Gene Expression of Grafted Bone in Calvarial Defect in Mice

การศึกษาลักษณะการแสดงออกของยีนของกระดูกปลูกถ่ายในกระโหลกศีรษะของหนู

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ABSTRACT

Bone is a dynamic tissue that coordinated balance between bone formation by osteoblasts and bone resorption by osteoclasts, under regulation of many systemic and local factors. The purpose of this study was to evaluate genes of interest indicate osteoblast and osteoclast activity in bone remodeling including Bcl2A1 and S100A4 after bone grafting with demineralized freeze-dried human bone (DFDBA) and deproteinized bovine bone mineral (DBBM), and compare them to natural bone healing using quantitative real-time polymerase chain reaction (qPCR). At 3 month , the DBBM group was shown significantly higher of S100A4 mRNA levels when compared with both control and DFDBA groups (P<0.05). Both 1 and 3 months periods, The control group was shown significantly higher of Bcl2A1 transcription than DBBM group (P<0.05). The results of this study suggest that DFDBA and DBBM could be used for the repair of bone defects.

บทคัดย่อ

กระดูกเป็นเนื้อเยื่อที่ประสานความสมดุลระหว่างการสร้างกระดูกโดยเซลล์สร้างกระดูก และการสลายกระดูกโดยเซลล์สลายกระดูกภายใต้ การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อประเมินการแสดงออกของยีนที่น่าสนใจบ่งชี้ถึงการทำงานของเซลล์สร้างกระดูกและเซลล์สลายกระดูกในการสร้างกระดูกใหม่ หลังการปลูกถ่ายกระดูกด้วยวัสดุปลูกถ่ายเอกพันธุ์ชนิดผ่านการละลายของแร่ธาตุภายใต้สภาวะแช่แข็งหรือดีเอฟดีบีเอ และ วัสดุปลูกถ่ายวิวิธพันธุ์ที่ได้จากวัวที่ผ่านการสลายโปรตีนหรือดีบีบีเอ็มเปรียบเทียบกับการรักษากระดูกธรรมชาติ โดยใช้ปฏิกิริยาลูกโซ่โพลิเมอร์แบบเรียลไทม์เชิงปริมาณ ซึ่งในเดือนที่ 3 กลุ่มดีบีบีเอ็ม แสดงระดับของเอ็มอาร์เอ็นเอของยีนเอสวันฮัลเดสเอโฟว์ สูงกว่ากลุ่มควบคุมและกลุ่มดีเอฟดีบีเออย่างมีนัยสำคัญทางสถิติ (P<0.05) และพบว่าแสดงระดับของเอ็มอาร์เอ็นเอของยีนบีซีแอลทูเอวันทั้งในเดือนที่ 1 และ 3 ของกลุ่มควบคุมมีการแสดงออกสูงกว่ากลุ่มดี-บีบีเอ็มอย่างมีนัยสำคัญทางสถิติ (P<0.05) ผลการศึกษาครั้งนี้ชี้ให้เห็นว่าดีเอฟดีบีเอและดีบีบีเอ็มสามารถนำมาใช้ในการซ่อมแซมข้อบกพร่องของกระดูกได้

Keywords: Xenograft, Allograft, Real-time PCR

คำสำคัญ: กระดูกปลูกถ่ายเอกพันธุ์ กระดูกปลูกถ่ายวิวิธพันธุ์ เทคนิคเรียลทามพีซีอาร์

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Introduction

Nowadays, a combination of conventional prosthesis and dental implant is considered as one of the first choices of prosthodontics treatment in edentulous patients. However, main problem in patients with long-termed tooth loss is lack of bone quality and quantity. Physiological bone remodeling is a highly coordinated process responsible for bone resorption and formation to maintain mineral homeostasis. Dimensional changes of residual ridge also occurred especially during first 6 months after tooth extraction (Antje, 1972; Araujo, Lindhe, 2005). Without ridge preservation, the extraction site may lose up to 50% of their width in first year (Robert, Brian, 2012). As a result, many grafting materials including autograft, allograft, xenograft and alloplast are used for bone preservation and augmentation. Autograft provides limited quantity, needs secondary surgery site preparation, prolongs operation time and may cause higher risk of donor site morbidity (Boyne et al., 1997; Burchardt, 1983). Due to limitation of autogenous bone graft. Allograft and xenograft have been used clinically as alternative grafting materials (Eppley et al., 2005; Norton et al., 2003).

