bone tissue have the same shape and size.[17] A large amount of these enzymes including MMP-2, MMP-9, MMP-13, MMP-14, tartate-resistance acid phosphatase (TRAP) and cathepsin K (CTSK) are released by mature osteoclasts to resorp bone.[20,21] Those enzymes are placed in lysosomes and released to the sealing zone near ruffled border of osteoclasts.[49] Knockout mice lacking TRAP have increased mineral density both interm of bone shape and remodeling.[50]

In a homeostatic equilibrium, the balance of resorption and formation is crucial. The new bone continuously replace the old bone and adapt according to mechanical load and strain.[51] In humans, resorption can occurs for about 2 weeks, the reversal phase continues for about 4 or 5 weeks, while formation may last up to 4 months until the new bone structure is completely created.[52]

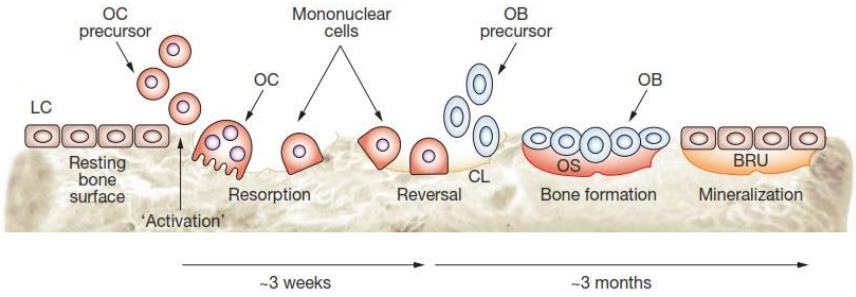


Figure 3 The sequence of bone remodeling in healthy individuals [53]

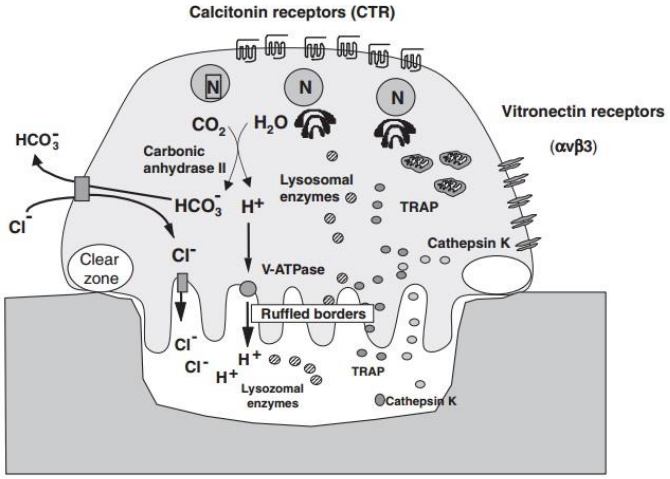


Figure 4 The role of CtsK and MMPs in bone resorption [49]

Osteocytes as mechanosensors

In the adult creatures, over 90–95% of all bone cells,[54] Osteocytes are non-proliferative. They are terminally differentiated cells of the osteoblast lineage that are locked inside both mineral bone matrix and newly formed osteoid.[46] They regularly live inside the mineralized matrix called lacunae. They connect to each other and the cells on the bone surface via long slender cytoplasmic processes that reside inside the tiny canals called canaliculi. These cellular projections not only allow osteocytes to communicate with each other but also with cells on the bone surface.[55] The communication with other cells is through gap junctions. The other way to communicate is by using signaling molecules releasing through the bone fluid of the lacuna-canaliculi system. The major function of osteocytes is being mechanosensor cells. They control bone remodeling by translating mechanical strain into biochemical signals to induce activity of osteoblasts and osteoclasts.[56] Osteocytes may also play a role in bone resorption by releasing of RANKL and OPG to stimulate osteoclast precursors.[53]

