CLINICAL ORAL IMPLANTS RESEARCH

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Gene expression and morphometric parameters of human bone biopsies after maxillary sinus floor elevation with autologous bone combined with Bio-Oss® or BoneCeramic®

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Key words: bone graft, clinical study, dental implant, gene expression, maxillary sinus floor elevation (MSFE), micro-computed tomography (microCT)

Abstract

Objectives: Although the clinical success of Bio-Oss® and BoneCeramic® has been corroborated by histologic and histomorphometric findings, the biological events that occur during healing after maxillary sinus floor elevation (MSFE) are unknown. Here, we evaluated biopsies of grafted bone with a mixture of autologous bone and Bio-Oss® or BoneCeramic® after two different healing time periods to understand the molecular process underlying bone formation after MSFE.

Material and methods: Seven patients, following a bilateral split-mouth design model and needing a MSFE to allow implant placement, were recruited for this study. Right or left sinuses were grafted with autologous maxillary bone combined either with Bio-Oss® or BoneCeramic®, respectively. Twenty biopsies were taken at the time of implant insertion after 4–5 months or 6–8 months of MSFE, and analyzed by micro-computed tomography (microCT) and gene-expression analysis.

Results: MicroCT analysis revealed no differences in the morphometric parameters or BMD either after 4–5 months or 6–8 months of MSFE between Bio-Oss® and BoneCeramic®. At molecular level, a higher expression of bone forming gene Runx2 was observed after 4–5 months of MSFE in the Bio-Oss® compared with the BoneCeramic® group.

Conclusions: Our results indicate that differences found at the molecular level between Bio-Oss® and BoneCeramic® are not translated to important differences in the 3D microstructure and BMD of the grafted bone.

Maxillary sinus floor elevation (MSFE) aims at achieving a bone volume sufficient for the insertion of endosseous implants in those patients with insufficient bone volume condition. During the procedure, a door hinge in the lateral side of the maxillary sinus wall is prepared and internally rotated, together with the Scheneiderian membrane, to a horizontal position forming a new sinus floor (Boyne & James 1980; Tatum 1986). MSFE stimulates the growth of new bone into the augmented site, but, despite the extended use of this procedure, the mechanism of the observed bone formation remains still unknown. It is tempting to assume that the osteoprogenitor cells of the Scheneiderian membrane (Srouji et al. 2010) are the main players in bone formation; in fact, it has been proved that no graft is needed for new bone formation if a void space is created and allows the formation of a blood clot (Ellegaard et al. 1997; Kumar et al. 2013; Lundgren et al. 2003; Riben & Thor 2012). However, other authors have reported that MSFE without graft material fails to achieve sufficient bone volume for implant installation (de Oliveira et al. 2013); thus, in cases in which a great increase on bone height from alveolar crest to sinus floor is needed, bone graft is placed between the maxillary bone and the elevated membrane to support the attachment, proliferation and migration of osteoprogenitor cells and thereafter initiate bone formation (McKee 2006).

Autologous bone graft is considered the "gold standard" as grafting material, given its excellent osteoinductive, osteoconductive, and osteogenic properties. However, its disadvantages, mainly associated morbidity,

limited availability, and unpredicted graft resorption, have led to the use as graft materials of a variety of allografts, xenografts, and alloplasts or combinations, showing good results in clinical (Hurzeler et al. 1996; Merkx et al. 2003; Raghoebar et al. 2005; Ramirez-Fernandez et al. 2013a,b; Tong et al. 1998) and animal studies (Calvo-Guirado et al. 2010; Calvo Guirado et al. 2013; Ramirez-Fernandez et al. 2011a,b; .

