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Gene Expression Analysis of Osteopontin and Osteocalcin in Maxillary Sinus Floor Augmentation Using Xenograft
การวิเคราะห์การแสดงออกของยีนออสติโอพอนติน และออสติโอแคลซิน ของกระดูกปลูกถ่ายวิวิธพันธุ์ที่ใช้ในการเสริมโพรงอากาศแม็กซิลลา

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ABSTRACT

Bone loss in maxillary sinus areas could complicate the implant surgery procedures. Bone substitutes are used for filling and correcting bony defect before placing dental implants. The aim of this study is to determine the differences in gene expression of Osteopontin (OPN), Osteocalcin (OCN) between bone grafting (Bio-oss®) and spontaneous healing group (control). Bone samples were collected from patients using trephine burs at Department of Oral Maxillofacial Surgery, Faculty of Dentistry, Chulalongkorn University (n=6). Real-time PCR method was used for studying gene expression. The result showed no statistical significant difference in gene expression for all groups ($p<0.05$). These findings suggest that Bio-oss® healing site has no difference in level of gene expression of OPN and OCN when compared to non-grafted healing site.

บทคัดย่อ

ในการปักรากเทียมสำหรับฟันบนบริเวณโพรงอากาศแม็กซิลล่า ในเคสที่มีกระดูกไม่เพียงพอ ทำให้การผ่าตัดมีความซับซ้อนมากขึ้น กระดูกทดแทนจึงถูกนำมาใช้เพื่อใช้เตรียมกระดูกก่อนปักรากเทียม วัตถุประสงค์ของงานวิจัยนี้ คือ เพื่อเปรียบเทียบการแสดงออกของยีน ได้แก่ ออสติโอพอนตินและ ออสติโอแคลซินระหว่างกระดูกขากรรไกรบนที่ได้รับการเสริมด้วยกระดูกวิวิธพันธุ์เทียบกับกระดูกขากรรไกรบนที่ไม่ได้เสริมกระดูกทดแทน กระดูกจากคนไข้ถูกเก็บด้วยหัวกรอเทรฟิไฟร์ที่ที่ภาควิชาศัลยศาสตร์ คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย (จำนวนตัวอย่าง 6 ชิ้น) เพื่อมาทำการวิเคราะห์การแสดงออกของยีนเป้าหมายโดยวิธีการเรียลไทม์ อาร์ที-พีซีอาร์ จากผลการทดลองพบว่า การแสดงออกของยีนเป้าหมายไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ กำหนดนัยสำคัญที่ระดับ 0.05 จึงสรุปได้ว่ากระดูกทดแทนมีการแสดงออกของยีนออสติโอพอนตินและ ออสติโอแคลซินไม่แตกต่างกับกระดูกจากกลุ่มที่ไม่มีการใส่กระดูกทดแทน

Keywords: Sinus lift, Gene expression, Xenograft

คำสำคัญ: การผ่าตัดยกโพรงอากาศแม็กซิลล่า การแสดงออกของยีน กระดูกวิวิธพันธุ์

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Introduction

The quantity and quality of the bone in the recipient site are essential to achieve the stability of dental implant (Raikar et al., 2017). Pneumatization of the maxillary sinus coupled with loss of alveolar bone often cause the need for bone preparation. The maxillary sinus floor augmentation is performed in order to enhance bone volume before the placement of dental implant. In brief, surgeon will create the access through the lateral wall of maxilla, lift the sinus membrane and place the bone graft (Danesh-Sani et al., 2016).

The bone substitute materials form solid scaffolds and prohibit the migration of unwanted cells, and only permit the migration of bone cells into the site to regenerate the hard tissue in deficient areas (Yamada, Egusa, 2018). There are various types of bone substitute materials which can be classified in two main categories; bone substitutes derived from biological products and synthetic products (Fernandez de Grado et al., 2018). Bovine bone is a biological products that is commonly used in oral surgery.

Evidence based on histomorphometry studies after sinus augmentation could be summarized that bovine bone graft was not interfere with the new bone formation process. The histological examination showed that graft materials were in direct contact with newly formed bone. No signs of acute inflammation were observed (Danesh-Sani et al., 2017). However, histomorphometry is unable to explain the function of gene involved with the bone healing process.

Objective of the study

To determine differences in gene expression levels of bone-formation genes (Osteopontin (OPN), Osteocalcin (OCN)) between bone grafting groups and spontaneous healing group (control).

Materials and Methods

1. Sample collection

Six patients (1 males, 5 females, mean age 49.33±11.02) with partially edentulism at maxillary sinus area and residual bone height less than 4 mm participated to this study and were enrolled between October 2019 to October 2020. Inclusion criteria were maxillary partial edentulous arch involving the premolar/molar areas in control group. In xenograft group, additional criteria were the presence of less than 4 mm of crestal bone between sinus floor and the alveolar ridge Exclusion criteria were patient who have systemic disease, uncontrolled metabolic diseases (diabetes mellitus, bone pathologies), psychiatric problems, smokers, patients treated with radiotherapy to the head/neck, patients with uncontrolled periodontal disease.

