GENE EXPRESSION AND IMMUNOHISTOCHEMISTRY ANALYSIS OF XENOGRAFT AND ALLOGRAFT IN MAXILLARY SINUS FLOOR AUGMENTATION

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การวิเคราะห์การแสดงออกของยีน และอิมมูโนฮิสโตเคมิสทรี ระหว่างกระดูกปลูกถ่ายวิวิธพันธุ์ และกระดูกปลูกถ่ายเอกพันธุ์ ที่ใช้ในการเสริมโพรงอากาศแม็กซิลลา

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อิษยา กังวานชัยกุล : การวิเคราะห์การแสดงออกของยีน และอิมมูโนฮิสโตเคมิสทรีระหว่างกระดูกปลูกถ่ายวิวิธพันธุ์ และกระดูกปลูกถ่ายเอกพันธุ์ที่ใช้ในการเสริมโพรงอากาศแม็กซิลลา. (GENE EXPRESSION AND IMMUNOHISTOCHEMISTRY ANALYSIS OF XENOGRAFT AND ALLOGRAFT IN MAXILLARY SINUS FLOOR AUGMENTATION) อ.ที่ปรึกษาหลัก : ผศ. ทพญ. ดร.ใจแจ่ม สุวรรณเวลา

คุณภาพของกระดูกเป็นปัจจัยที่สำคัญ และส่งผลถึงความสำเร็จในการทำรากฟันเทียม ส่วนสูงของกระดูกในบริเวณฟันหลังบนในบางกรณีก็ไม่เพียงพอ และทำให้เกิดความเสี่ยงต่อการปักรากเทียม การผ่าตัดเสริมโพรงอากาศแม็กซิลล่าด้วยกระดูกทดแทนจึงถูกนำมาใช้ เพื่อแก้ไขกระดูกในตำแหน่งนั้น วัตถุประสงค์ของงานวิจัยนี้ คือ เพื่อเปรียบเทียบการแสดงออกของยีน ได้แก่ ทูเมอร์เนคโครซิส แฟกเตอร์-แอลฟา, รันทรีเลททรานสคริปชันแฟคเตอร์ทู, คอลลาเจนวันเอวัน, แอลคาไลน์ฟอสฟาเตท ระหว่างกระดูกขากรรไกรบนที่ได้รับการเสริมด้วยกระดูกวิวิธพันธุ์ (ไบโอ-ออส) เทียบกับกระดูกขากรรไกรบนที่เสริมด้วยกระดูกเอกพันธุ์ (ชัว-ออส) โดยมีผู้ป่วยที่เข้ารับการผ่าตัดเสริมโพรงอากาศแม็กซิลล่าทั้งหมด 13 คน และมีผู้ป่วยที่เข้ารับการฝังรากเทียมที่ตำแหน่งฟันหลังบนโดยไม่ได้ผ่าตัดเสริมกระดูก เพื่อเป็นตัวเปรียบเทียบในการวิเคราะห์ทางอิมมูโนฮิสเตเคมี จำนวน 1 คน กระดูกจากผู้ป่วยจะถูกเก็บด้วยหัวกรอเทรฟไฟรด์ ขนาดเส้นผ่านศูนย์กลางภายใน 2 มิลลิเมตร เพื่อมาทำการวิเคราะห์การแสดงออกของยีนโดยวิธีการเรียลไทม์ พีซีอาร์ (จำนวน 10 ตัวอย่าง) และวิธีอิมมูโนฮิสโตเคมี ดูการติดสีของแอลคาไลน์ฟอสฟาเตท ในบริเวณที่มีกิจกรรมการสร้างกระดูก (จำนวน 4 ตัวอย่าง) จากผลการทดลองวิธีเรียลไทม์ พีซีอาร์พบว่า การแสดงออกของยีนรันทรีเลททรานสคริปชันแฟคเตอร์ทูในกลุ่มกระดูกเอกพันธุ์มีค่าสูงกว่ากระดูกวิวิธพันธุ์ ระดับนัยสำคัญน้อยกว่า 0.05 แต่การแสดงออกของยีนทูเมอร์เนคโครซิส แฟกเตอร์-แอลฟา และคอลลาเจนวันเอวันไม่แตกต่างกันอย่างมีนัยสำคัญระหว่างกลุ่ม การย้อมสีอิมมูโนฮิสโตเคมีของตัวอย่างทั้งสองกลุ่มแสดงถึงระดับการติดสี และตำแหน่งการติดสีของแอลคาไลน์ฟอสฟาเตทคล้ายคลึงกัน จึงสรุปได้ว่าการแสดงออกของยีนทูเมอร์เนคโครซิส แฟกเตอร์-แอลฟา และคอลลาเจนวันเอวันไม่แตกต่างอย่างมีนัยสำคัญระหว่างกระดูกที่เสริมด้วยไบโอ-ออส และชัว-ออส แต่พบว่าในชัวออสมีการแสดงออกของยีนรันทรีเลททรานสคริปชันแฟคเตอร์ทูสูงกว่า อย่างไรก็ตาม ผลจากวิธีอิมมูโนฮิสโตเคมีแสดงผลที่เหมือนกันระหว่างกระดูกทดแทนทั้งสองกลุ่ม

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Isaya Kungvarnchaikul : GENE EXPRESSION AND IMMUNOHISTOCHEMISTRY ANALYSIS OF XENOGRAFT AND ALLOGRAFT IN MAXILLARY SINUS FLOOR AUGMENTATION. Advisor: Asst. Prof. JAIJAM SUWANWELA, D.D.S. Ph.D.

Quality of bone is an important factor contributing to the success of dental implants. The bone height in maxillary posterior area sometimes is not enough and might pose risks for placing dental implants. Maxillary sinus augmentation with bone substitutes is used for filling and correcting bony defects. This study aims to determine the differences in gene expressions of TNF-alpha, RUNX2, COL1A1, ALP between xenograft (Bio-Oss®) and allograft (SureOss®). Patients who needed two-stage maxillary sinus floor augmentation were included (n=13). Patient who had dental implant at posterior maxilla without bone substitutes was also used in immunohistochemistry analysis (IHC) (n=1). Bone samples were collected using trephine bur 2 mm internal diameter. Quantitative real-time PCR (qPCR) method was used for studying gene expression (n=10). Immunohistochemical staining of ALP was used to visualize the area of osteogenic activity (n= 4). The results of qPCR showed higher RUNX2 expression of allograft when compared to xenograft (p<0.05). Although, TNF-alpha, COL1A1 expression was not statistically different between groups. Immunohistochemistry staining of both samples also showed similar intensity and expression of ALP. The data suggested that gene expression levels of TNF-alpha and COL1A1 were not significantly different between bone grafted with Bio-Oss® and SureOss®. However, the expression of RUNX2 was significantly higher in SureOss®. Nevertheless, result from IHC study showed similar characteristics between both groups.

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

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Chapter I

INTRODUCTION

Introduction

Placing dental implant in maxillary posterior site is quite challenging because of the nature of bone in that area (1). After tooth loss, not only the bone continues to resorb, but the sinus cavity also expands into the alveolar bone (2). As a result, bone quantity and quality are compromised. To avoid complication after implant surgery, surgeon will perform two-stage maxillary sinus floor augmentation if the residual bone height is less than 4 mm (3). Maxillary sinus floor augmentation with bone substitute materials can increase bone volume, which enhances primary stability of dental implant and will finally increase the survival rate of dental implant (4).

There are many biomaterials available in the market. Bone substitute materials are produced to help the problems related to autogenous bone. The xenograft from bovine bone is commonly used by dental surgeons. Allograft from human donor is also a preferred choice for dental procedures. Histomorphometric studies comparing bone grafting materials in aspect of new bone formation, remaining graft, and soft tissue components are widely reported (5). However, molecular biology knowledge behinds the bone remodeling process after grafting is still limited.