The data from our previous study using two types of bone grafts, demineralized freeze-dried human bone (DFDBA; OraGraft™) and deproteinized bovine bone mineral (DBBM; Bio-oss®) in mouse calvarial defects shown that there were significant activation of osteoblast genes and slow resorption of grafting materials comparing with natural bone grafting in micro CT (Kangwannarongkul et al., 2018).

Therefore, the purpose of this study is to investigate different bone grafts which are DFDBA and DBBM, comparing with natural bone healing in mouse calvarial defects by using the gene expression to explain the bone remodeling process.

Objective of the study

To study the expression of gene that associate in osteoblast cell ; S100A4 and osteoclast cell ; Bcl2A1 after bone grafting using DFDBA and DBBM comparing natural bone healing.

Materials and methods

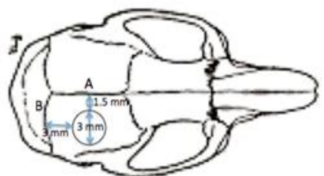
This research is the continuation of the previous study : Gene expression and micro-computed tomography analysis of grafted bone using deproteinized bovine bone and freeze-dried human bone by Thanyaporn Kangwannarongkul, DDS., MSc. Graduated student, Department of Prosthodontics, Faculty of dentistry, Chulalongkorn University (Kangwannarongkul et al., 2018).

Animal used for experiments

A total of mice from previous study were used.These mice were obtained from National Laboratory Animal Center of Salaya campus, Mahidol University (Bangkok,Thailand). The experiment was approved by Animal Care and Use Committee of Chulalongkorn University (ethical approval number 1432001). The mice were housed in light and temperature controlled facilities and given food and water ad libitum. Mice were divided into 3 groups according

to the type of graft;Group 1: bare defect as control, Group 2: deproteinized bovine bone [Bio-oss®; Geistlich Pharma AG, Wolhusen, Switzerland], Group 3: demineralized freeze-dried human bone [OraGraft™; LifeNet, Virginia, USA]

Harvesting and transplantation of bone graft (all the surgical procedures were done from previous study) Under general anesthesia, Nembutal® (Pentobarbital), was prepared with a phosphate buffered saline into a dilution of 1:10 and the concentration of 4 mg/kg [or 8μL of dilution/wt (g)] were used. The anesthesia was injected into peritoneum layer, and the surgical field was prepared with alcohol and povidone iodine after the mouse's hair was removed with blade. Afterwards, subcutaneous of skull was injected by 0.2 ml of 1% lidocaine with 1:100,000 epinephrine. A 1.5 mm midline incision was made on the skull, and the soft tissue and periosteum were elevated. Under constant saline irrigation, the 3-mm diameter circular defects, and 0.2 mm in depth , were created on both left and right side of parietal bone by trephine burs . The cavity was created on each side of the the parietal bone 1.5 mm away from sagittal and 3 mm from lambdoid suture (Figure 1). Then, the defect was randomly filled with the bare defect control , Bio-oss® (Geistlich Pharma AG, Wolhusen, Switzerland) and OraGraft™ (LifeNet,Virginia, USA) as shown in the table (Table 1). The bone graft was then inserted into skull cavity and stitched up with nylon 3-0.



A = sagittal suture B = lambdoid suture

Figure 1 Location of where the defect was created.

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

Table 1 The table show transpantation bone grafting of mice. C = Control B = Bio-oss® D = DFDBA

Mice No.	Left defect		Right defect
1,10	C1, C7		B1, B7
2,11	C2, C8		D1, D7

Table 1 The table show transpantation bone grafting of mice. C = Control B = Bio-oss® D = DFDBA (Cont.)