The experimental protocol was approved by the ethic committee of Chulalongkorn University (Study code : HREC-DCU2020-012). Written informed consent to participate was obtained from all participants. The bone samples examined in this study were obtained from healthy patient who

undergone maxillary sinus augmentation procedure. In all cases, samples were taken at the time of implant placement, 7 to 9 months after the grafting procedure. The bone samples were retrieved by trephine bur (internal diameter 2mm) and immediately submerged in RNA-stabilizing reagent and frozen at -80 degree Celsius.

2. RNA isolation

Total RNA was isolated from the bone tissue using lysis reagent : RiboEx™ (GeneAll®, Korea) according to the manufacturer's instructions. In brief, after removing the RNA-stabilizing agent, lysis reagent was added to each power bead tube. The homogenization step was done by using PowerLyzer 24 Homogenizer machine. The lysate from homogenization step was mixed vigorously with chloroform and centrifuged at 12000g for 15min at 4°C. The aqueous phase of the samples was collected and mixed with 70% ethanol. After incubation at room temperature, the extract was centrifuged using RNeasy mini kit protocol. Finally, the RNA pellet was dissolved in RNase-free water. Concentration and purity of RNA were determined using the NanoDrop (ND-2000; ThermoScientific,). RNA samples with an A260/A280 ratio <1.8 were excluded.

3. Quantitative polymerase chain reaction

The single-stranded cDNA required for the polymerase chain reaction analysis was synthesized using 400 ng of extracted total RNA as a template for reverse transcription. Reverse transcription was carried out using precision nanoscript2 reverse transcription kit (Primer design, London, UK). Primer design was performed with Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Osteocalcin and Osteopontin were selected to represent biological process associated with bone formation. Quantitative polymerase chain reaction (qPCR) was performed using qPCRBIO SyGreen Mix (PCR Biosystems, London, UK) in 10 μl reactions on the CFX96 platform (Bio-rad Laboratories, Inc., California, USA). Quantities of target genes were normalized using the reference gene 18S. The normalized relative quantities were calculated using the delta Cq method and assuming nearly 100% PCR efficiency. The performance and reporting of the gene expression analysis adhered to the MIQE guidelines. Cq values were subsequently used to determine deltaCq values, and differences in Cq values were used to quantify the relative amount of PCR product, expressed as the relative change by applying the equation.

Table 1 Primer Sequences Used in This Study

Gene		Primer sequences (5'to 3')
18s	forward	GGC GTC CCC CAA CTT CTT A
	reverse	GGG CAT CAC AGA CCT GTT ATT
OCN	forward	CTT TGT GTC CAA GCA GGA GG
	reverse	CTG AAA GCC GAT GTG GTC AG
OPN	forward	AGG AGG AGG CAG AGC ACA
	reverse	CTG GTA TGG CAC AGG TGA TG

22nd NGRC การประชุมวิชาการเสนอผลงานวิจัยระดับบัณฑิตศึกษาแห่งชาติ ครั้งที่ 22
March 25, 2021 วันที่ 25 มีนาคม 2564 ณ มหาวิทยาลัยขอนแก่น

MMP12-4

Statistical analysis

The statistical analysis was performed using IBM SPSS statistics (Version22) predictive analysis software. For statistical analysis, Levene's test was used first and revealed normally distributed data. Thus, the parametric data are presented as means. Mean values and standard deviations were calculated for each gene in two experimental groups. The group were compared using a t test for unpaired data (xenograft vs control). Significance value was set at $p < 0.05$.

Results

OCN gene expression

An independent t-test was conducted to explore differences of OCN gene expression between the bone grafting groups (n=3) and the spontaneous healing groups (n=3). An alpha level of .05 was utilized. Descriptive statistics are in Table 2. All group were normally distributed. Variances were homogenous, $F(4) = 1.03$, $p = .367$. Hence, equal variances were assumed. There was not a significant difference in gene expression levels were observed, $t(4) = 3.5$, $p = .744$, ns. A graphical representation of means expression fold change and standard error is displayed in Figure 1. These results suggest that the bone grafting groups has same expression of OCN gene compared to the spontaneous healing group.