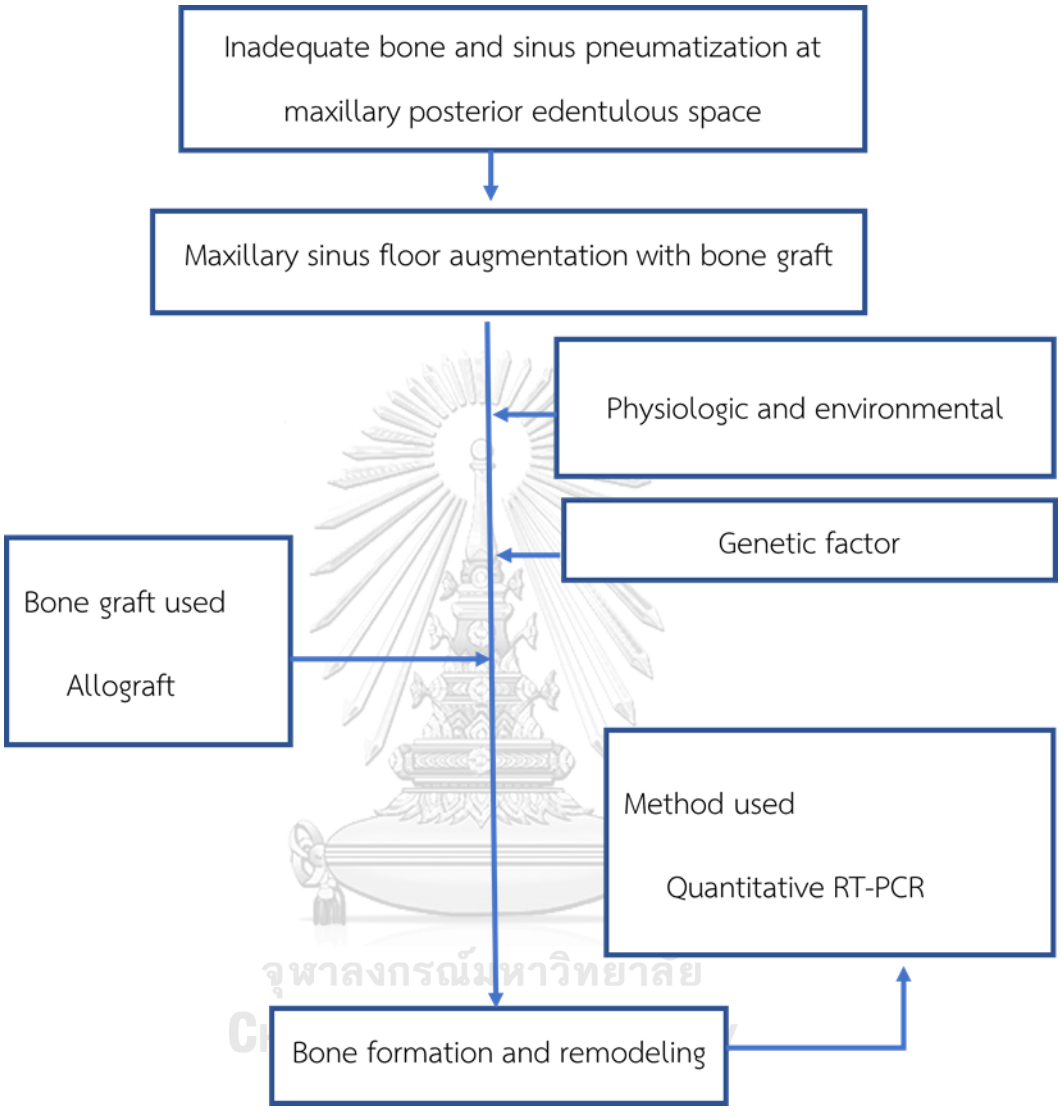
Bone remodeling occurs by bone cells receive a sign of inflammation from microenvironment after bone injury. Mediators of inflammation such as TNF-alpha (proinflammatory cytokine) stimulates the formation of IL-1 cytokine. When osteoblasts receive the inflammatory signals, they will produce RANKL. RANKL will bind to RANK on the surface of nearby monocytes and helps promote the osteoclast formation and function (6). Apart from RANKL, TNF-alpha synergistically supports the formation of osteoclast and helps in bone resorption. Following bone resorption, osteoblast differentiation is initiated. Regulation of osteoblast differentiation is controlled by transcription factors such as Runx2 and WNT/β-catenin, Osterix etc. (7).

RUNX2, a major transcription factor can induced the expression of extracellular bone matrix protein like collagen type1 (COL1A1) (8) . At the last stage of osteoblastic differentiation, alkaline phosphatase (ALP) expression is detected in mineralized tissue (9).

To achieve better understanding about the effect of graft to the expression of gene related to bone formation and bone remodeling. Gene expression analysis with real-time quantitative PCR is chosen to perform in this project. In addition, immunohistochemical staining is used for visualizing the expression of osteogenic activity in bone tissue.

The gene expression study of specific target genes that related to function of bone cells at different periods could enlighten the clinicians in term of using grafting materials that is suitable for the case based on the data received from relative gene expression and immunohistochemistry between different types of bone grafts.

Conceptual framework



Research questions

1. Are there any differences in the gene expression levels of TNF-alpha, RUNX2, COL1A1 between bone grafted with xenograft (Bio-Oss®) and bone grafted with allograft (SureOss®) in maxillary sinus floor augmentation procedure?
2. Are there any differences in immunohistochemical staining of ALP between bone grafted with xenograft (Bio-Oss®) and bone grafted with allograft (SureOss®) in maxillary sinus floor augmentation procedure?

Research objectives

1. To examine the differences in the gene expression levels of TNF-alpha, RUNX2, COL1A1 between bone grafted with xenograft (Bio-Oss®) and bone grafted with allograft (SureOss®) in maxillary sinus floor augmentation procedure
2. To examine the differences in immunohistochemical staining of ALP between bone grafted with xenograft (Bio-Oss®) and bone grafted with allograft (SureOss®) in maxillary sinus floor augmentation procedure

Research hypothesis

1. **H_0** There are no differences in the gene expression levels of TNF-alpha, RUNX2, COL1A1 between bone grafted with xenograft (Bio-Oss®) and bone grafted with allograft (SureOss®) in maxillary sinus floor augmentation procedure.

   H_1 There are differences in the gene expression levels of TNF-alpha, RUNX2, COL1A1 between bone grafted with xenograft (Bio-Oss®) and bone grafted with allograft (SureOss®) in maxillary sinus floor augmentation procedure.

2. **H_0** There are no differences in immunohistochemical staining of ALP between bone grafted with xenograft (Bio-Oss®) and bone grafted with allograft (SureOss®) in maxillary sinus floor augmentation procedure. **H_1** There are differences in immunohistochemical staining of ALP between bone grafted with xenograft (Bio-Oss®) and bone grafted with allograft (SureOss®) in maxillary sinus floor augmentation procedure.

Proposed benefits

1. Knowledge in biology of gene expression related to bone formation, bone remodeling.
2. Knowledge of how to select bone grafting materials in molecular aspect.

Chapter II

REVIEW OF LITERATURE

Statistics of partially edentulous patient and dental implant

The 8th Thai National Oral Health Survey in 2017 reported that the percentage of elderly people (age 60-74 years) who had four pairs of opposing posterior teeth were 39.4% (10). This could be explained that about 60 percent of elderly people had lost some of their posterior teeth. Tooth loss can cause a number of problems if they are not replaced. Not only the opposing tooth will slowly extrude to the edentulous space, the adjacent tooth will move and collapse into to the space, which would cause occlusion problem.

Dental implant is a prosthesis that fixed into the jaw bone and replace the missing tooth. It can function more like a natural tooth when compares to removable prosthesis. A systematic review concludes that long-term dental implant survival rate is 96.4% (11). The high survival rate helps growing patient acceptance of dental implants. The use of dental implant has been rising especially for the elderly people. Dental implant prevalence in the United stated of America had increased considerably for 17 years (1999-2016) (12).

Implant osseointegration

Osseointegration is the direct contact between an implant and bone. From this definition, osseointegration depends on two main factors, implant and bone. If dental implant has adequate primary stability, it will continue to develop secondary stability which is known as osseointegration. Primary stability of dental implant is related to bone quality and quantity (13).

After implant placement, osteotomy site is filled with blood. Platelets that are released from injured blood vessels help form blood clot and stimulate inflammatory cells and growth factors. Various growth factors are necessary in forming fibrin scaffold and new blood vessels. Later, osteoprogenitor cells will migrate into the injured site

and promote bone healing. Peri-implant bone healing could occur via two mechanisms, distance osteogenesis and contact osteogenesis. Contact osteogenesis is the bone healing process that initiated from surface of implant. On the other hand, distant osteogenesis is the bone healing process that initiated from the osteotomy site (14).

Implant site that will received dental implant must have adequate bone quantity and quality. The site should also have enough vascularity for the cells to migrate and promote bone formation. The previous studies concluded that in the areas that received bone augmentation by bone substitutes, types of bone grafts did not have an impact on the primary stability of dental implants. When consider other factors related to bone augmentation procedure, such as period of healing, it was not related to primary stability as well. Although, native bone was the main area for initial integration of bone and implant to occur, the bone substitutes did not seem to interfere the process of osseointegration (15).

Bone remodeling

Bone remodeling is stimulated by bone cells that sense damage in bone after injury. Bone remodeling is controlled by an intervention of cells and their substances through multiple mechanisms. Each cell has a specific role during the process (16).

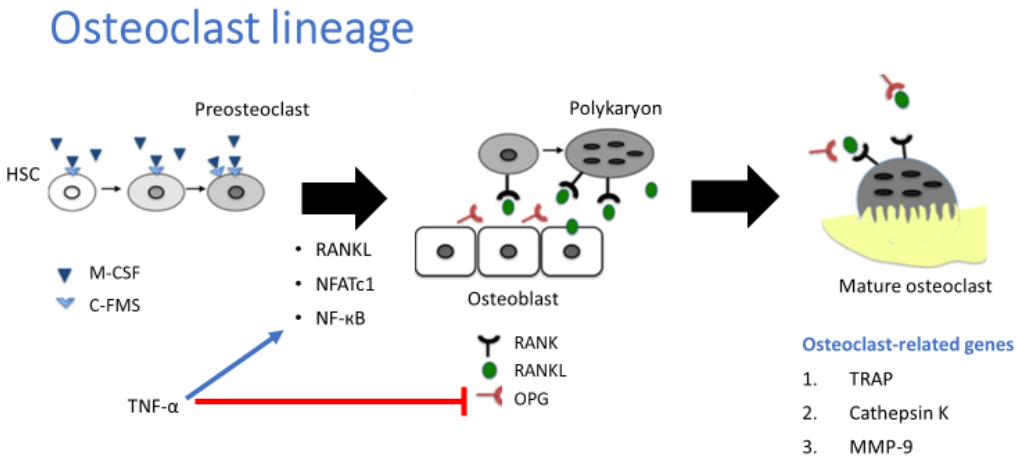


Figure 1 Genes involved in osteoclast differentiation

Bone remodeling occurs in the BMU (Basic multicellular units) (16). The initial phase includes the recruitment of inflammatory mediators such as IL-1, TNF-alpha, inflammatory cells, growth factors, osteoclast precursors. IL-1 and TNF-alpha can enhance osteoclastogenesis (17). Osteoclast precursors will differentiate into osteoclasts and initiate bone resorption. The most important signals involved in this step are M-CSF and RANKL, produced by osteoblasts (17). They promote the differentiation of osteoclast precursors and prolong the life of activated osteoclasts (18). RANK connects with RANKL and provides RANK signaling that activates transcription factors essential for osteoclastogenic cascade including NF-kβ, c-fos, NFATC1 (19, 20). Osteoclast function is to resorb the bone matrix through the production of proteolytic enzyme, such as Cathepsin K. It is a protease that degrade collagen and other bone matrix proteins. MMP enzymes works in the same function as Cathepsin K (21).

