Gene Expression and Microcomputed Tomography Analysis of Grafted Bone Using Deproteinized Bovine Bone and Freeze-Dried Human Bone

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Purpose: *Bio-Oss and demineralized freeze-dried bone allograft (DFDBA) are two commercial bone grafts that have been associated with clinical success for many years. However, there are few in vivo studies regarding their healing mechanism. The purpose of this study was to investigate the level of bone formation using microcomputed tomography (micro-CT) and gene expression in mouse calvaria at 1 and 3 months after bone grafting with deproteinized bovine bone and freeze-dried human bone, and compare them to natural bone healing.* ***Materials and Methods:*** *Thirty-six mice were divided into three groups (n = 6 per group) according to the type of bone graft used: group 1 (control)—an empty defect without bone graft; group 2—treatment with deproteinized bovine xenograft (Bio-Oss); group 3—treatment with DFDBA. The bone graft was inserted into two 3-mm calvarial defects created on both sides of the parietal bone. At 1 and 3 months, the mice were sacrificed and bone volume was evaluated using micro-CT and gene expression analysis using reverse transcription polymerase chain reaction (RT-PCR).* ***Results:*** *Micro-CT analysis demonstrated that the parietal bone of mice grafted with Bio-Oss had significantly greater bone volume than both the DFDBA and control groups at both 1 and 3 months. The expression of bone marker genes (Runx2, Osterix [Osx], alkaline phosphatase [ALP], osteopontin [OPN], and osteocalcin [OCN]) were significantly increased from 1 month in both Bio-Oss and DFDBA groups at 3 months. Runx2 and Osx had significantly higher expression in the Bio-Oss and DFDBA groups compared to the control group at 3 months. No statistically significant difference was observed among groups after 1 month.* ***Conclusion:*** *These results showed that both bone graft materials promoted bone regeneration. Bio-Oss demonstrated high osteoconductive properties.* Int J Oral Maxillofac Implants 2018;33:541–548. doi: 10.11607/jomi.6234

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The combination of a conventional prosthesis and implant placement is one of the first choices considered for prosthodontic treatment of edentulous patients. However, the main problem for patients with long-term tooth loss is a lack of bone quality and quantity. Dimensional changes of the residual ridge also occur, especially during the first 6 months after tooth extraction.[1–3] Without ridge preservation, the extraction site may lose up to 50% of its width within the first year.[4] To solve this problem, scientists have been developing bone grafting materials. A number of these have been used both in oral and maxillofacial surgery, eg, autograft, allograft, xenograft, and alloplast. Autograft is well known as the "gold standard" of grafting materials due to its osteoconductive, osteoinductive, and osteogenic properties and non-immunologic response.[5] However, it comes in limited quantity, requires a secondary surgical site, prolongs operation time, and may cause higher risk of donor site morbidity.[6,7] Therefore, the use of alternative bone materials such as allograft and xenograft has increased.[8,9]

Allograft is a human bone graft that is harvested from another person; it is freeze-dried to preserve and sterilize the material. Three types of bone allograft are available: fresh frozen bone allograft, freeze-dried

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Fig 1 Bone grafts filled into the 3-mm-diameter calvarial defects: Bio-Oss on the left and DFDBA on the right side.

bone allograft (FDBA), and demineralized freeze-dried bone allograft (DFDBA). FDBA and DFDBA are commonly used materials because they have provided good clinical results for many years.[10,11] FDBA is a source of type I collagen, which is a major component of bone, while DFDBA contains demineralized inorganic substance. DFDBA can also release bone morphogenic protein (BMP), which provide the osteoinductive properties in this graft. Urist and Strates claimed that DFDBA possessed osteoinductive properties different from FDBA.[12] This contradicted a later study in 1996, which found no osteoinductive properties from both grafts.[13] Histologic analysis by Wood and Mealey revealed that bone grafted with DFDBA in humans for 19 weeks showed significantly more bone formation and lower amounts of graft material than FDBA.[4] Presently, the osteoinductive properties of FDBA and DFDBA are still not predictable.