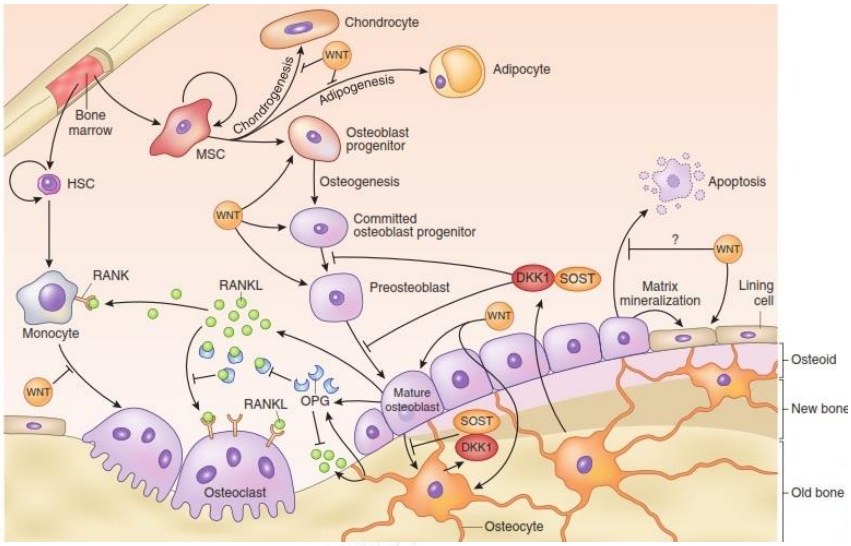


Figure 5 The Interaction between bone cells [57]

Proinflammatory cytokines involved in osteoclastic function

Osteoblasts and osteoclasts play a key role in bone remodeling. Osteoblasts release a soluble mediator called a cytokine to stimulate osteoclastic bone resorption. Cytokines are soluble polypeptide products of cells of the immune system. Purified cytokines have been given the name “interleukin”. Interleukin-1beta (IL-1β) is one of the important mediators of bone resorption.[58] IL-1β stimulates osteoclasts to resorb bone in vivo.[22,23] Dewhirst et al. found that the amino-terminal sequence of IL-1β was identical to that of osteoclast-activating factor (OAC).[59] Multinucleated cells (MNC) formation, which is osteoclast characteristic, is increased by adding IL-1β into human marrow culture.[60] IL-1β increases MMP production in endothelial cells, synovial cells and chondrocytes.[61]

Real time RT-PCR

There are several methods that have been used to study gene expressions. The polymerase chain reaction (PCR) is a common method for amplifying DNA. For mRNA-based PCR, reverse transcriptase enzyme is used to reverse-transcribed the RNA sample to complementary DNA (cDNA). Then the cDNA is used as a template for exponential amplification using PCR. This process is called reverse transcription polymerase chain reaction (RT-PCR). The real-time reverse transcription polymerase chain reaction (RT-PCR) is one of the standard techniques for the quantification of nucleic acids.[62,63] Real time RT-PCR is also referred to as real-time quantitative PCR, quantitative real-time PCR and quantitative PCR. During each cycle of the PCR reaction, detection probe or non-probe based chemistry is used to detect the production of amplification products. SYBR green dye is widely used as the non-probe based chemistry. After the binding of fluorescent SYBR green dye to the double strand cDNA PCR products is excited, detector can measure the intensity of fluorescence signal from each cycle. The quantification output can be calculated from the copy number of unknown target relative to an external standard curve. The external standard curve is the linear regression of the threshold cycle (C_T) of the unknown sample dilution series, while the internal standard is the comparison of the C_T of the target sample to the reference gene. Therefore, the product can be compared between samples.[64]

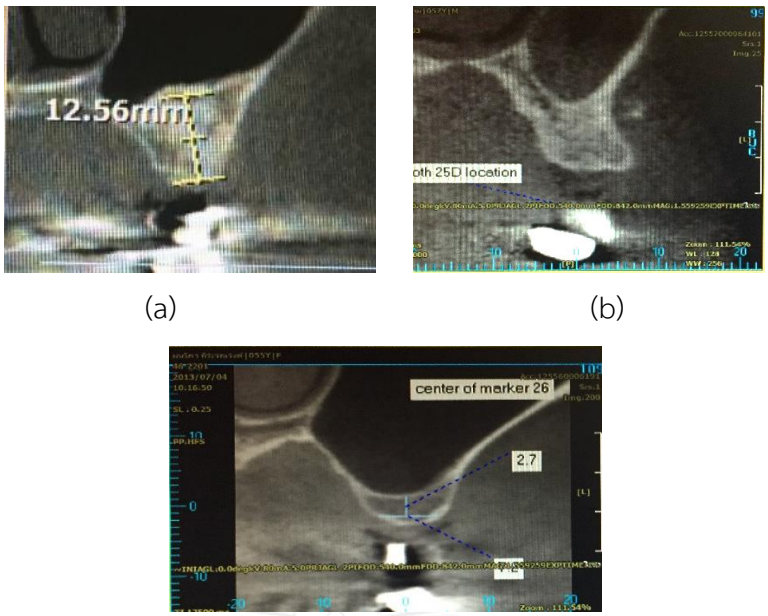
In this study, six specific genes associated with bone remodeling and inflammation: RANKL, OPG, IL-1β, MMP-9, TRAP, and RUNX2, will be observed by the real time RT-PCR technique.