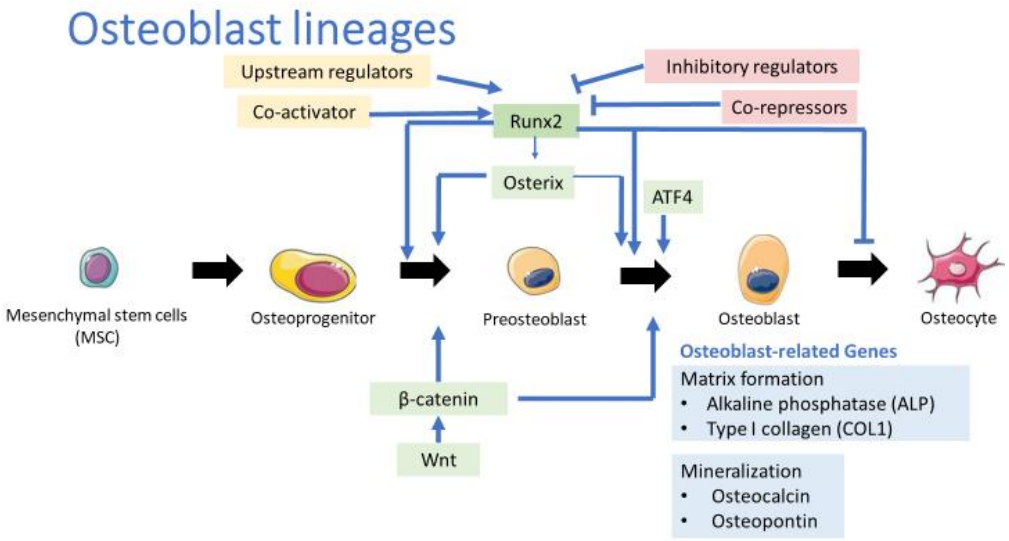


Figure 2 Genes involved in osteoblast differentiation

The transition phase occurs when the osteoblast precursor cells are recruited to the osteoclast resorption site. Macrophages clear off the resorptive lacunae. Bone matrix derived growth factor (TGF-β) inhibits the production of RANKL from osteoblast, and induces proliferation of osteoblast precursors in the bone (19) . Other

coupling mechanism of osteoclast and osteoblast is not clearly understood. The termination phase is when osteoblast differentiation reaches to the last step (17). The new bone matrix is consisting of protein such as collagen type I. This phase lasts about 3 months in humans (22).

Osteoclast differentiation is suppressed via osteoprotegerin (OPG) produced by osteoblast during progress of bone formation. Osteoblast differentiation is controlled by the master transcription factor RUNX2 (23). RUNX2 is indispensable for mesenchymal stem cell differentiation to the osteoblast lineage. The fully differentiated osteoblast is characterized by co-expression of ALP, type I collagen, osteocalcin, osteopontin (24).

Genes involved in bone remodeling process that used in this study

TNF-alpha

Tumor necrosis factor-alpha is a multifunctional cytokine, which works independent of RANKL to stimulate osteoclast differentiation. TNF-alpha supports osteoclast function by intervene with DNA and collagen synthesis, so the bone mineralization process is inhibited. It also stimulates macrophage colony-stimulating factor (M-CSF) which supports osteoclast differentiation in the early stage. Data from in vitro and in vivo studies could be summarized that TNF-alpha promotes bone resorption, and works together with other cytokines such as IL-1 and RANKL, which cause synergistic effect on osteoclast differentiation (17).

RUNX2

Runt-related transcription factor 2 (RUNX2) binds to DNA binding proteins (such as AP1, Smads) and together activate the other genes related to osteoblast differentiation. It is a master gene for osteoblast differentiation by activates the differentiation of mesenchymal stem cells toward the preosteoblast. RUNX2 function is important for the early stages of osteoblast differentiation and osteoblast proliferation, while it is downregulated in the mature osteoblast (25). It controls the

activity between osteoblast and osteoclast through its capability to activate the osteoprotegerin (OPG) promoter. The production of OPG will inhibit osteoclast formation by interfering with RANKL-RANK pathway.

COL1A1

The pro-alpha1 chains of type I collagen, whose triple helical structures contain two chains of alpha1 and one chain of alpha2, is encoded by the COL1A1 gene. Collagen type I is a major protein in bone organic extracellular matrix, however it can also be found at most connective tissue, as a result the protein is not bone-specific. The protein performs the function of cell adhesion, proliferation, and differentiation of the osteoblast phenotype. Bone strength depends on collagen extracellular matrix protein. When COL1A1 presents with other bone matrix protein, it can be considered as an indicator of osteoblastic differentiation (26).

ALP

Tissue nonspecific alkaline phosphatase (TNAP) is controlled by the ALP gene. This enzyme function is involved with the initiation of bone mineralization process, by induced osteoblast to synthesis extracellular matrix protein, mainly type I collagen. For this process to be complete, intracellular calcium and extracellular phosphate are necessary for hydroxyapatite crystals formation. ALP expression is found throughout the healing process and highly expressed at late stage of mineralized bone formation (27).

Mechanism of bone regeneration and healing

Bone augmentation with bone graft is a procedure used to restore the missing bone. Grafting is the process of implanting a healthy tissue or scaffold to the recipient site, so that it will enhance the regenerative capacity of the bone and lead to new bone formation (28). The mechanism of bone graft regeneration might be categorized into 3 categories.

1. Osteogenesis

Osteogenesis is the ability of the graft to produce new bone by viable donor cells inside transplanted bone graft (29). The transplanted autogenous graft have osteogenesis properties especially the graft that was taken from cancellous marrow. Osteoblast inside cancellous marrow could differentiate and form new bone.

2. Osteoconduction

Osteoconduction is the ability of the graft to act as a scaffold and passively allow localized bone cells and perivascular tissue to reside, differentiate, finally form new bone. The porous structure of bone graft facilitates the bone formation process. Both natural bone graft and synthetic bone graft have this properties. The rate of osteoconduction for synthetic bone graft is slower than natural bone graft due to its lack of osteoinductive properties (30).

3. Osteoinduction

Osteoinduction is the ability of the graft to induce mesenchymal stem cells to differentiate into osteoblast. Three main factors that determine the bone formation of osteoinductive biomaterials are macrostructure, micro/nano structure, chemical composition. Macrostructure characteristics include form, geometry and porosity of materials, which effect how cells and nutrient infiltrate inside. Micro/nano structure is related to surface roughness and grain size of materials. Chemical composition of materials could results in ion release especially the calcium phosphate based ceramics (30).

Classification of bone graft

Ideal characteristics of bone graft should have good biocompatibility and facilitate bone regeneration. Bone graft can be categorised based on their origin into 4 groups (31).

1. Autogenous bone graft

It is a bone obtained from the individual patient, so extra surgery site is required. The graft can be transferred from one site to place in another site. They possess osteoconductive, osteogenic, osteoinductive properties. The advantage of this graft is that its structures composed of mineral, collagen, viable osteoblast, BMP. The graft is easily revascularized and rapidly incorporated, which results in the early production of new bone (28, 31).

2. Allograft

It is a bone obtained from the same species usually cadaver. It has to undergo a lot of different treatments to eliminate the immune response to the recipient. The treatment that the manufacturer used is bone lyophilization and demineralization. When compared to autogenous bone, this graft is comparable in the properties except that it does not provide the necessary osteoinductive properties. The process that used for sterilization the graft is not only eliminate the cause of infection but also destroy the proteins that responsible for those properties. This graft resolves several disadvantages of autogenous graft (32).

SureOss®

c

Figure 3 SureOss® Freeze dried bone allograft

SureOss® is a Freeze Dried Bone Allograft (FDBA) comprised of 100% cortical bone. Freeze-Dried cortical bone promotes new bone formation by osteoinduction which depends on the biological property of the graft to stimulate ingrowth of new blood vessels and facilitate mesenchymal cells migration to the graft site. Cortical bone provides dense lamellar structure and acts as the scaffold for bone cells. Recommended use of SureOss® cortical bone by manufacturer: Hans Biomed Corp., is for the procedures such as maxillary sinus floor augmentation, socket and ridge preservation. Bone particles come in 2 forms. The smaller size comes as powder : 200-850 µm and the larger size comes as chip : 850-1000 µm (33).

3. Xenograft

It is the bone from animals and is processed to eliminate the organic components chemically or by heat. The mineral part of bone is conserved. The most common xenografts are derived from bovine. High temperature or chemical extraction is used to remove the organic component and their antigenicity. The remaining inorganic components give excellent sources of calcium and natural matrix that use to support the osteogenic cells (32). Resorption rate is slow or maybe not completely degradable (34).

Bio-Oss®

Figure 4 Geistlich Bio-Oss® bovine bone substitute

Bio-Oss® is the xenograft produced from bovine bone. It is processed through the stepwise heat treatment up to 300°Celsius, followed by the strong alkaline

chemically cleansing step with sodium hydroxide. The particle size of this material is 1-2 mm (35).