Xenograft is bone graft taken from another species, ie, bovine or pig.[14] It undergoes a heating process under 300˚C to remove cells and organic contents.[15] Only osteoconductive properties are described for this type of bone graft. One product readily available on the market is Bio-Oss. It comes from bovine bone, has a porous structure similar to human bone, and can resist compressive forces of up to 35 MPa. Bio-Oss has been widely used for its high osteoconductive properties, slow resorption rate, and biocompatibility.

Clinically, the different healing patterns of natural bone and bone grafts can be distinguished by radiographic examination. However, there is still a lack of research on how the bone grafting materials alter osteoblast gene expression. Therefore, this study aims to investigate bone regeneration in an animal model after placing two types of commonly used bone grafts, xenograft (Bio-Oss) and human allograft (Oragraft), and compare them to the healing of normal bone.

MATERIALS AND METHODS

Animals

Thirty-six 8-week-old C57BL/6MLac mice weighing 25 to 30 g were used in this study. The experiment was approved by the Animal Care and Use Committee of Chulalongkorn University. The mice were housed in light- and temperature-controlled facilities and given food and water ad libitum.

Surgical Procedure

The sedative, pentobarbital (Nembutal), was diluted with a phosphate-buffered saline in a ratio of 1:10 and a concentration of 4 mg/kg (or 8 µL of dilution/wt [g]) as used.[16] After the sedative was injected into the peritoneum layer, the mice's hair was removed with a blade, and the scalp cleaned with alcohol and povidone iodine. Next, 0.2 mL of 1% lidocaine with 1:100,000 epinephrine was injected into the subcutaneous tissue of the skull. An incision 1.5 mm in length was made to visualize the parietal bone. A 3-mm-diameter cavity was precisely created on both the right and left sides of parietal bone, 1.5 mm away from the sagittal suture and 3 mm from the lambdoid, using a hand drill and trephine burs with normal saline coolant. The procedure was performed gently in order to avoid dura mater injury. The exact amount (10 mg) of the two types of bone graft, with mean particle size ranging between 250 and 1,000 µm, as commercially available, was randomly filled into the skull cavity, packed gently using a cotton pellet soaked with normal saline, and stitched up with nylon 3-0. The defects were divided into three groups according to the type of graft and control (Fig 1):

- Group 1: bare defect as control
- Group 2: deproteinized bovine bone (Bio-Oss, Geistlich Pharma AG)
- Group 3: demineralized freeze-dried human bone (Oragraft, LifeNet)

Microcomputed Tomography Imaging

At 1 month and at 3 months after surgery, the animals were sacrificed and dissected. Their calvaria were removed and immediately immersed in 10% formalin overnight (n = 6 per group). They were then rinsed with PBS before being analyzed with microcomputed tomography (micro-CT) imaging (uCT 35, Scanco Medical AG) in a standard-resolution scanning mode. To position the calvaria, a holder 20 mm in width and 75 mm in height was used. The following micro-CT settings were used: 70 kVp, 114 µA, 8 W, voxel size 20 µm. A threshold of 212 was used for analysis of mineralization. The morphology was observed and bone volume was calculated into mean ± SD (mm^3).

Table 1 The Specific Primers for Real-Time PCR

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Runx2	TCC TTC ACT CCA AGA CCC TA	TCA GAT ACC ATG GGT GCT TC
Osx	GAT TCC TGG GGT ATG TAG GA	TGG GAA ACA GGA ATA TGG GC
ALP	GGC TCT CTT CAC TCC AAG AT	GAA GGA AGC TAC CAA CTG CT
OCN	TGG GAA ACA GGA ATA TGG GC	GCA GAT TGT GAG ACC TTC AG
OPN	TGA AAG TGA CTG ATT CTG GC	CCT TTT CTT CAG AGG ACA CA
18S rRNA	GTG ATG CCC TTA GAT GTC C	CCA TCC AAT CGG TAG TAG C

RNA Extraction and Real-Time Polymerase Chain Reaction

Bone samples were collected using a 5-mm-diameter trephine bur and stored in a cryotube (SPL Life Science). Samples were submerged in liquid nitrogen immediately prior to RNA extraction. Total RNA isolation was done using Qiazol reagent (Qiagen, Inc) according to the manufacturer's protocol. RNA purity and concentration was checked by Nanodrop spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific).