Mice No.	Left defect		Right defect
3,12	B2, B8		D2, D8
4,13	B3, B9		D3, D9
5,14	C3, C9		C4, C10
6,15	C5, C11		B4, B10
7,16	C6, C12		D4, D10
8,17	B5, B11		D5, D11
9,18	B6, B12		D6, D12

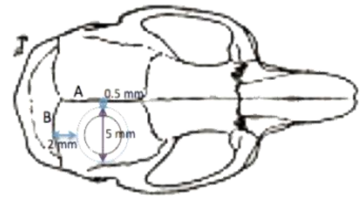


Figure 2 Location of colledted sample bone

RNA extraction

RNA was prepared from previous study.[8] Briefly, Bone samples adjacent to the defect will be collected then lyzed and grounded. After that purified RNA will be obtained by RNeasy mini spin column then the amount of extracted RNA will be determined by a Nanodrop machine (NanoDrop 2000, Thermo Fisher Scientific, Inc., USA). The Nanodrop machine is used to detect the amount of nucleic acid and protein based on surface tension and light absorption principle.

Real-time polymerase chain reaction

Complementary DNA(cDNA) will be reverted from 50 ng of RNA by Sensiscript® reverse transcription (Qiagen, Qiagen, Inc., USA) system using methods provided by manufacturer. RNA sample will be mixed with 10µM Oligo-dT primer, 5mM dNTP mix, 10x Buffer RT, 10 unit/µl RNase inhibitor and Sensiscript reverse

transcriptase then incubated in 37° for 60 minutes by thermocycler (Bio-Rad CFX96TM real time -PCR system; Bio-Rad laboratories, Inc., USA).

The cDNA will be analyzed for the gene expression including Bcl2A1 and S100a4 by SYBR® FAST Universal kit (Kapa Biosystem, Inc., USA) with Bio-Rad CFX96TM real time -PCR system (Bio-Rad laboratories, Inc., USA). Rn18s will be used as an internal control. The primers used for RT-qPCR are shown in the table below (Table 2).

Table 2 Primer design

Gene		Primer sequences
Bcl2A1	Forward	5`-CTTCAGTATGTGCTACAGGTACCCG-3`
	Reverse	5`-TGGAAACTTGTTTGTAAGCACGTACAT-3`
S100a4	Forward	5`-CCACAAGTACTCGGGCAAAG-3`
	Reverse	5`-GTCCCTGTTGCTGTCCAAGT-3`
Rn18s	Forward	5`-AGGGGAGAGCGGGTAAGAGA-3`
	Reverse	5`-GGACAGGACTAGGCGGAACA-3`

Statistical analysis

The differences of the relative gene expression among groups and within each group were evaluated with independent t-test with significant level of 5%.

Results

Both 1 and 3 months periods, The control group was shown significantly higher of Bcl2A1 transcription than DBBM group, but not significantly mRNA levels with DFDBA group. At 1 month, the gene expression of S100A4 in control group and DBBM group had significantly higher than DFDBA group. At 3 month , the DBBM group was shown significantly higher of S100A4 mRNA levels when compared with both control and DFDBA groups. (figure3)

The gene expression of both genes between 1 and 3 months were shown significant decrease of mRNA levels of Bcl2A1 gene in all groups. hile DFDBA group was shown significant increase of mRNA levels of S100A4 gene. (figure4)

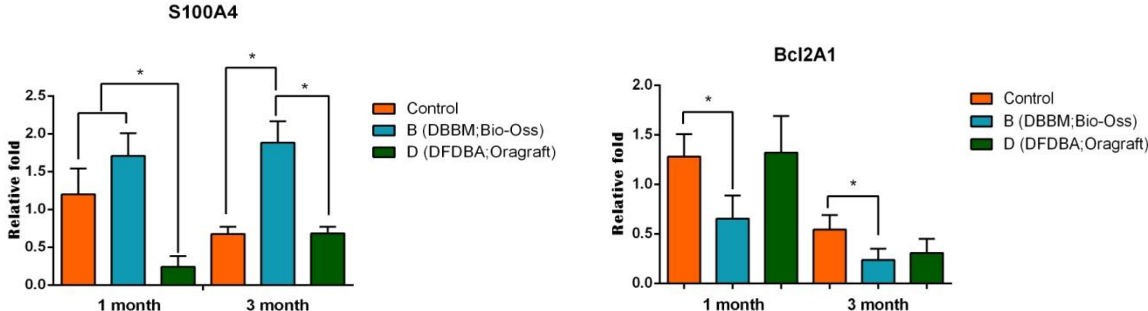


Figure 3 The expression of Bcl2A1 and S100A4 gene among groups. Value present by means ± SD. (* Indicated significant difference in relative gene expression at $p < 0.05$).