CHAPTER III RESEARCH METHODOLOGY

Subject selection and bone samples

Subjects were older than 20 years. Any systemic and local factors interfering with implant surgery were defined as exclusion criteria: presence of uncontrolled diabetes, immune diseases, or other contraindicated systemic conditions, history of radiation or chemotherapy, uncontrolled periodontal disease, history of using bone-related drug such as bisphosphonates, history of severe craniofacial accident, and history of abnormal craniofacial morphology. Informed consent for study participation was obtained from each patient. The ethical review committee at Chulalongkorn University, which works in accordance with Helsinki declaration, approved the study design.

A total of 11 Subjects (10 females and 1 male) with an age ranging from 21 to 66 participated in this study. Fourteen bone samples were collected from 11 subjects, seven samples were from subjects who had a lateral window technique of MSFA procedure using solely xenograft “ABB” (figure 6a) and 7 samples were from subjects who had normal healing after tooth extraction at the maxillary posterior edentulous area (figure 6b). Subjects who had a lateral window technique of MSFA had the residual crestal bone height less than 5 mm from the CBCT scan using CT guided stent (figure 6c). This CT guided stent was also used to locate the position of implant placement. Bone samples were collected during receiving dental implant surgeries at Faculty of Dentistry, Chulalongkorn University. Healing period was at least 6 months before implant placement.



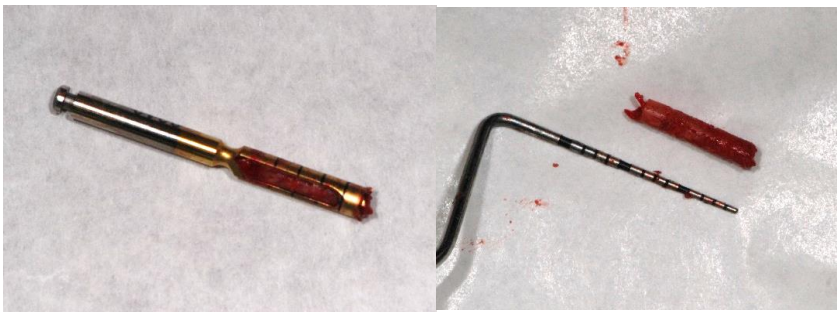
(a) (b)

(c)

Figure 6 The CBCT scan of maxillary edentulous bone crest

Bone collecting

Samples of bone tissue were harvested from each of the donor regions by using a 2 mm internal diameter trephine bur through dental implant surgeries (Figure 7a). Steriled periodontal probes were used to push immediately the bone tissues out of the burs into the cryovial tubes (Figure 7b). They were frozen in liquid nitrogen until the RNA extraction (figure 7c, d). Subject who had two surgical sites, each sample was kept separately.



(a) (b)

(c) (d)

Figure 7 The bone collecting

Bone homogenization and cell lysis

Bone homogenization was done in lysis reagent (Qiazol®, Qiagen, Inc., USA) by the Bead-Based Homogenizer (PowerLyzer™, Mo Bio Laboratories, Inc., USA). The bone sample was transferred from a cryovial tube to a metal bead tube. The metal bead tube was pre-chilled in liquid nitrogen to reduce the heat while homogenizing.[62] The Lysis reagent was also pre-chilled on ice. The metal bead tube containing bone particles was added one ml of the pre-chilled lysis reagent. Then, the bone was homogenized in homogenizer 3,500 RPM speed for 2 cycles of 30 seconds and 30 seconds pause between cycles.

Figure 8 The homogenizer and metal bead tube

RNA isolation

The spin-column based method was used to extract the mRNA according to the manufacturer's instructions (PureLink® RNA Mini Kit, Life Technologies, Inc., USA). Followed the manufacturer protocol.

Phase Separation

After the bone homogenization and cell lysis, the RNA was isolated using the following protocol:

1. Incubate the lysate with lysis reagent at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes.
2. Transfer the lysate from the metal bead tube to a sterilized RNase-free tube.
3. Add 0.2 ml chloroform per 1 ml lysis reagent used and vertex the tube for 15 seconds. Then, leave it incubate at room temperature for a few minutes.
4. Centrifuge the sample at 12,000 *g* for 15 minutes at 4°C.

After centrifugation, the mixture separates into 3 different layers: lower, interphase and upper. The lower layer is red phenol–chloroform phase. While the upper layer is colorless aqueous phase which contains the RNA.

5. Transfer the colorless upper phase about 600 μl to a sterilized RNase-free tube.
6. Add an equal volume of 70% ethanol. Vortex 10 seconds for thorough mix and proceed to binding, washing and elution steps.