Two-step reverse transcription qualitative polymerase chain reaction (RT-qPCR) was used in this study. First, RT was done by using the Sensiscript RT kit (Sensiscript, Qiagen, Inc). Second, the qPCR was done by using the KAPA SYBR FAST qPCR kit (Kapa Biosystems, Inc) with real-time RT-PCR (CFX96 system, Bio-Rad). Primer3 and BLAST were used for designing primers (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primer sequences are shown in Table 1. The following parameters were used in qPCR: enzyme activation at 95˚C for 3 minutes, denaturation for 40 cycles at 95˚C for 1 to 3 seconds, annealing and extension at 60˚C for 20 more seconds per plate read, dissociation at 95˚C for 5 seconds. For each gene, all samples were amplified in duplicate in one run. Negative control reactions with no sample (RNase free water) were included in each run. Analysis of relative gene expression was performed using the $2^{-\Delta\Delta Ct}$ method. 18S rRNA was used as a housekeeping gene to normalize the expression data. The qRT-PCR was used as a method to evaluate the five specific genes (alkaline phosphatase [ALP], osteopontin [OPN], osteocalcin [OCN], runt-related transcription factor 2 [Runx2] and Osterix [Osx]) after bone graft placement.

Data Analysis

SPSS version 17.0 (SPSS Inc) was used for data analysis. The differences in bone volume and the differences in the relative gene expression among groups were evaluated using a one-way analysis of variance (ANOVA), followed by post hoc Tukey Honestly Significant Difference test with a significance level of 5%. The differences of the relative gene expression within each group were evaluated with independent *t* test with a significance level of 5%.

RESULTS

One of 36 mice treated with both Bio-Oss and DFDBA was lost during the operation period.

Micro-CT Imaging of Bone Regeneration

Three-dimensional micro-CT images from at 1 month showed bone formation in all groups at the defect margins. The Bio-Oss grafts had more remaining particles within the defect compared to the DFDBA grafts (Fig 2). At 3 months, bone formation from defect margins increased in all groups compared to bone formation at 1 month. However, the residual grafts inside the defects were markedly decreased, especially in the DFDBA group (Fig 3). No defects were closed completely at the end of the experiment.

At 1 month, mean bone formation was 0.25 ± 0.08 mm^3 (1.5% bone volume [BV] of the total volume [TV]) in the control group, followed by 0.5 ± 0.12 mm^3 (3.14% BV/TV) in the DFDBA group and 2.0 ± 0.45 mm^3 (12.64% BV/TV) in the Bio-Oss group. At 3 months, mean bone formation was 0.33 ± 0.13 mm^3 (1.95% BV/TV) in the control group, 0.48 ± 0.2 mm^3 (2.47% BV/TV) in the DFDBA group, and 1.06 ± 0.7 mm^3 (6.26% BV/TV) in the Bio-Oss group (Fig 4).

Gene Expression of Bone Markers

At 1 month, no difference in gene expression of ALP, OPN, OCN, Runx2, and Osx was found, both in Bio-Oss and DFDBA compared to the control. There also was no significant difference in the gene expression between Bio-Oss and DFDBA at 1 month. At 3 months, the upregulation of Runx2, Osx, and ALP compared to the control were found in Bio-Oss samples, and the upregulation of Osx, ALP, and OPN compared to control were found in DFDBA. No difference was observed for the expression of OCN compared to control in any of the samples at both 1 and 3 months. At 3 months, Bio-Oss increased Runx2 expression significantly compared