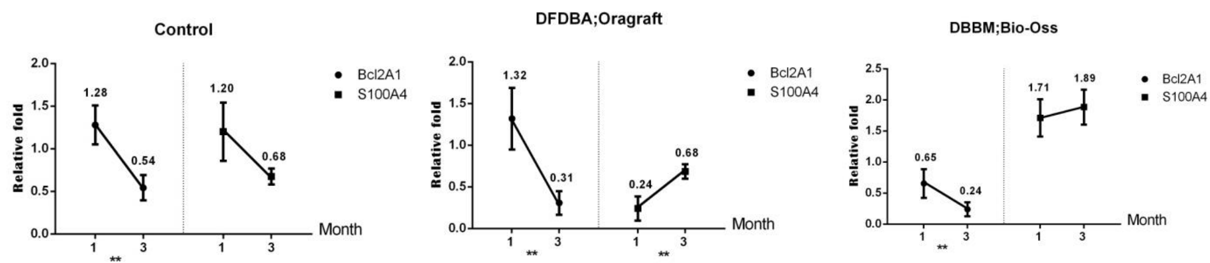


Figure 4 The gene expression of Bcl2A1 and S100A4 gene between 1 and 3 months. Value present by means ±SD. (** Indicated significant difference in relative gene expression at $p < 0.05$).

Discussion

OraGraft™ (LifeNet, Virginia, USA) is DFDBA, a bone graft material form individual of the same species used for ridge maintenance and chronic facial reconstruction. It is advantageous for getting rid of immune response and disease transmission(Prasanna et al., 2013). The result of demineralization treatment that uses hydrochloric acid to increase osteoinductive potential passes through the collagen matrix (Tong et al., 1998). It exhibited an increase in bone by approximately 87.1% within 6 weeks. In a rabbit brain, it created a defect from the dentist hand and completed the bone fill within 12 weeks (Clokie et al., 2002). Also, in the present study shown the mRNA levels of Bcl2A1 gene between 1 and 3 months were shown significant decreased while mRNA levels of S100A gene increased in DFDBA group. Bcl2A1 is associated with osteoclast differentiation (Haijun et al., 2012) and S100A4 is an intracellular calcium-binding protein expressed by osteoblastic cells. It has a negative effect on mineralization and osteoblast differentiation (Wagner et al., 2003; Pierre et al., 2005). The S100 immunohistochemical staining accentuated the structural and constitutive properties of the bone graft, as well as new bone properties of the bone graft as well as new bone properties of S100 protein (Pierre et al., 2005).

Specific S100 proteins that presented interested interesting properties for immunohistochemistry were used S100A6 , which is expressed with fibroblast. The osteoblast and osteoclast surrounding xenograft were also seen using S100A6 immunoreaction (Pierre et al., 2005). In present study , we should to study of morphogenic data for confirm the result of mRNA level of Bcl2A1 and S100A4.

In bone remodeling process has many interested gene that expression when osteoclast and osteoblast activity. we should to study more and try to use another technique example, microarray , is a technology in which thousands of nucleic acids are bound to a surface and is used to measure the relative concentration of nucleic acid sequences in a mixture via hybridization and subsequent detection of the hybridization events. Therefore, it is used to simultaneously measure expression levels of thousands of RNA transcription which is ideal for finding difference genes expression between biological groups (Carinci et al., 2006).

Conclusion

Both materials have the potential to increase the expression of osteoblast related genes and decrease the expression of osteoclast related genes in vivo compared to natural bone healing. The results of this study suggest that DFDBA and DBBM could be used for the repair of bone defects.

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