Several studies that use Bio-Oss® show some promising results comparable to autogenous bone graft (36). From the histologic study, it can be seen that Bio-Oss® particles do not interfere with the bone healing process after maxillary sinus floor augmentation. In addition, it also supports new bone formation (37). A study about gene expression in Bio-Oss® compared with maxillary posterior edentulous bone conclude that mRNA expression of bone marker genes between both groups is not difference (38).

4. Alloplast

It is a group of chemically synthetic bone grafts including, hydroxyapatite, calcium phosphates cement, β-tricalcium phosphate, bioactive glass, biocompatible composite polymer. The main disadvantage of alloplast is its unpredictability in allowing bone formation (39).

Differences between allograft and xenograft

1. Physicochemical properties of different grafting materials

Allograft has lower crystallinity compared to xenograft which results in its fast resorption rate and its fast regenerative mechanism. Organic content in allograft might facilitate the osteogenic activity. Other properties like hydrophilicity are varied which depends on the manufacturing process. In summary, the differences that can be found between bone graft are percent of calcium component, particle sizes, crystallinity (40).

2. Histology and histomorphometric results of different grafting materials

The systematic review by Danesh-Sani, S. A. et al., summarized the effect of different types of graft materials on histomorphometric variables at 3-time phase. The difference in percentage of new bone was not found between allograft and xenograft at all time point. The percentage of residual graft was less in allograft group after 9

months of healing. Connective tissue component in allograft was higher than xenograft after 4.5 months of healing (5).

3. Micro-CT results of different grafting materials

In vivo study using mouse model by Kangwannarongkul et al. (41), reported that bone formation at 3 months compared to 1 month was increased for both xenograft and allograft group. The residual grafts inside the defects was decreased in both groups. In the other study, bone chamber model in rat was used to evaluate osteoconductive properties of different bone grafts by micro CT analysis , which found that bone volume was greater in chambers filled with allograft compared to the xenograft group (42).

4. Immunohistochemistry results of different grafting materials

Hawthorne AC et al. (43), compared allografts with autografts using immunohistochemistry and found a similar pattern for both groups, except for TRAP staining that is related to bone resorption. They concluded that both grafts were different in graft resorption process. Galindo- Moreno et al. (44), compared a mixed of autogenous bone with xenograft and a mixed of autogenous bone with allograft found that RUNX2 expression was higher in the samples grafted with a mixture of xenograft, but it was not statistically significant difference.

5. Gene expression results of different grafting materials

Gene expression analysis of the bone marker genes in mouse calvaria defect based on RT-PCR shown that the differences between xenograft and allograft were found in the expression of RUNX2 and OPN gene. For RUNX2, xenograft showed higher expression than allograft. For OPN expression, the result was opposite to RUNX2 expression (41).

Maxillary sinus floor augmentation

Sinus augmentation is used to correct bone deficiency in the maxillary posterior teeth area before implant placement (45). The principle of this surgery is to increase

bone volume, so the implant can be place safely. When primary stability is compromised by severe atrophy of residual bone, a two-step procedure is suggested.

Maxillary sinus floor augmentation is preferred to be done via two access, lateral window access and vertical access which is known as crestal sinus floor elevation technique. Both techniques are proved to be predictable and widely used to treat bone defect and fill up bone volume for implant placement. It is reported that implant survival rates for sinus augmentation using a lateral window technique showed similar survival rates as the non-grafted posterior maxilla. Lateral window technique with delay implant placement is used if remaining bone is less than 4 mm (46).

When the lateral window technique is compared to crestal sinus floor elevation technique, the advantage of the second technique is the less morbidity. However, it may be limited to mild and moderated vertical bone loss. Stern, A., & Green, J (2012) suggested that this technique should be used for the case that have remaining bone more than 8 mm (46).

Implant placement in maxillary sinus augmentation area

It is commonly found that some patients do not have adequate bone for dental implant procedure in posterior maxilla area, so bone graft augmentation is used to correct this problem. Short dental implants (6 mm) are another option for mild vertical bone loss case.

A 10-year retrospective follow-up study of effectiveness in maxillary sinus floor elevation found that the procedure did not effect to survival rate of dental implant. The factor that related to implant failure was the remaining bone height. For 0-5 mm height, the height was related to long term implant survival, other than that it was not correlated statistically. The study could not find the correlation between type of graft and long-term implant survival (47) .

Methods to study the effect of bone graft in maxillary sinus floor augmentation

Evaluation of regenerated bone subsequent to graft placement has been analyzed by the following methods.

1. Histologic and histomorphometric analysis

It is the most common approach to study the changes after grafting. This helps evaluate the healing patterns and the percentage of new bone formation, connective tissue and remaining bone graft (48). The disadvantage is the analysis of a few sections cannot be used as a representative of an entire sample.

The proportion of total bone area to total area is measured to find out the extent of new bone formation. Total bone area (TB) is defined as the original bone area plus new bone formation area, while the total area (T) is the total bone area that also includes the soft tissue area.

2. Radiographic analysis

The use of 2-D radiographic analysis is to evaluate the amount of graft height changes. Different types, composition, and age of the graft affected radiographic features. The use of 2-D radiographic analysis has some limitations compared to 3-D radiographic analysis. Micro-CT (3-D radiographic analysis) aids in the examination of bone volume and bone structure after bone regeneration. Data from the micro-CT method could help in distinguishing between the graft material and the mineralized bone, comparing their volumes, and visualizing graft resorption gradient (49).

3. Gene expression analysis

It is the method based on molecular biological evaluation. Gene encodes the protein and protein dictate cell function. Information from a gene that specific to the cell could be studied with real-time polymerase chain reaction (50). Relative gene expression is the method to explore the changes in the expression of a target gene compared to reference gene. Gene expression studies such as microarray or RNA

sequencing are used for simultaneously explore the patterns of expression of several genes.

Technique for study gene expression

1. Quantitative Real-Time PCR

Real-time PCR is a technique developed from the conventional PCR used to quantify a number of genetic components in the sample (50). Two-step quantitative reverse-transcription PCR (qRT-PCR) starts with cDNA synthesis from template RNA with reverse transcriptase enzyme. Next, cDNA will be diluted and transferred into PCR tube. Master mix that contains DNA polymerase enzyme, dNTP and dye is added. After that, the tubes are placed inside the real-time machine. Real time PCR reaction is completed by the repeated cycles of changing temperature incubation.

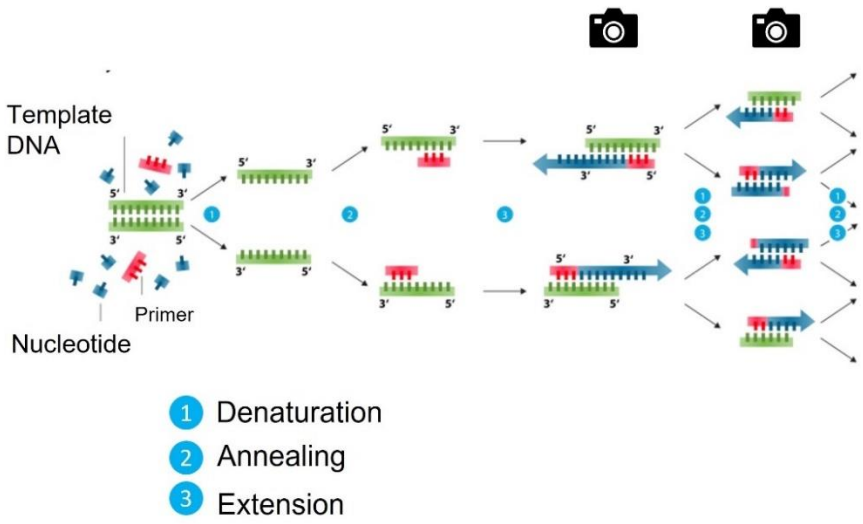


Figure 5 Steps in polymerase chain reaction (51)

There are three main steps for each cycle in real-time PCR.

1) Denaturation— High temperature about 94-98°c is used to separate the double-stranded DNA.
2) Annealing— The temperature is reduced to about 55°c to 60°c, so the primer of target gene can anneal to the single stranded of template DNA.

3) Extension— Primer extension occurs by DNA polymerase, dNTPs and can be detected by real-time machine using double stranded DNA-binding dye.

To analyze the data from qPCR, the researcher often uses the relative quantification method (The comparative threshold method) in which the expression of a gene of interest in the sample (treated) and the reference gene (a baseline) is compared (52). The quantity of target gene in the sample, normalized to an endogenous reference gene related to the normalized calibrator, is then given by $2^{-\Delta\Delta Cq}$, where $\Delta\Delta C_q = \Delta C_q$ (Sample) $-\Delta C_q$ (Calibrator) and ΔC_q is the C_q of the target gene subtracted from the C_q of the reference gene. For accurate results, amplification efficiencies of the reference gene and target gene should be above 90%.

2. Immunohistochemistry technique

Immunohistochemistry is used to investigate the changes in the composition of key proteins in the tissue of interest. This is a technique based on using the specific antibodies to visualize and localize the interested antigen in cells or tissue sections. It develops from the antigen-antibody reaction and is used to specify a particular cell type.

Conducting immunohistochemistry is comprised of several essential steps (53, 54). These include proper handling of the specimen, appropriate fixation to maintain adequate antigenicity of the tissue. The specimen is embedded in a paraffin block. Antigen retrieval is performed before the addition of the unlabeled primary antibody followed by a labeled secondary antibody. Incubation and washing the specimen is proceeded as the protocol suggested.

Fedchenko (55) reviewed and summarize the six ways to interpreted and analyzed IHC data which is to,

1. Describe the morphological parameters
2. Count the number of stained cells/structures

3. Count the percentage of stained cells in the area
4. Score difference force of IHC data qualitatively
5. Create and use semiquantitative scoring
6. Calculate objective parameters and use automated systems.