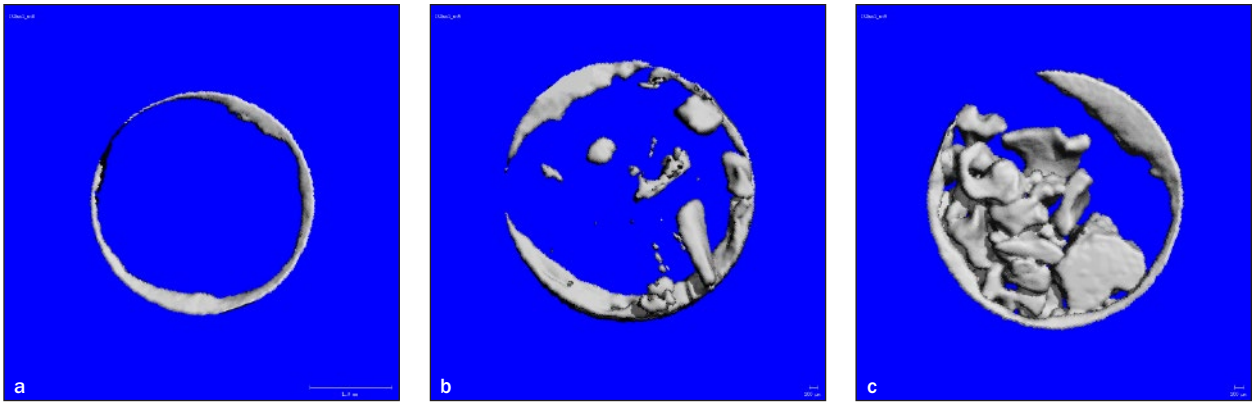

Fig 2 Three-dimensional micro-CT images of defects at 1 month: *(a)* control, *(b)* DFDBA, *(c)* Bio-Oss.

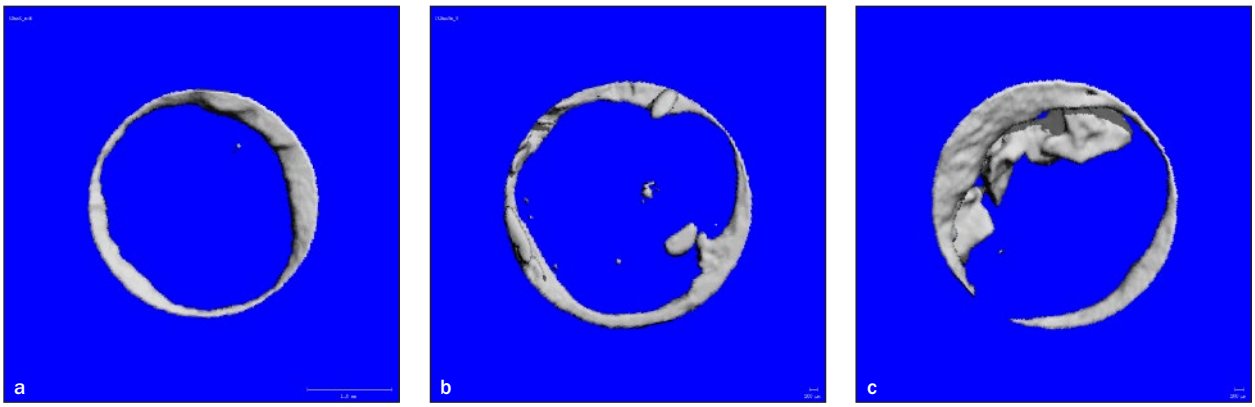

Fig 3 Three-dimensional micro-CT images of defects at 3 months: *(a)* control, *(b)* DFDBA, *(c)* Bio-Oss.