OPN gene expression

An independent t-test was conducted to explore differences of OPN gene expression between the bone grafting groups (n=3) and the spontaneous healing groups (n=3). An alpha level of .05 was utilized. Descriptive statistics are in Table 2. All group were normally distributed. Variances were homogenous, $F(4) = 6.116$, $p = .069$. Hence, equal variances were assumed. There was not a significant difference in gene expression levels were observed, $t(4) = 1.2$, $p = .297$, ns. A graphical representation of means expression fold change and standard error is displayed in Figure 1. These results suggest that the bone grafting groups has same expression of OPN gene compared to the spontaneous healing group.
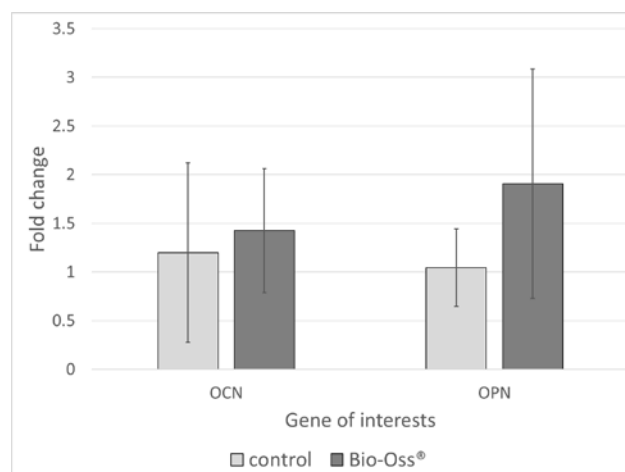

Figure 1 The expression of bone marker gene among groups. Value present by means ± SE.

Table 2 Analytical description of patient data and results

Group	Number of samples	Mean Fold change of OCN	SD	Mean Fold change of OPN	SD
Control	3	1.20	.92	1.05	.40
Bio-Oss®	3	1.43	.64	1.91	1.18

Discussion and conclusions

The relative gene expression was used to compare the expression of related genes from bone samples at the maxillary sinus floor augmentation and from normal tooth extraction socket at the maxillary sinus area. The expression of genes involved in osteoblast differentiation and bone matrix formation was examined because osteoblasts are unique bone-forming cells and are crucial regulators for differentiation of osteoclasts.

The main finding was that no significant changes in the messenger RNA (mRNA) levels of bone formation genes were found between both groups which suggested that there were no differences in the bone-forming capacity. In other word, when compared bone grafted with xenograft and spontaneous bone healing, bone grafted with xenograft did not inhibit any bone formation process, however, it also did not speed the bone formation process.

Osteopontin (OPN) is a glycoprotein that is first identified as a major non-collagenous sialoprotein in bone produced by osteoblast. OPN can bind to various extracellular molecules, including Collagen type I and osteocalcin. Moreover, OPN plays a role in anchoring osteoclasts to the mineral matrix of bone, serves to initiate the process by which osteoclasts develop their ruffled borders to begin bone resorption (Gorski, 2011; Standal et al., 2004).

Osteocalcin (OCN) is the most abundant non-collagenous protein in bone secreted solely by osteoblasts. In its carboxylated form it binds calcium directly and thus concentrates in bone. Osteocalcin are used as a marker of the mature osteoblast, also known as a marker of matrix mineralization (Boskey, Robey, 2013; Tsao et al., 2017). In this study, the gene expression analysis indicated that grafted samples displayed expression of osteopontin more than osteocalcin. While in the control group, the expression of osteocalcin is more than osteopontin. The meaning of this could be interpreted as in grafted group the bone remodeling is occurring more than mineralization process. Meanwhile, in the control group, the mineralization is take place more than the bone remodeling.

Prior histomorphometry studies that assessed new bone volume and non-mineralized tissue of bone graft found that almost all the biomaterials were surrounded by bone after grafting (Danesh-Sani et al., 2017). Some biomaterials were more resorbable than others. Bio-Oss® is a xenograft that showed slow remodeling process. However, in case of maxillary sinus augmentation, this effect is considered helpful for maintaining bone volume in order to restore defects (Yildirim et al., 2001).

22nd NGRC การประชุมวิชาการเสนอผลงานวิจัยระดับบัณฑิตศึกษาแห่งชาติ ครั้งที่ 22
March 25, 2021 วันที่ 25 มีนาคม 2564 ณ มหาวิทยาลัยขอนแก่น

MMP12-6

The strength of gene expression study is that it use a specific method to study function of bone cells in molecular level. By observing relative gene expression between bone samples, this would lead to a more understanding in the biomolecular process of bone healing. Limitation of this study is the time to collect the sample resulted in the small sample size and the study design has inter-subject variability due to unavailable of the subject who has edentulous space at both side. Further investigation could compare expression of bone marker genes in the differences types of grafts (xenograft vs graft from other origins) or different brand of xenograft and expression of genes that involved in inflammatory process and osteoclast function.

In this study, the performance of commonly used bone substitutes is evaluated by measuring gene expression level of interested genes. Gene expression analysis in vivo showed that Bio-Oss and control have no significant differences in the expression of genes for osteoblast differentiation. The present study was conducted to explored the feasibility of recruiting inadequate bone support patients that need maxillary sinus lift in faculty of dentistry, Chulalongkorn university (pilot study) and to prepared for a future study that aims to evaluate the effect of using bone graft as an intervention for bone healing in a larger sample sizes.

Acknowledgements

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