Chapter III

RESEARCH METHODOLOGY

Patient selection and bone biopsy

All patients enrolled in this study must be satisfied with the following entry criteria:

1. Age between 46-65 years old.
2. No smoking habit.
3. No systemic disease that could affect the normal healing process.
4. No current pregnancy.
5. No history of cancer-based treatment like chemotherapy.

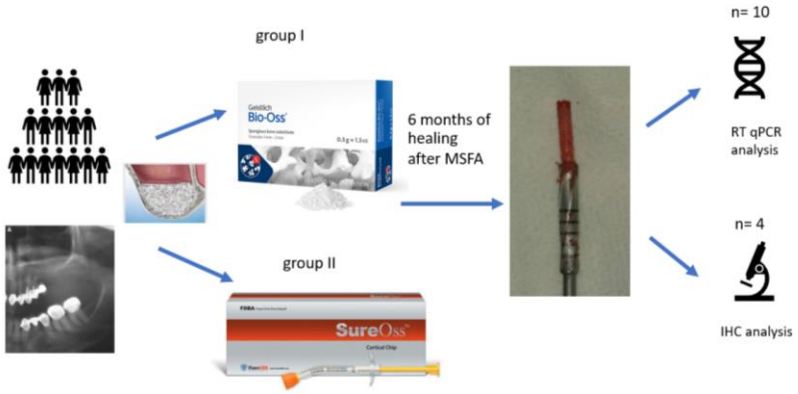


Figure 6 Workflow of this study

Thirteen randomly selected patients who were candidates for maxillary sinus augmentation, opted for two-stage procedure and one patient who had dental implant at posterior maxilla without bone substitutes were participated in this study. The consent papers of all patient were collected at the beginning of this project. The ethics committee of the faculty of dentistry, Chulalongkorn university approved the study protocol (study code: HREC-DCU 2020-012). All patients had a cone beam computed tomography scan prior to surgery. Under local anesthesia, first a crestal incision was created in the edentulous area with buccal releasing incisions. Full thickness flaps were raised to expose the lateral wall of the maxillary sinus. Next, lateral opening to access

the maxillary sinus was achieved with round bur under sterile saline solution irrigation. Then, sinus membrane was detached and elevated to the height of the desired augmentation. Finally, the bone graft materials were added inside the prepared space. The space was close with membrane and was left to heal. The second stage was performed after the first stage had been completed for at least six months. The bone tissues were collected at the second stage using a trephine bur (internal diameter 2 mm, length 10 mm). Bone sample which will undergo gene expression analysis was then transferred from trephine bur into the cryovial tube contained RNA*later*™ (Invitrogen™, Thermo Fisher Scientific, USA) using a periodontal probe. The tube was kept into a liquid nitrogen tank immediately. For the immunohistochemistry analysis, each bone sample was kept in a tube containing a solution of neutral buffer formalin.

Bone homogenization & cell lysis

Figure 7 Reagent and device used for bone homogenization

The preservative reagent RNA*later*™ was removed from the bone sample. The bone sample was moved from a cryovial tube to a metal bead tube which prechilled in liquid nitrogen. The lysis reagent (RiboEx™, GeneAll®, Korea) which pre-chilled on ice was added into a PowerBead tubes (Metal 2.38 mm) (Qiagen, USA). Next, bone homogenization was achieved using a bead-beating technique in Bead-based homogenizer (PowerLyzer, Mo Bio Laboratories, Inc., USA) at 3500 RPM speed for 2 cycles of 30 seconds and 30 seconds pause between cycles.

RNA isolation

Phase separation

This was performed using RiboEx™ recommended protocol, which is stated as followed.

1. Leaving the sample at room temperature for 5 minutes, to allow complete dissociation of the nucleic acid and proteins.
2. Add 0.2 µL of chloroform, shake the tube strongly for about 15 seconds.
3. Incubate the tube for 2-3 minutes at room temperature.
4. Set a centrifuge (Allegra® X-22R, Beckman Coulter, Indianapolis, USA) for 4 degree Celsius and centrifuge a sample at 12,000 x *g* for 15 minutes.
5. After centrifugation, the sample in the tube was separated into three different layers. The colorless upper layer contains the RNA (Figure 8).

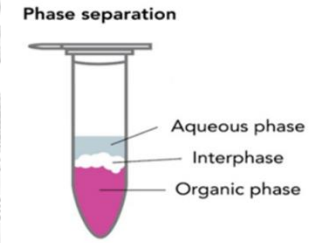


Figure 8 RNA obtained from phase separation

6. Transfer the supernatant about 400µL to a new RNase free tube and add an equal volume of 70% ethanol and mix well by vortexing.

Binding, washing, and elution

This was performed using RNeasy® Mini Kit (Qiagen, USA) , the protocol is stated as followed.

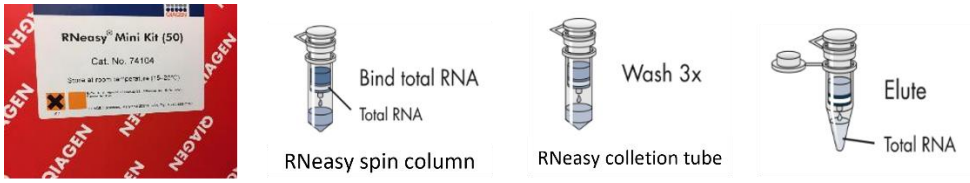


Figure 9 RNA isolation using RNeasy® Mini Kit

1. Transfer 700μL of the sample from phase separation to the spin cartridge (with the collection Tube)
2. Centrifuge at 12,000 x *g* for 15 seconds at room temperature in order to remove the lysate from RNA. Discard the lysate flow through. Repeat step 1.
3. Wash bonded RNA by adding 700 μL of Buffer RW1 to the spin cartridge and centrifuge at 12,000 x *g* for 15 seconds. Discard the flow-through and the collection tube
4. Place the spin cartridge into a new collection tube and add the 500 μL of prepared Buffer RPE (Buffer RPE with ethanol). Centrifuge at 12,000 x *g* for 15 seconds. Discard the flow-through and repeat this step once.
5. Dry the membrane with attached RNA by centrifuging the Spin Cartridge at 12,000 x *g* for 1-2 minutes. Discard the collection tube.
6. Insert the spin cartridge into a recovery tube then add 30 μL of RNase-free water to help elute the RNA. Incubate the tube at room temperature for 1 minute.
7. Centrifuge the spin cartridge inserted in the recovery tube at 12,000 x *g* for 2 minutes at room temperature to elute the RNA from the membrane into the recovery tube.
8. Finally, ultrapure RNA is in the recovery tube ready to be use or store at -20 degrees Celsius.

Analyzing RNA yield and quality

Figure 10 NanoDrop2000c spectrophotometer

The spectrophotometer instrument "NanoDrop 2000c"(Thermo Scientific, Massachusetts, USA) was used to quantify the purity and concentration of the sample by measure absorbance across specific wavelengths.

To accurately assess sample purity 260/280 ratios were analyzed. Pure nucleic acid yield 260/280 ratio of approximately near 2.0 for RNA was quantified to be used in the next procedure.

RT-PCR

Two-step real-time PCR was used in this study. First, RNA was reverse transcribed into cDNA. Next, cDNA was amplified so the gene of interest could be detected.

Reverse-transcription protocol

Reverse transcription is the process that transcribes RNA templates into cDNA molecules. iScript™ Reverse Transcription Supermix (BIO-RAD, USA) was used following manufacturer protocol.

Figure 11 iScript™ Reverse Transcription Supermix

Table 1 iScript™ Reverse Transcription Supermix protocol for a single cDNA synthesis reaction

Component	Volume/reaction
RNA template (1 µg-1 pg total RNA)	x µL
iScript RT Supermix	4.0 µL
Nuclease-free water	x µL
Total Volume	20 µL

1. Calculate the amount of RNA template to add in the reaction. Use as maximum amount of RNA as possible.
2. Add 4 µL of iScript RT Supermix to each sample on ice (Table 1).
3. Close a lid of each samples, mix tubes by briefly vortexing followed by a pulse spin.
4. Use Eppendorf® (Mastercycler gradient) thermocycler for incubate the samples follow the protocol list in table 2.

Table 2 iScript™ Reverse Transcription Supermix Protocol for setting thermocycler

Reaction	Temperature	Duration
Priming	25°c	5 min
Reverse transcription	46°c	20 min
RT inactivation	95°c	1 min

5. Prepare the dilution of cDNA before use in qPCR procedure (cDNA concentration for qPCR = 5ng/ul) and keep the cDNA stock at -20 °C refrigerator.

Primer design and preparation

The properties of primers used must satisfy all following criteria.

1. Primer Length is about 18-24 base pairs.
2. A primer contains with 40- 60% of GC content.
3. Melting temperature of primer is between 50-60°c.

NCBI primer-blast software were used for designing the primers (http://www.nchi.nim.nih.gov/tools/primer-blast/) (56, 57). The primer sequences were shown in the table 3. 18s was used as a reference gene to normalize the expression data. The dry primers were dissolve with RNase free water and were kept at -20°C refrigerator. The stocked primers were diluted in 10 µM concentration for use in the real-time PCR reaction.