Binding, Washing and Elution

1. Transfer up to 700 µl of the mixture from phase separation step to a spin cartridge (with a collection tube).

2. Centrifuge at 12,000 g for 15 seconds at room temperature. Discard the flow-through and re-insert the spin cartridge into the same collection tube.

3. Repeat Steps 1–2 until the entire sample has been processed.

4. Add 700 µl Wash Buffer I to the spin cartridge. Centrifuge at 12,000 g for 15 seconds at room temperature. Discard the flow-through and the collection tube. Insert the spin cartridge into a new collection tube.

5. Add 500 µl Wash Buffer II with ethanol to the spin cartridge.

6. Centrifuge at 12,000 g for 15 seconds at room temperature. Discard the flow-through, and reinsert the spin cartridge into the same collection tube.

7. Repeat Steps 5–6 once.

8. Centrifuge the spin cartridge and collection tube at 12,000 g for a few minutes at room temperature to dry the membrane with attached RNA. Discard the collection tube and insert the new spin cartridge into a recovery tube.

9. Add 80 µl RNase-Free Water to the center of the spin cartridge.

10. Incubate at room temperature for 1 minute.

11. Centrifuge the spin cartridge with the Recovery Tube for 2 minutes at ≥12,000 g at room temperature. Discard the spin cartridge. Always keep the purified RNA on ice if used within a few hours. For long-term storage, store the purified RNA at –80°C.

12. Proceed to next step (RT-PCR step).

Finally, a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Inc., USA) was used to assess the amount (quantity) and purity (quality) of mRNA. Nucleic acids and proteins have the absorbance range at 260 and 280 nm. The ratio of these wavelengths were used to determine RNA purity. The A260/280 ratio ~2.0 is accepted as pure for RNA. The samples that had the A260/280 ratio less than 1.8 were excluded.

Figure 9 NanoDrop 2000

RT-PCR

In this study, two-step real time PCR was used. Firstly, the reverse transcription kit (Sensiscript®, Qiagen, Inc., USA) was used to reverse transcribe RNA to cDNA. Then, the cDNA was used as a template in PCR process using the real time PCR Master Mix (SYBR® FAST Universal kit, Kapa Biosystems, Inc., USA). The protocols were set according to the manufacturer's instructions.

A Bio-Rad CFX96TM real time RT-PCR system (Bio-Rad laboratories, Inc., USA) was used to analyze expression levels of RUNX2, RANKL, OPG, MMP-9, TRAP, and IL-1β. Primers for the genes were designed following primer designing tool (Primer3 and BLAST, http://www.ncbi.nlm.nih.gov/tools/primerblast/) (Table 2). GAPDH was used as a reference gene for normalization and amplified in a separate tube.[63]

Table 2 Primer sequences used in this study

Gene		Primer sequences
RANKL	forward	GCCAGTGGGAGATGTTAGAC
	reverse	ATAGCCCACATGCAGTTTCT
OPG	forward	TCAGGTTTGCTGTTCCTACA
	reverse	GTTCTTGTGAGCTGTGTTGC
IL-1β	forward	GCTCTGGGATTCTCTTCAGC
	reverse	AAGTCATCCTCATTGCCACT
MMP9	forward	CCTTCTACGGCCACTACTGT
	reverse	CCAGTACTTCCCATCCTTGA
TRAP	forward	GCTATCTGCGCTTCCACTAT
	reverse	GAGGCCTCGATGTAAGTGAC
RUNX2	forward	CAGCCCAGAACTGAGAAACT
	reverse	ACAGATGGTCCCTAATGGTG
GAPDH	forward	TGAAGGTCGGAGTCAACGGAT
	reverse	TCACACCCATGACGAACATGG

Reverse-transcription protocol

1. Thaw template RNA, 10x Buffer RT, dNTP Mix, Oligo dT primer solutions, and RNase-free water by placing them on ice. Slightly vortex to thoroughly mix.

2. Prepare a master mix according to Table 3. All the components except the template RNA were mixed, vortexed and centrifuged gently. Always keep tube on ice.

3. Dispense 19.0 µl of the master mix solution into PCR tubes.

4. For the final components, add template RNA 1.0 µl to the individual PCR tubes.

. 5. Incubate at 37°C for 60 min in the thermocycler.

6. Keep the reverse transcription reactions on ice and proceed to PCR step.

Table 3 Reverse transcription reaction components by using Sensiscript® reverse transcription kit.