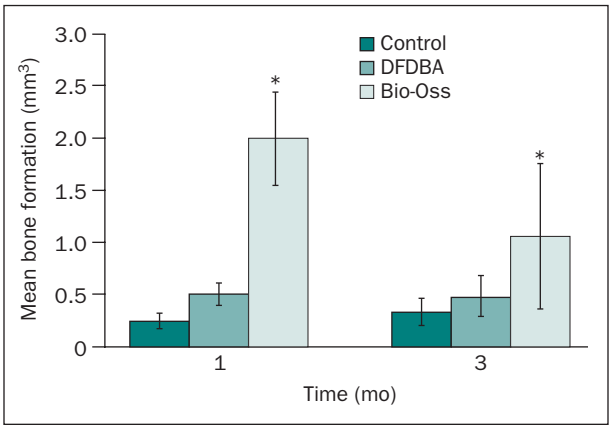

Fig 4 Mean bone formation (bone volume/total volume, mm^3) of bone grafts at 1 and 3 months. *Indicates significant difference in bone volume at $P < .05$.

to DFDBA. The expression of OPN was significantly upregulated in DFDBA compared to Bio-Oss. Figure 5 shows the relative mRNA levels of bone marker genes (ALP, OPN, OCN, Runx2, and Osx).

The difference in gene expression at 1 month and 3 months was evaluated within each group. In the control group, all genes except OPN decreased at 3 months, with a statistically significant difference in Runx2 and Osx. In the Bio-Oss and DFDBA groups, all genes increased at 3 months, with a significantly significant difference in ALP and OPN found in Bio-Oss and a significantly significant difference in Osx and OPN found in DFDBA (Fig 6).

DISCUSSION

Bio-Oss and DFDBA have been widely used for many years, as they have a history of good clinical outcomes. Therefore, Bio-Oss and DFDBA were chosen as representative for xenograft and allograft, respectively, and compared in efficiency in bone formation.[4,7,11,17,18] In vivo studies often use animal models to evaluate the bone regeneration process and bone substitute interaction. Mice are commonly used due to their low cost, high reproducibility, and easy handling and maintenance. In consideration of the implantation site, the calvarial defect serves as a model of intramembranous ossification. It has the same embryonic origin as the maxilla and body of the mandible. This study used 3-mm critical-size defects in accordance with Aalami et al; their study concluded that mice calvarial bone defect sizes 3, 4, and 5 mm were critical to adult mice.[19]