Table 3 Primer used in this study

Gene	Forward primer (5′-3′)	Reverse Primer (5′-3′)
18s	GGC GTC CCC CAA CTT CTT A	GGG CAT CAC AGA CCT GTT ATT
TNF-α	CTG ACA TCT GGA ATC TGG AG	AAG GAA GTC TGG AAA CAT CT
RUNX2	CAC TGG CGC TGC AAC AAG A	CAT TCC GGA GCT CAG CAG AAT AA
COL1A1	CCA GAA GAA CTG GTA CAT CAG CAA	CGC CAT ACT CGA ACT GGA ATC

Quantitative polymerase chain reaction

Figure 12 Luna® Universal qPCR master mix

Table 4 qPCR reaction components for Luna® Universal qPCR master mix

Component	Volume/reaction	Final concentration
Nuclease-free water	6.5 µL	
10 µM forward primer	0.5 µL	250 nM
10 µM reverse primer	0.5 µL	250 nM
Luna® Universal qPCR Master mix	5 µL	1x
Template DNA	2.0 µL	10 ng
Total volume	10 µL	

The reaction was prepared under manufacturer protocol, which stated as followed

1. Thaw Luna Universal qPCR Master mix and other reaction components on ice (table 4). After the reagents has completely thawed, mix each component by gentle vortexing and centrifuge briefly to collect the master mix.
2. Aliquot master mix into qPCR strips.
3. Add DNA template to qPCR strips.
4. Seal the reaction strips with flat, optically transparent flat caps.
5. Centrifuge strips shortly to take off bubbles and collect liquid (1 minute at 300 rpm).

6. Program the real-time instrument using following conditions (Table 5). Use the SYBR® scan mode setting on the CFX96 real-time instrument (BIO-RAD, USA).

Table 5 Reaction setup for Luna® Universal qPCR master mix

Cycles	Temperature	Duration	Cycle step
1	95°c	60 seconds	Initial denaturation
40	95°c	15 seconds	Denaturation
	55-60°c	30 seconds	Annealing/extension
	65-95°c Increment 0.5°c	5 seconds	Melt curve analysis

Figure 13 CFX96 real-time instrument

Gene expression analysis

The expression of three target genes was normalized with the reference genes, 18s. No template control (RNase free water) and inter run calibrator were included in each run. The Cq data from BIO-RAD CFX Manager™ software, was transferred into Microsoft Excel. Microsoft excel was used to perform the relative quantification which determines the change in gene expression relative to a reference sample. The mean Cq values from each gene were provided by Bio-Rad CFX96™. The ΔCq for each gene

was calculated by subtracting the Cq of the target from the control sample. The amplification efficiency is nearly 100%.

Livak stated that the relative gene expression = $2^{-\Delta\Delta Cq}$

Whereas $\Delta\Delta C_q = \Delta C_{q\ target} - \Delta C_{q\ control}$

$= (C_{q\ target} - C_{q\ ref})_{treatment} - (C_{q\ target} - C_{q\ ref})_{no\ treatment}$

The analyzed data using this equation were presented as the fold change in gene expression normalized to a reference gene and relative to inter-run calibration control.

Immunohistochemistry protocol

Bone samples were processed according to the following protocols.

1. Bone samples were fixed in 4% formaldehyde before embedding in paraffin. Finally, the tissues were sectioned using a microtome.
2. The tissue sections were deparaffinized in two changes of xylene for 5 minutes then rehydrated with serial of alcohol including absolute alcohol, 95% alcohol for 5 minutes. All sections were then placed in distilled water.
3. Antigen retrieval was performed with the use of citrate phosphate buffer and microwave. During the process kept the slices from drying out.
4. Inactivated endogenous peroxidase with 3% hydrogen peroxide at room temperature for 10 minutes. Rinsed the section with Tris-buffered saline (TBS).
5. Following that the slides were dry, used a PAP pen to draw circles around the tissue section. Nonspecific blocking was achieved by using bovine serum albumin to cover the tissue evenly for 30 minutes.
6. A solution of primary antibody (ALP antibody, sc-166261, Santa Cruz, dilute 1:50) was added in the sections and incubated for overnight at 4 degrees Celsius. After that sections were washed with TBS three times for 5 minutes.

7. A solution of secondary antibody was added in sections and incubated for 60 minutes at room temperature. After that sections were washed with TBS three times for 5 minutes.
8. The sections were stained with DAB chromogen for 10 minutes at room temperature. All the sections were rinsed in distilled water for 5 minutes to stop DAB action.
9. The sections were counterstained with hematoxylin for 1 minutes. Then they were rinsed with running tap water for 1 minute.
10. The sections were dehydrated and mounted with coverslips.

Data analysis

The mRNA expression of the different genes for two groups (1. MSFA with Bio-Oss, 2. MSFA with SureOss™) were analyzed by using 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 statically significant. Descriptive statistic was used to calculate mean and standard deviation for each sample group. IHC results of ALP was provided with analytical description of the images.

CHAPTER IV

RESULTS

Patient data

Fourteen patients were participated in this study. There were 2 males, 5 females in Bio-Oss® group patients, which age were between 51-62 years (mean ages 56.29±3.82 years) and had mean healing time of 7.43±0.79 months. There were 5 males 1 females in SureOss® group patients, which age were between 46-65 years (mean ages 58.17±7.22 years) and had mean healing time 7.5±1.76 months (Table 6).

Table 6 Data of patients

No.	Graft type	Sex	Teeth No.	Age	Duration (month)	Lab
1	Bio-Oss®	M	16	60	9	qPCR
2	Bio-Oss®	F	16	51	7	qPCR
3	Bio-Oss®	F	25	53	7	qPCR
4	Bio-Oss®	F	25	62	7	qPCR
5	Bio-Oss®	F	26	57	8	qPCR
6	SureOss®	M	26	61	6	qPCR
7	SureOss®	M	16	46	9	qPCR
8	SureOss®	M	16	65	8	qPCR
9	SureOss®	F	16	61	6	qPCR
10	SureOss®	M	16	53	10	qPCR
11	Bio-Oss®	M	16	56	7	IHC
12	Bio-Oss®	F	15	55	7	IHC
13	SureOss®	M	16	63	6	IHC
14	Normal bone	M	25	46	7	IHC

Nucleic acid quantification results

Quantification of RNA in the extracted samples was achieved by using NanoDrop™2000 spectrophotometer. Minimum concentration of RNA was 31.8 ng/ µl and maximum concentration of RNA was 169.2 ng/ µl. The absorbance ratio 260/280 of all samples were more than 1.89. The absorbance ratio 260/230 of all samples were more than 1.19 (Table 7).

Table 7 Nucleic acid quantification results from NanoDrop™2000

Graft type	Sample ID	Conc.	Unit	A260	A280	260/280	260/230
Bio-Oss®	1	40	ng/µl	1.114	0.59	1.89	1.19
Bio-Oss®	2	48.9	ng/µl	0.953	0.504	1.89	1.33
Bio-Oss®	3	46.2	ng/µl	1.1	0.563	1.95	1.12
Bio-Oss®	4	57.7	ng/µl	1.343	0.668	2.01	1.74
Bio-Oss®	5	47.9	ng/µl	1.208	0.64	1.89	1.21
SureOss®	6	33.9	ng/µl	0.81	0.415	1.95	1.57
SureOss®	7	169.2	ng/µl	4.322	2.123	2.04	2.08
SureOss®	8	46.4	ng/µl	1.131	0.561	2.02	1.91
SureOss®	9	83.9	ng/µl	2.381	1.158	2.06	1.75
SureOss®	10	31.8	ng/µl	0.814	0.424	1.92	1.19

Quantitative real-time PCR data

Data from table 8 show that target gene were expressed in descending order of COL1A1, RUNX2, TNF-alpha respectively in all samples. Graphical presentation of this data is shown in figure 14. This Cq data were used for calculate the relative gene expression using Livak method. Inter-run calibration (IRC) was one sample that was included in every plate to remove inter-run variation. IRC sample is a pooled cDNA of all individual samples.

Table 8 The Cq mean of all samples

Graft type	Sample ID	18S	COL1A1	RUNX2	TNF-alpha
Bio-Oss®	1	10.50	19.51	26.42	29.88
Bio-Oss®	2	9.68	17.28	24.89	29.51
Bio-Oss®	3	9.21	14.32	23.36	27.23
Bio-Oss®	4	9.05	14.57	24.06	27.81
Bio-Oss®	5	11.04	18.84	26.62	31.26
Average Bio-Oss®		9.90	16.90	25.07	29.14
SureOss®	6	10.48	17.00	25.26	27.71
SureOss®	7	11.06	17.46	25.72	29.01
SureOss®	8	10.55	15.08	23.62	28.87
SureOss®	9	9.22	14.25	22.97	28.48
SureOss®	10	10.23	15.39	23.71	29.08
Average SureOss®		10.31	15.84	24.26	28.63
IRC	IRC	10.19	15.92	24.42	28.48

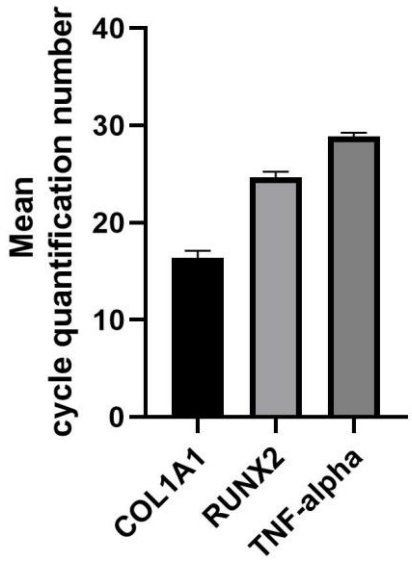


Figure 14 Mean cycle quantification of all target genes used in this study, the lesser the value, the higher the expression of gene.