(The following protocol is used when < 50 ng RNA)

Component	Volume/reaction	Final concentration
10x Buffer RT	2.0 µl	1x
dNTP Mix (5mM each dNTP)	2.0 µl	0.5mM each dNTP
Oligo-dT primer (10 µM)	2.0 µl	1 µM
Sensiscript Reverse Transcriptase	1.0 µl	
RNase-free water	12.0 µl	-
Template RNA	1.0 µl	<50 ng (per reaction)
Total volume	20.0 µl	-

PCR protocol

1. KAPA SYBR® FAST qPCR Master Mix was used to prepare a PCR master mix consisting of the appropriate volumes according to the table 4. The reaction mix was mixed gently.

2. Transfer 19 µl of PCR master mix and add 1 µl of cDNA template (from the previous step) to each well of a PCR tube.

3. Set and Run the qPCR following the protocol as described in table 5.

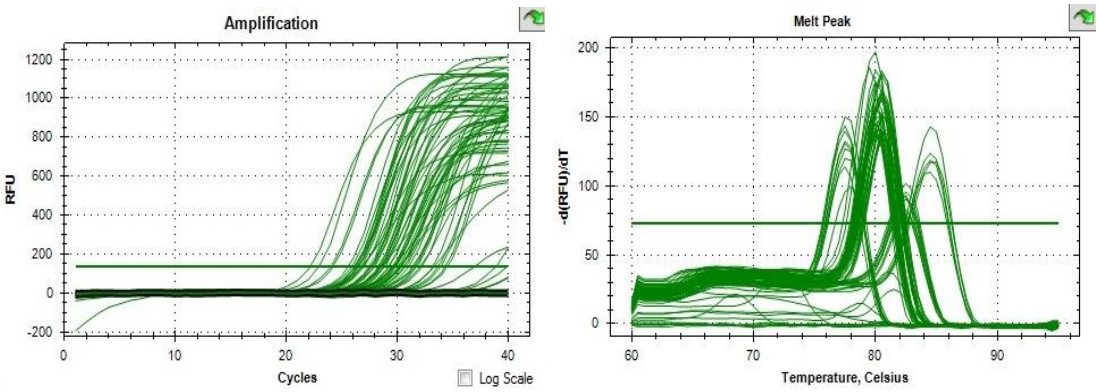
Table 4 qPCR reaction components by using KAPA SYBR® FAST qPCR Master Mix

Component	Volume/reaction	Final concentration
PCR-grade water	8.2 µl	N/A
2x KAPA SYBR® FAST qPCR Master Mix Universal	10 µl	1x
10 µM Forward Primer	0.4 µl	200 nM
10 µM Reverse Primer	0.4 µl	200 nM
Template DNA	1.0 µl	<20 ng
Total volume	20 µl	-

Table 5 The protocol of quantitative RT-PCR using KAPA SYBR® FAST qPCR Master Mix Universal kit

Step	Temperature	Duration	Cycles
Enzyme activation	95°C	3 min	Hold
Denature	95°C	1-3 sec	40
Anneal/extend	60°C	≥ 20 sec+ Plate Read	
Dissociation (Melt curve)	60.0-95.0 °C,	5 sec + Plate Read	increment 0.5 °C until reach 95.0 °C

During the PCR cycle, the fluorescence detection data would be plotted as shown in Figure 10 (a) and (b)



(a) (b)

Figure 10 Real time amplification plot (a) and melting curve (b)

(a) The linear scale amplification plot of the fluorescence detection during the PCR cycle. The baseline is the noise level in early cycles, the baseline threshold is adjusted to a value above the background and placed within the linear region of the amplification curve, which represents the detectable log-linear range of the PCR. The cycle that the fluorescence detection reaches the baseline threshold is described as a threshold cycle (C_T). (b) Melting curve is an assessment of the dissociation-characteristics of double-stranded DNA during heating. The samples with the same primer should have approximately the same peak.

Gene expression analysis

The expressions of six target genes were normalized with the reference gene, GAPDH. Negative control reactions (RNase free water) were included in each run. The gene expression analysis software (CFX Manager™, Bio-Rad, USA) was used to perform the relative quantifications according to the delta delta C_T from the C_T value for any samples normalized to the reference genes[65]

The relative quantity (ΔCq) for any sample (GOI) is calculated with this formula:

$$\textbf{Relative Quantity}_{\text{sample (GOI)}} = E_{\text{GOI}}^{(C_{q(MIN)} - C_{q(sample)})}$$

Where:

• E = Efficiency of primer and probe set. This efficiency is calculated with the formula (% Efficiency * 0.01) + 1, where 100% efficiency = 2

• Cq (MIN) = Average Cq for the Sample with the lowest average Cq for GOI

• Cq (sample) = Average Cq for the Sample

• GOI = Gene of interest (one target)