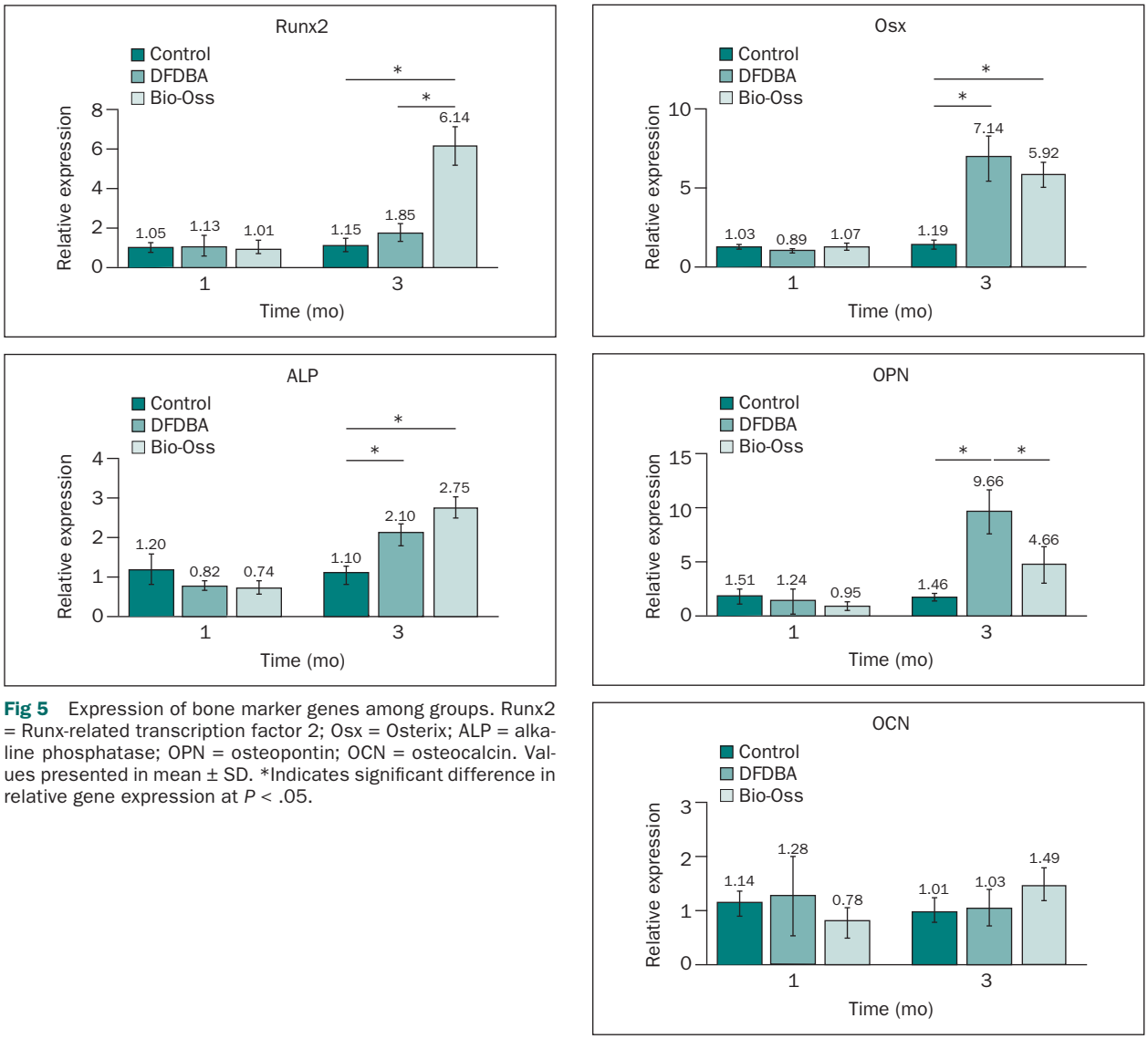

Fig 5 Expression of bone marker genes among groups. Runx2 = Runx-related transcription factor 2; Osx = Osterix; ALP = alkaline phosphatase; OPN = osteopontin; OCN = osteocalcin. Values presented in mean ± SD. *Indicates significant difference in relative gene expression at $P < .05$.

From the micro-CT analysis, it was found that bone formation in all groups started from the defect margins. Bio-Oss groups had greater bone volume than DFDBA at both 1 and 3 months. This corresponded with a previous study in which Bio-Oss showed osteoconductive properties and good biocompatibility with intraoral tissue.[20] A histomorphometric study of sinus grafting with Bio-Oss in chimpanzees demonstrated that organic bovine bone was resorbed and replaced with new bone within 1.5 years.[17] Some studies reported that organic bovine bone still remains after 44 months in humans.[21,22] The present study found that although there was a reduction of Bio-Oss graft particles within the first month, new vital host bone gradually occupied this space. However, at 3 months, no defect was completely filled with new bone. The period of time required for the graft to be completely replaced by new bone cannot be predicted due to the time constraints of this study.

One of the questions of the present study was whether the grafting materials interfere with or influence the bone healing process. Previous studies have found their molecular data to be unclear. This in vivo study placed focus on specific genes related to bone formation using a mouse model. It was found that Bio-Oss and DFDBA had upregulated ALP in the 3-month group compared to the control. ALP was detected in the initial stage of bone formation. Thus, both materials can help promote early bone mineralization. Runx2 was significantly upregulated in the 3-month Bio-Oss group. It is essential for osteoblast differentiation from mesenchymal stem cells to premature osteoblasts, but inhibits osteoblast maturation.[23] The level of expression of Osx had significant upregulation at 3 months, both in Bio-Oss and DFDBA. Osx is a downstream gene to Runx2 that is required for the differentiation of premature osteoblasts into mature osteoblasts.[24] Runx2 interacts with Osx and can upregulate the expression