Quantitative real-time PCR results

TNF-alpha gene expression

The difference in TNF-alpha gene expression of the Bio-Oss® xenograft groups (n=5) and the SureOss® allograft group (n=5) was compared using an independent t-test. A p-value lower than 0.05 was considered significant. Descriptive statistics are shown in Table 9. After statistic calculation, equal variance of data were assumed. The data showed that no significant difference in level of TNF-alpha gene between groups, t(8) = 1.556, p= 0.158. Figure 15 shows a graphical representation of the mean fold change expression and standard deviation. These findings showed that the Bio-Oss® xenograft group and SureOss® allograft group groups had the same TNF-alpha gene expression.

RUNX2 gene expression

The difference in RUNX2 gene expression of the Bio-Oss® xenograft groups (n=5) and the SureOss® allograft group (n=5) was compared using an independent t-test. A p-value lower than 0.05 was considered significant. Descriptive statistics are

shown in Table 9. After statistic calculation, equal variance of data were assumed. The data showed a significant difference in level of RUNX2 gene between groups, $t(8) = 2.429$, $p = 0.041$. Figure 15 shows a graphical representation of the mean fold change expression and standard deviation. These findings implied that the RUNX2 gene expression in the Bio-Oss® xenograft group was lower than the SureOss® allograft group.

COL1A1 gene expression

The difference in COL1A1 gene expression of the Bio-Oss® xenograft groups (n=5) and the SureOss® allograft group (n=5) was compared using an independent t-test. A p-value lower than 0.05 was considered significant. Descriptive statistics are shown in Table 9. After statistic calculation, equal variance of data were assumed. The data showed that no significant difference in level of TNF-alpha gene between groups, $t(8) = 1.530$, $p= 0.164$. Figure 15 shows a graphical representation of the mean fold change expression and standard deviation. These findings showed that the Bio-Oss® xenograft group and SureOss® allograft group groups had the same COL1A1 gene expression.

Table 9 Analytical description of data and results

Group	Number of samples	Mean Fold change of TNF-alpha	SD	Mean Fold change of RUNX2	SD	Mean Fold change of COL1A1	SD
Bio-Oss®	5	0.60	.38	0.57	.29	0.66	.64
SureOss®	5	1.10	.62	1.35	.66	1.32	.72

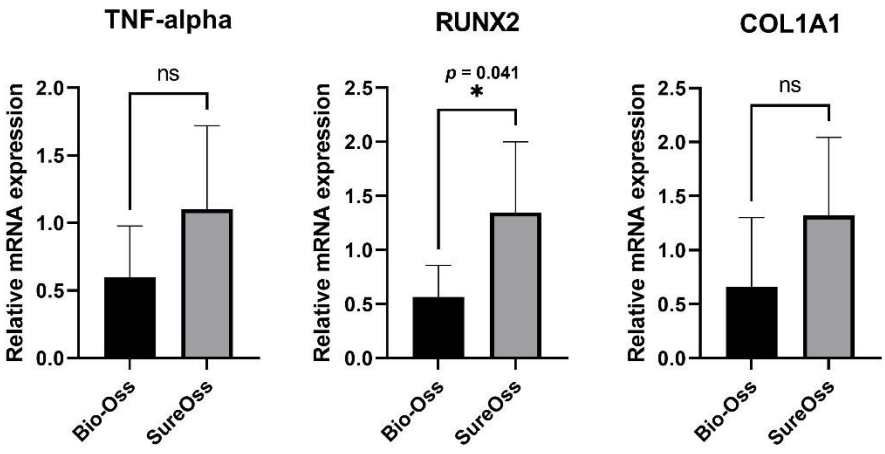


Figure 15 The expression of target genes among groups. Value present by means ± SD. (* = statistically significant)

Figure 15 shows that in all target gene, the higher expression is found in SureOss® allograft group, but the significant difference between groups is only found in RUNX2 expression.

Immunohistochemistry result

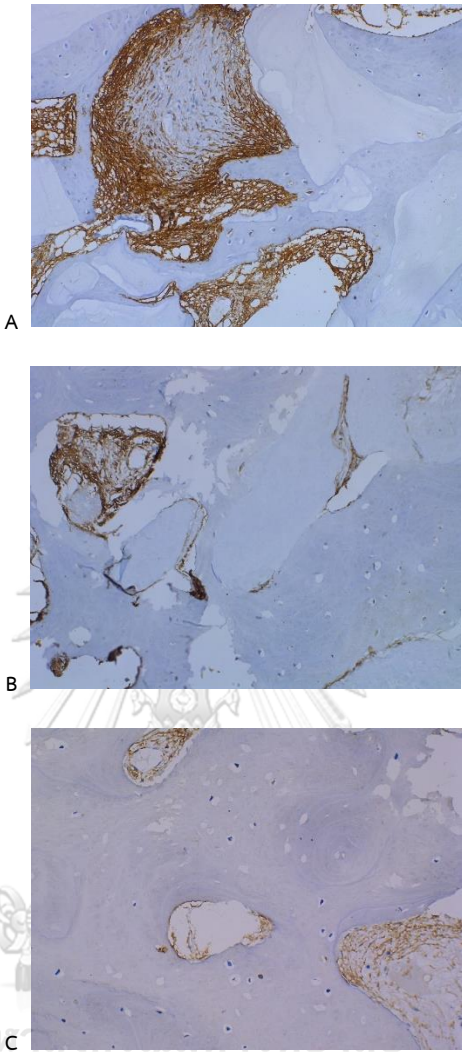


Figure 16 Immunohistochemical staining of ALP (20x)

(A) Bio-Oss® (B) SureOss® (C) Normal bone

ALP Similar labeling was found in all grafted bone groups. No stain was detected in osteocyte and bone lamellae in the area of bone (purple color) and bone graft (pale purple color with no cell in bone lacune). Positive signal of ALP was visualized in connective tissue, which showed intense staining in the area near to the margin of bone graft or bone matrix (Figure 16).

CHAPTER V

DISCUSSION

In the present study, the analysis of gene expression of grafted bone from maxillary sinus area using quantitative real-time PCR and immunohistochemistry was described. Three genes of interest were chosen for real-time qPCR study. TNF-alpha was representative of proinflammatory cytokine and RUNX2 and COL1A1 were osteoblast related genes representative. Real-time PCR was used to measure and compare gene expression of xenograft (Bio-Oss®) and allograft (SureOss®) grafted bone. Immunohistochemical staining of alkaline phosphatase (ALP) was used to visualize marker protein that located in bone tissue samples.

The findings from this study showed that bone grafted with allograft (SureOss®) expressed higher RUNX2 when compared to bone grafted with xenograft (Bio-Oss®). However, TNF-alpha, COL1A1, and ALP expressions were not significantly difference between groups. COL1A1 and ALP were expressed at the matrix mineralization stage and both grafted bone showed similar expression. The high expression of COL1A1 demonstrates that both bone grafts have entered the stage of bone healing, where matrix mineralization activity occurs.

Bone healing that occurs after maxillary sinus floor augmentation is initiated by pro-inflammatory cytokine such as TNF-alpha. TNF-alpha not only functions as pro-inflammatory cytokine but also works independently with RANKL to induce osteoclast differentiation. In this study, TNF-alpha showed the lowest expression compared to other target genes which involved with osteoblast differentiation. This could be interpreted that both grafted bone had passed the stage of osteoclast differentiation. This result is similar to Z Lin et al.'s study, which showed that inflammatory cytokine genes were highly expressed at the early stage and decreased at later stage of bone healing (58). Expression of TNF-alpha between grafted bone were not significantly difference, due to the fact that both grafting materials were considered a foreign body

to the host as demonstrated by in vitro study of Humidat, A et al which indicated that the increasing of TNF-alpha was induced by bone graft, (59).

Bone formation begins when bone resorption decreases. Bone formation is occurred by osteoblast differentiation, that is stimulated mainly by RUNX2 transcription factor. RUNX2 induces the multipotential mesenchymal stem cells to differentiate into preosteoblasts. According to the result, RUNX2 expression in bone grafted with allograft was statistically higher than bone grafted with xenograft. A possible explanation may have to do with the difference of mechanism of bone regeneration between both grafts. Allograft which has additional osteoinductive property facilitates osteoblast differentiation at the early stage. This finding is in agreement with the previous study which found that RUNX2 expression is higher in the graft that has higher osteoinductive property (60).

Type1 collagen is the specific protein in extracellular matrix (Bone matrix protein). COL1A1 is secreted by osteoblast-lineage cells. COL1A1 expression was highly expressed by both immature and mature osteoblasts (61). Based on this study, the difference of COL1A1 expression of both grafted groups was detected, however it was not significant. Furthermore, both bone grafts showed the highest level of this COL1A1 gene compared to other target genes used in this study. The data suggest that both bone grafts used in this study could lead to matrix mineralization process of bone. Although, there are no previous study comparing the expression of COL1A1 between xenograft and allograft. The trends of COL1A1 expression might be in the same direction with other bone matrix protein expression such as alkaline phosphatase.

The activity of bone cells can be visualized by specific immunohistochemical staining methods. In this study, expression of ALP gene was used to detect bone matrix protein in the samples. ALP was found at the surface of bone area where the bone graft was not attached to and not found in bone cells. The result of immunostaining for both grafts showed similar pattern and same level of stain intensity. According to

Milani, S. et al., ALP staining in demineralized bovine bone was located at the surface of mineralized lamellar bone, but not found in bone cells. They suggested that when mature osteoblast differentiated to osteocytes or when bone remodeling cycle was terminated, ALP quantities would be decreasing (62).

The limitations of this study were that first, the two-stage maxillary sinus augmentation had waiting time period for bone healing. This leaded to the difficulty to collect the samples to reach the planned sample sizes. Second, due to the low concentration of RNA in bone tissue, the process of isolation needed to be precise since the RNA products that did not meet expected quality, had to be excluded from the study. Last, spilt mouth design which was the best method to compare between bone grafts could not be achieved. This could lead to variation of results.