The normalized expression is calculated by the following formula, which uses the calculated Relative Quantity (RQ) calculation:

$$\textbf{Normalized Expression}_{\text{sample (GOI)}} = \frac{RQ_{\text{sample (GOI)}}}{(RQ_{\text{sample (REf}_n)})^{\frac{1}{n}}}$$

Where:

• RQ = Relative Quantity of a sample

• Ref = Reference target in a run that includes one or more reference targets in each sample

• GOI = Gene of interest (one target)

Data analysis

The mRNA expression of the different genes between the two groups (1. MSFA with xenograft group 2. Maxillary posterior edentulous bone group) were analyzed by using the independent t-test at significant level of 0.05 using SPSS (SPSS version 22.0; IBM, Chicago, IL).

A p-value of < 0.05 is considered to be statistically significant. Descriptive statistic was used to calculate mean and standard deviation for each sample group. (n=7)

CHAPTER IV RESULTS

The subjects that participated in this study had a normal wound healing with no infection after MSFA or implant placement surgeries. 11 Subjects (10 females and 1 male) with an age ranging from 21 to 66. The average subject age of the MSFA and the MPEB group was 55.1 and 53.7 years, respectively. The average height the residual crestal bone before MSFA was 2.7 mm. Descriptive statistics and independent t-test of the mRNA expression levels of RUNX2, RANKL, OPG, MMP-9, TRAP, and IL-1β, as well as the ratio of RANKL/OPG are shown in Table 6.

Table 6 Descriptive statistics and independent t-test of the genes expression levels

Gene	Group (N=5)	Mean	SD	Sig.
RANKL	MSFA	1.64	1.05	.343
	MPEB	1.18	0.63	
OPG	MSFA	1.52	1.14	.334
	MPEB	1.04	0.48	
IL-1β	MSFA	1.32	0.78	.193
	MPEB	0.83	0.54	
MMP9	MSFA	0.79	0.39	.614
	MPEB	0.88	0.28	
TRAP	MSFA	0.68	0.33	.104
	MPEB	0.96	0.28	
RUNX2	MSFA	1.11	0.64	.351
	MPEB	1.81	1.79	
RANKL/OPG ratio	MSFA	1.18	0.29	.858
	MPEB	1.22	0.47	

The analysis of mRNA expression of the different genes between the two groups (1. MSFA with xenograft group 2. MPEB group) did not reveal any difference in expression. No significant differences in gene expression levels were observed by using the independent t-test at significant level of 0.05 using SPSS (SPSS version 22.0; IBM, Chicago, IL). (Figure 11).

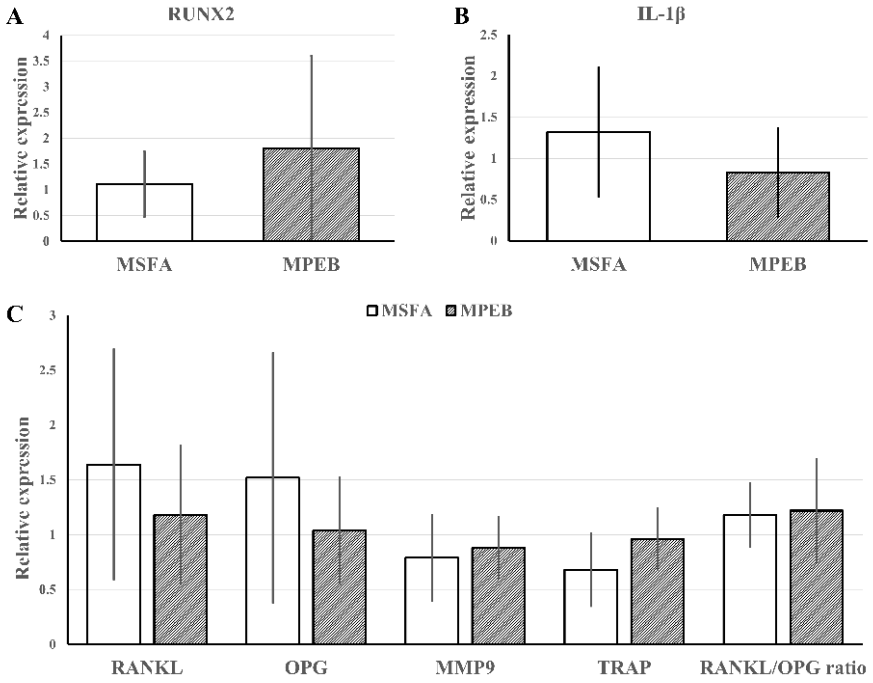


Figure 11 The relative expression of six target genes and the ratio of RANKL/OPG (+/-1 SD)