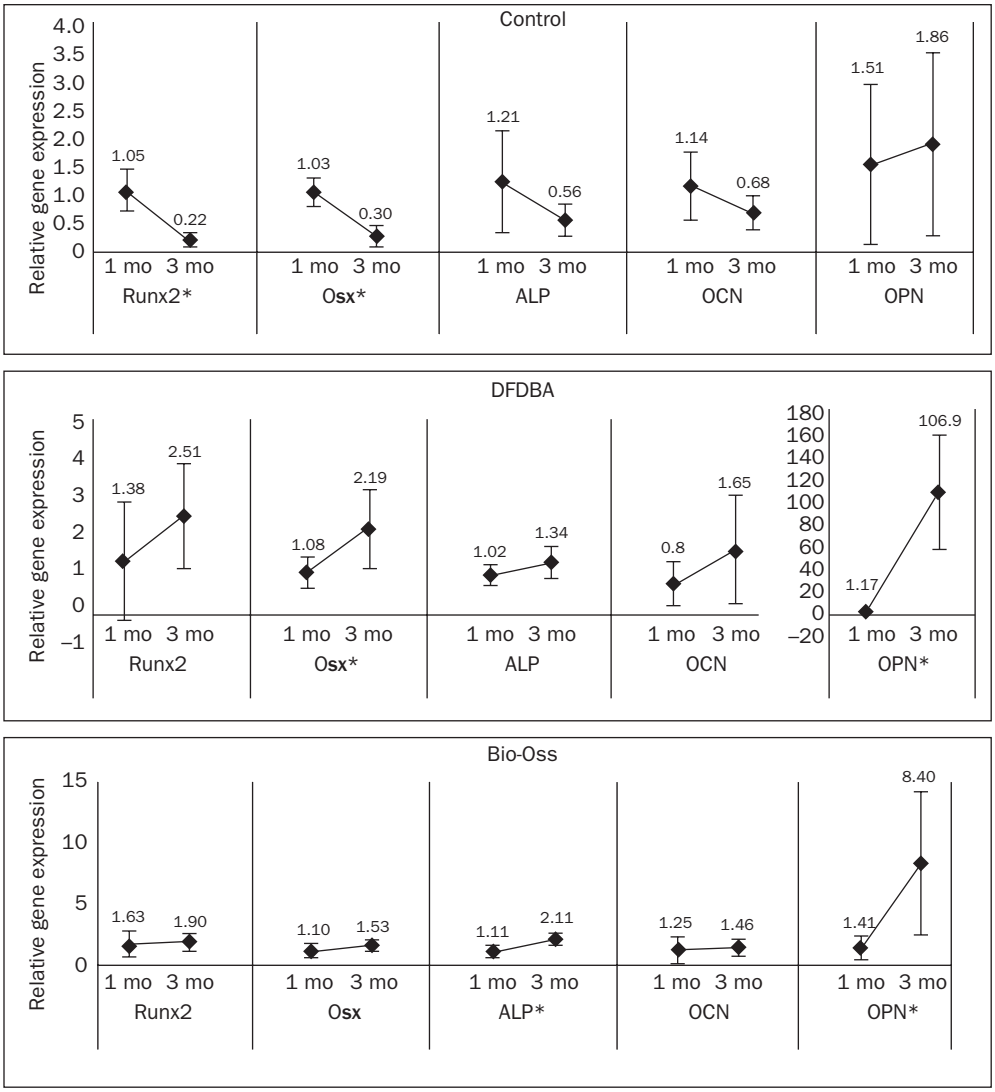

Fig 6 Difference in expression of bone marker genes at 1 and 3 months. Value presented in mean ± SD. *Indicates significant difference in relative gene expression at $P < .05$.

of OCN, a specific bone marker found at the late stage of cell maturation.[25] Monjo et al revealed that OCN is the best predictive marker for osseointregration of titanium implants in the animal model.[26] According to the present study, Runx2 and Osx had increased expression at 3 months, while no difference in OCN expression was found. This may have been due to the short time period of this study.