Future research with different time to collect bone tissue could be done to see the possibility of early dental implant operation. Clinical study to compare the quality and quantity of different types of bone grafts used in implant site preparation is also an interesting project.

CHAPTER VI

CONCLUSION

In the present study, the data suggested that gene expression levels of pro-inflammatory gene (TNF-alpha) and matrix mineralization gene (COL1A1) were not significantly different between bone collected from patients who undergone maxillary sinus elevation with Bio-Oss® and SureOss®. However, the expression of RUNX2, master transcription factor for osteoblast lineage, was higher in SureOss®. Nevertheless, result from immunohistochemical study showed similar characteristic between both groups.

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APPENDIX

The Cq , Cq mean, Cq std., Melt temperature

of no template control and Inter-run calibrator control

Target	Sample	Biological Set Name	Cq	Cq Mean	Cq Std. Dev	Melt Temperature
18s	NTC		32.96	32.96	0.000	75.00
18s	NTC		32.80	32.80	0.000	81.50
col1	NTC		32.08	32.08	0.000	82.00
col1	NTC		30.82	30.82	0.000	82.00
runx2	NTC			0.00	0.000	None
runx2	NTC			0.00	0.000	None
tnf-alpha	NTC			0.00	0.000	None
tnf-alpha	NTC			0.00	0.000	None
18s	Pos Ctrl		10.08	10.08	0.000	82.00
18s	Pos Ctrl		10.79	10.79	0.000	82.00
col1	Pos Ctrl		16.09	16.09	0.000	82.00
col1	Pos Ctrl		15.58	15.58	0.000	82.00
runx2	Pos Ctrl		24.27	24.27	0.000	84.50
runx2	Pos Ctrl		24.40	24.40	0.000	85.00
tnf-alpha	Pos Ctrl		28.32	28.32	0.000	82.50
tnf-alpha	Pos Ctrl		28.23	28.23	0.000	82.50

The Cq , Cq mean, Cq std., Melt temperature of Bio-Oss® group

Target	Sample	Biological Set Name	Cq	Cq Mean	Cq Std. Dev	Melt Temperature
18s	1	Bio-Oss	10.79	10.77	0.029	82.00
18s	1	Bio-Oss	10.75	10.77	0.029	82.00
col1	1	Bio-Oss	19.34	19.42	0.126	82.00
col1	1	Bio-Oss	19.51	19.42	0.126	82.00
runx2	1	Bio-Oss	26.20	26.34	0.185	84.50
runx2	1	Bio-Oss	26.47	26.34	0.185	84.50
tnf-alpha	1	Bio-Oss	29.54	29.65	0.153	82.50
tnf-alpha	1	Bio-Oss	29.76	29.65	0.153	82.50
18s	2	Bio-Oss	10.08	9.94	0.189	82.00
18s	2	Bio-Oss	9.81	9.94	0.189	82.00
col1	2	Bio-Oss	17.30	17.19	0.159	82.00
col1	2	Bio-Oss	17.08	17.19	0.159	82.00
runx2	2	Bio-Oss	24.62	24.77	0.220	84.50
runx2	2	Bio-Oss	24.93	24.77	0.220	84.50
tnf-alpha	2	Bio-Oss	29.53	29.30	0.326	82.50
tnf-alpha	2	Bio-Oss	29.07	29.30	0.326	82.50
18s	3	Bio-Oss	9.31	9.46	0.208	82.00
18s	3	Bio-Oss	9.60	9.46	0.208	82.00
col1	3	Bio-Oss	14.41	14.23	0.255	82.00
col1	3	Bio-Oss	14.05	14.23	0.255	82.00
runx2	3	Bio-Oss	23.38	23.27	0.166	84.50
runx2	3	Bio-Oss	23.15	23.27	0.166	84.50
tnf-alpha	3	Bio-Oss	27.09	27.06	0.031	82.50
tnf-alpha	3	Bio-Oss	27.04	27.06	0.031	82.50
18s	4	Bio-Oss	9.07	9.33	0.367	82.00
18s	4	Bio-Oss	9.59	9.33	0.367	82.00
col1	4	Bio-Oss	14.36	14.48	0.166	82.00
col1	4	Bio-Oss	14.60	14.48	0.166	82.00
runx2	4	Bio-Oss	24.14	23.95	0.272	84.50
runx2	4	Bio-Oss	23.76	23.95	0.272	84.50
tnf-alpha	4	Bio-Oss	27.73	27.57	0.229	82.50
tnf-alpha	4	Bio-Oss	27.41	27.57	0.229	82.50
18s	5	Bio-Oss	11.35	11.25	0.143	82.00
18s	5	Bio-Oss	11.15	11.25	0.143	82.00
col1	5	Bio-Oss	18.66	18.73	0.097	82.00
col1	5	Bio-Oss	18.80	18.73	0.097	82.00
runx2	5	Bio-Oss	26.58	26.51	0.098	84.50
runx2	5	Bio-Oss	26.44	26.51	0.098	84.50
tnf-alpha	5	Bio-Oss	31.08	31.09	0.019	82.50
tnf-alpha	5	Bio-Oss	31.11	31.09	0.019	82.50

The Cq , Cq mean, Cq std., Melt temperature of SureOss™ group

Target	Sample	Biological Set Name	Cq	Cq Mean	Cq Std. Dev	Melt Temperature
18s	6	SureOss	10.73	10.75	0.038	82.00
18s	6	SureOss	10.78	10.75	0.038	82.00
col1	6	SureOss	16.81	16.90	0.131	82.00
col1	6	SureOss	16.99	16.90	0.131	82.00
runx2	6	SureOss	25.10	25.16	0.093	84.50
runx2	6	SureOss	25.23	25.16	0.093	84.50
tnf-alpha	6	SureOss	27.50	27.51	0.018	82.50
tnf-alpha	6	SureOss	27.52	27.51	0.018	82.50
18s	7	SureOss	11.06	11.32	0.362	82.00
18s	7	SureOss	11.57	11.32	0.362	82.00
col1	7	SureOss	17.53	17.38	0.212	82.00
col1	7	SureOss	17.23	17.38	0.212	82.00
runx2	7	SureOss	25.78	25.61	0.235	84.50
runx2	7	SureOss	25.45	25.61	0.235	84.50
tnf-alpha	7	SureOss	28.82	28.79	0.043	82.50
tnf-alpha	7	SureOss	28.76	28.79	0.043	82.50
18s	8	SureOss	10.97	10.84	0.188	82.00
18s	8	SureOss	10.71	10.84	0.188	82.00
col1	8	SureOss	15.14	14.99	0.217	82.00
col1	8	SureOss	14.83	14.99	0.217	82.00
runx2	8	SureOss	23.31	23.50	0.270	85.00
runx2	8	SureOss	23.70	23.50	0.270	85.00
tnf-alpha	8	SureOss	28.70	28.65	0.066	82.50
tnf-alpha	8	SureOss	28.61	28.65	0.066	82.50
18s	9	SureOss	9.18	9.45	0.384	82.00
18s	9	SureOss	9.73	9.45	0.384	82.00
col1	9	SureOss	14.16	14.16	0.004	82.00
col1	9	SureOss	14.17	14.16	0.004	82.00
runx2	9	SureOss	22.97	22.87	0.138	85.00
runx2	9	SureOss	22.78	22.87	0.138	85.00
tnf-alpha	9	SureOss	28.33	28.31	0.018	82.50
tnf-alpha	9	SureOss	28.30	28.31	0.018	82.50
18s	10	SureOss	10.50	10.46	0.055	82.00
18s	10	SureOss	10.42	10.46	0.055	82.00
col1	10	SureOss	15.37	15.30	0.090	82.00
col1	10	SureOss	15.24	15.30	0.090	82.00
runx2	10	SureOss	23.39	23.57	0.249	85.00
runx2	10	SureOss	23.74	23.57	0.249	85.00
tnf-alpha	10	SureOss	28.83	28.90	0.098	82.50
tnf-alpha	10	SureOss	28.97	28.90	0.098	82.50

Descriptive statistics of the gene expression levels of TNF-alpha, RUNX2, COL1A1

		N	Mean	Std. Deviation	Std. Error Mean
Relative expression of TNF-alpha	Bio-Oss®	5	.5980	.37897	.16948
	SureOss®	5	1.1020	.61743	.27612
Relative expression of RUNX2	Bio-Oss®	5	.5680	.28900	.12924
	SureOss®	5	1.3460	.65535	.29308
Relative expression of COL1A1	Bio-Oss®	5	.6600	.64354	.28780
	SureOss®	5	1.3220	.72220	.32298

Independent T-Test of the gene expression levels of TNF-alpha, RUNX2, COL1A1

Independent Samples Test

		Equality of Variances		t-test for Equality of Means						
									Interval of the	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Relative expression of TNF-alpha	Equal variances assumed	.839	.387	-1.556	8	.158	-.50400	.32399	-1.25112	.24312
	Equal variances not assumed			-1.556	6.639	.166	-.50400	.32399	-1.27863	.27063
Relative expression of RUNX2	Equal variances assumed	3.444	.101	-2.429	8	.041	-.77800	.32031	-1.51664	-.03936
	Equal variances not assumed			-2.429	5.499	.055	-.77800	.32031	-1.57941	.02341
Relative expression of COL1A1	Equal variances assumed	.022	.886	-1.530	8	.164	-.66200	.43260	-1.65958	.33558
	Equal variances not assumed			-1.530	7.896	.165	-.66200	.43260	-1.66187	.33787

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