The relative expression was analyzed by a Bio-Rad CFX96TM real time RT-PCR system (Bio-Rad laboratories, Inc., USA). (A) Osteoblast related gene: RUNX2 (B) Inflammatory related gene: IL-1β (C) Osteoclast related genes: RANKL, OPG, MMP9, TRAP, and the ratio of RANKL/OPG No differences in genes expression level were observed by the independent t-test at significant level of 0.05 using SPSS (SPSS version 22.0; IBM, Chicago, IL). (MSFA = maxillary sinus floor augmentation, MPEB = maxillary posterior edentulous bone)

CHAPTER V DISCUSSION

The present study is the first in which the expression levels of a number of genes were analyzed in bone samples of the subjects that received MSFA with anorganic bovine bone (ABB, Bio-Oss®). ABB has been studied and used as grafting material for maxillary sinus floor augmentation. It is a xenograft that consists of deproteinized, sterilized cancellous bovine bone. Histologic and histomorphometric methods have often been used to evaluate the clinical success of ABB[6-10], but analysis of gene expression has seldom been done. Quantitative real time PCR shown that autografts was better than ABB in order to stimulate bone marrow stromal cell migration, proliferation, and differentiation assessed by genes coding for bone markers including Runx2, Collagen I, and Osteocalcin.[24] Although, autograft has osteoinductive potential, short term "collapse" of autograft due to its high rate of resorption is an obstacle to maintain vertical height needed for the MSFA procedure. MSFA using ABB yields a good result as a stable scaffold with no apparent "slumping" over time because of its slow resorption rate.[66] Because of its slow resorption and osteoconductive ability, Bio-Oss® is suitable for a stable scaffold and promotes bone-forming cells at the periphery to penetrate the graft material and form new bone. [8,9,38] Sinus augmentation using ABB performed by lateral approach technique yielded high success and survival rates.[67,68] Newly formed bone and osteoclasts were observed in closed contact with ABB particles after grafting; a phenomenon still seen after many years.[7]

Because of the residual ABB particles, we could clearly observe from the CBCT scan images that the alveolar bone of a group after MSFA with ABB had more radiopaque appearance than a maxillary posterior edentulous bone group. In this study, the average of residual ridge height was 2.7 mm. It almost completely eliminated by round carbide bur that the surgeon used to locate the position of

implant placement. After that we collect the grafted bone with a 2 mm internal diameter trephine bur. Each sample of bone tissue was aseptically harvested from the posterior maxillary region. Despite the difference of radiographic appearance, ABB particles do not interfere with the normal osseous healing process after MSFA and promote new bone formation in tooth extraction sockets in a similar degree as ungrafted sockets.[11] The results show no statistically significant difference between any of gene expression analyzed. Similar levels of expression were found for the osteoblast related, osteoclast related, and proinflammatory genes.

Bivariate regression showed that there were positive associate in RANKL OPG and IL-1β gene expression level in MSFA group. RANKL OPG and IL-1β were likely to express more in MSFA group; however, there were not statistically significant. (See appendix, table 13) According to the study of Piattelli M et al[7], osteoclasts can be founded in close contact with the remaining grafted particles of ABB. RANKL, expressed on the surface of osteoblasts, enhances the preosteoclasts differentiation into mature osteoclasts.[45] IL-1β stimulates osteoclasts to resorb bone.[22,23] While the cell-to-cell interaction between RANK and RANKL was increased, OPG was also released by osteoblast in order to inhibit mature osteoclasts.[47] Both RANKL and OPG had higher expression, so the RANKL/OPG ratio was likely to express the same. On the other hand, MMP-9 and TRAP which are released by mature osteoclasts to resorp bone were likely to express less in MSFA group.[20,21]

Our data indicate that following a healing period of at least 6 months ABB particles do not affect expression of the analyzed genes. We assume that a homeostatic situation had been reached, a situation comparable to tissue without ABB. Because the remaining grafted particles of ABB has slow resorption, they might induce higher numbers of osteoclasts than tissue without grafted particles. However, that osteoclasts did not release the higher resorption enzymes. Because of the limited number of the patients following a lateral window technique of MSFA procedure using

solely xenograft “ABB”, we were not able to analyze the level of proteins in order to compare these with our gene expression results. Yet, there are many histological studies that appear to confirm our findings on osteoblastic and osteoclastic activity.

CHAPTER VI CONCLUSION

In the present study, we have shown that gene expression levels of RUNX2, RANKL, OPG, MMP-9, TRAP, and IL-1β, as well as the ratio of RANKL/OPG were not different between bone obtained from patients following MSFA with ABB and bone obtained from the MPEB when healing period was at least 6 months.