Another investigated gene, OPN, was significantly increased in the 3-month DFDBA group. The OPN gene is produced by osteoblasts and a possible role of OPN in osteoclast attachment and function has been suggested.[27] Merry et al, using an in situ hybridization technique to investigate the OPN in human bone tissue, showed that OPN was highly expressed in pre-osteoblasts and decreased markedly in mature osteoblasts.[28] From these findings, it can be suggested that both Bio-Oss and DFDBA can promote osteoblast differentiation.

Comparison of gene expression at 1 and 3 months was performed. Several genes in the control group had decreased expression at 3 months (statistically significant difference was found in Runx2 and Osx), while both Bio-Oss and DFDBA groups had increased gene expression at 3 months. This implies that the use of bone graft materials can prolong specific bone marker genes in the in vivo mouse model.

Micro-CT has gained recognition for use in studying small osseous and soft tissue structures of animals. It can be used in bone research to analyze the degree of mineralization and new bone formation.[29] This technique provides three-dimensional images of bone without destroying its structure and allows for the accurate visualization of the anatomy and morphology of tissues. Furthermore, the process is much faster compared to the processing time required in conventional histologic procedures.[30] Previous studies confirm that micro-CT is a repeatable and reproducible technique

for providing information on bone formation.[31] Duke et al demonstrated that micro-CT scans are an important corollary to histologic studies by evaluating the use of implants in healing of bony defects.[32] Therefore, micro-CT was selected for the present study to provide quantitative data on bone volume.

In this study, the Bio-Oss group had superior bone volume compared to the DFDBA and control groups at both 1 and 3 months. In clinical studies, Bio-Oss and DFDBA both have shown efficiency in decreased pocket depths and gains in clinical attachment levels in intrabony defects in humans.[11] In a histologic study, Mokbel et al found that DFDBA had a significantly greater mean bone formation than Bio-Oss in rat calvarial bone defect models.[8] Recently, Paknejad et al found no difference between DFDBA and Bio-Oss in their histologic evaluation of bone formation in rabbit calvaria after 30 days of placement.[33] In another comparative study between Bio-Oss and DFDBA in rabbit calvaria, it was found that DFDBA had a high resorption rate, but this did not affect the new bone formation.[34] Although the DFDBA showed less bone volume in micro-CT, gene expression still increased in real-time PCR. This could be because of the confounding variable of the differences between the graft components. For DFDBA, 40% of the mineral content is removed by acid, resulting in lower detection in micro-CT. However, micro-CT cannot replace conventional histology. Future histologic analysis would be beneficial, as it could provide qualitative data (discriminate immature bone, inflammatory cells, residual graft particles) and further information to confirm the present results. This is the first in vivo study that investigated the relationship between genetic and phenotypic data of bone formation.

The limitation of this study was that it was performed in the mouse model. The mouse's small size and low vascularization leads to limitation in surgical precision. Therefore, the amount of bone formation observed in this experiment may be lower compared to the intraoral situation. It is understood that there are differences between humans and the mouse model that might effect the result of grafting. However, previous studies have shown that the gene activities in the mouse and humans are related and the basic gene structure has been conserved in both species throughout evolutionary time.[35–37] Therefore, the mouse is a good model for studying human biology. Moreover, studies in humans have shown similar histologic results to mouse model studies of these bone grafting materials.[38]

CONCLUSIONS

This study comparatively studied two commercially available bone grafts in their level of bone formation and genetic responses after grafting in bone defect models. Bio-Oss had higher bone formation in the defect sites when compared to DFDBA in micro-CT. It was found that both materials have the potential to increase the expression of osteoblast-related genes in vivo compared to natural bone healing.

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