After a healing period of 6 months ABB particles do not have an effect on the expression of genes associated with bone remodeling and inflammation.

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APPENDIX

Table 7 Bone samples from MSFA

sample	area	Gender (Female/Male)	Age (year)	Residual crestal bone (mm)	Healing time (month)	Nucleic acid concentration (ng/ μl)	260/280
1	14	F	21	3.1	5	29.96	2.02
2	26	F	57	2.7	10.5	23.3	1.98
3	27	F	57	2.4	10.5	28.13	1.98
4	26	M	65	2	12	14.5	1.8
5	26	F	58	2.7	9	29.23	1.91
6	15	F	64	3	6	87.33	2.01
7	16	F	64	3	6	36.27	1.99

Table 8 Bone samples from MPEB

sample	area	Gender (Female/Male)	Age (year)	Healing time (month)	Nucleic acid concentration (ng/ μl)	260/280
1	25	F	21	>6	11.7	2.06
2	25	F	57	10.5	8.63	1.99
3	25	F	57	>6	23.13	1.88
4	14	M	65	>6	15.08	1.83
5	26	F	58	>6	10.63	1.81
6	26	F	64	>6	17.8	2.01
7	26	F	64	12	9	1.9

Table 9 The ratio of residual crestal bone to implant length from MSFA group

Sample	Residual crestal bone (mm)	Implant length (mm)	Residual crestal bone/ Implant length
1	3.1	11	0.28
2	2.7	11	0.25
3	2.4	11	0.22
4	2	9	0.22
5	2.7	11	0.25
6	3	11	0.27
7	3	11	0.27

Table 10 Descriptive statistics of the gene expression level of RUNX2, RANKL, OPG, MMP-9, TRAP, and IL-1β, or in the ratio of RANKL/OPG (MSFA group)

	N	Mean	Std. Deviation	Asymp. Sig. (2-tailed) of One-Sample KS test
RANKL	7	1.638405	1.0546752	.150
OPG	7	1.523907	1.1442134	.200
IL-1β	7	1.327301	.7831589	.146
MMP9	7	.789564	.3944947	.200
TRAP	7	.677863	.3295510	.200
RUNX2	7	1.112488	.6363862	.091
RANKL/OPG	7	1.183751	.2853757	.043

Table 11 Descriptive statistics of the gene expression level of RUNX2, RANKL, OPG, MMP-9, TRAP, and IL-1β, or in the ratio of RANKL/OPG (MPEB group)

	N	Mean	Std. Deviation	Asymp. Sig. (2-tailed) of One-Sample KS test
RANKL	7	1.180072	.6297476	.127
OPG	7	1.041451	.4797483	.200
IL-1β	7	.832746	.5355713	.200
MMP9	7	.884116	.2782439	.200
TRAP	7	.964579	.2779442	.046
RUNX2	7	1.810198	1.7911902	.069
RANKL/OPG	7	1.221501	.4681785	.200

Table 12 Independent t-test of the gene expression level of RUNX2, RANKL, OPG, MMP-9, TRAP, and IL-1β, or in the ratio of RANKL/OPG

		Levene's Test for Equality of Variances		t-test for Equality of Means		
		F	Sig.	t	df	Sig. (2-tailed)
RANKL	Equal variances assumed	3.867	.073	.987	12	.343
	Equal variances not assumed			.987	9.796	.347
OPG	Equal variances assumed	10.401	.007	1.029	12	.324
	Equal variances not assumed			1.029	8.046	.334
IL-1β	Equal variances assumed	.480	.502	1.379	12	.193
	Equal variances not assumed			1.379	10.605	.196
MMP9	Equal variances assumed	1.420	.256	-.518	12	.614
	Equal variances not assumed			-.518	10.785	.615
TRAP	Equal variances assumed	.884	.366	-1.760	12	.104
	Equal variances not assumed			-1.760	11.668	.105
RUNX2	Equal variances assumed	2.490	.141	-.971	12	.351
	Equal variances not assumed			-.971	7.491	.362
RANKL/ OPG	Equal variances assumed	3.872	.073	-.182	12	.858
	Equal variances not assumed			-.182	9.918	.859

Table 13 Association of the gene expression level of RUNX2, RANKL, OPG, MMP-9, TRAP, and IL-1β, or in the ratio of RANKL/OPG (MSFA group)

Gene expression	β coefficient (95% Confidence Interval)
RANKL	.274
OPG	.285
IL-1β	.370
MMP9	-.148
TRAP	-.453
RUNX2	-.270
RANKL/OPG	-.053

Note: Analyses employed bivariate regression at significant level (0.05)

VITA

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