In our search for the ideal graft material, we have previously proved a harvesting procedure through the use of a Safescraper that allows the obtention of autologous bone from the maxillary malar area through the same incision performed during the MSFE procedure (Caubet et al. 2011). This device allows obtaining cortical bone chips between 900 and 1700 μm in length and 100 mm thick with almost no donor site morbidity. These chips contain living osteocytes (mean viability 45–72%) that allow proper bone regeneration for implant placement (Caubet et al. 2011; Zaffe & D'Avenia 2007). The autologous bone obtained in this way combined with commercially available bone graft substitutes leads to satisfactory bone regeneration decreasing the healing time compared with using bone graft substitutes alone (Galindo-Moreno et al. 2007; Simunek et al. 2008; Tadjoedin et al. 2002). The mixture leads to the addition of the advantages of each graft. On one hand, autologous grafting accelerates bone regeneration due to its content in osteoprogenitor cells (exclusive to this type of graft) and to its richness in growth factors (Hallman et al. 2002). On the other hand, inorganic bone graft substitutes are highly osteoconductive and, given its slower resorption compared with autologous bone, act as a template for new bone formation during the entire regenerative process (Hallman et al. 2002; Schlegel et al. 2003). Bio-Oss® is a bone graft substitute made of bovine bone of which all organic components are removed, but maintains the natural architecture of bone (Richardson et al. 1999). Bio-Oss is physically and chemically comparable to the mineralized matrix of human bone, with osteoconductive properties and high biocompatibility (Jensen et al. 1996), being one of the best documented biomaterials. BoneCeramic® is a fully synthetic bone graft substitute in particulate form composed of biphasic calcium phosphate consisting of 60% hydroxyapatite (HA), and 40% beta-form of tricalcium phosphate (b-TCP), which has shown osteoconduction and similar results than Bio-Oss in sinus floor augmentation (Cordaro et al. 2008; Schmitt et al. 2013). Other commercial synthetic bone grafts with similar HA/b-TCP ratio have been tested for their suitability as a bone substitute in animal models (Calvo-Guirado et al. 2012, 2013).

MSFE allows taking bone biopsies from patients after graft consolidation and before dental implant placement. This procedure is known as delayed implant placement, and it is recommended in cases with lower bone heights (<4 mm). Furthermore, the treatment of patients requiring a MSFE procedure at both sites of the maxilla enables the use of a bilateral split-mouth design model, allowing to study simultaneously different conditions in the same patient (Farre-Guasch et al. 2013). Despite the clinical success of Bio-Oss® and BoneCeramic® has often been corroborated by histologic and histomorphometric findings, the biological events that occur during healing after MSFE are still unknown. Here, with the aim of giving some light to the molecular process underlying bone formation after MSFE, we evaluated biopsies of grafted bone with a mixture of autologous bone and Bio-Oss® or BoneCeramic® after two different healing time periods following a bilateral split-mouth design model by means of micro-computed tomography (microCT) and gene-expression analysis of bone formation, resorption, and anti-inflammatory markers.

Materials and methods

Patient selection

Patients older than 18 years with a bone height requiring a MSFE procedure to place one or more dental implants in both sites were eligible for inclusion in the study. The residual bone height of the lateral-posterior segments of the edentulous maxilla below the floor of the maxillary sinus had to be ≤4 mm, as measured by CT scans. Furthermore, average residual bone width had to be at least in average 6 mm as measured by the CT scans. In total, seven patients (3 men and 4 women) were included in the study. The mean age of the patients was 53 years, ranging from 38 to 59 years. The study was conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki and was approved by the Ethics Committee of Balearic Islands (CEI-IB). All participants provided written informed consent. Systemic and local exclusion criteria were defined as any factors interfering with implant surgery: smokers of more than 15 cigarettes per day, severe liver or kidney disease, history of radiotherapy in head and neck region, concomitant chemotherapy to treat malignant tumors, decompensated diabetes, active periodontal disease, diseases of the oral mucosa (planus lichen in the treatment area), previous destructive surgery or contraindication ORL for sinus lift, poor oral hygiene, postexodoncia period <2 months, and uncooperative patients and bisphosphonates in the previous 3 months.

Surgical procedures

Antibiotics were administered to the patient 24 h before surgery (875/125 mg amoxicillin/clavulanate potassium and 300 mg clindamicine in allergics to peniciline). Smoking patients were advised to cease smoking.

First surgical stage: Sinus floor elevation and bone graft harvesting

Local anesthesia solution of articain with 0.5% mg epinephrine was injected into the buccal and palatal maxillary area. The incision was made on the top of the alveolar ridge, or slightly on the palatal side, through the keratinized, attached mucosa. This way the wound closure can be solid and with sufficient overlap to deal with a possible dehiscency.

The start of the preparation was normally with the bone scraper. Bone from the lateral wall of the sinus was collected as a part of the antrostomy. The preparation was finished with a large round diamond bur that cannot easily damage the membrane or perforate the bony wall. The Schneiderian membrane was lifted using special sinus floor elevation instruments [designed by Tatum (Tatum 1986)] that worked in different directions with different angles and blades. Bone from malar and maxillomalar buttress was harvested with the bone scraper by pushing the end of the device toward the bone surface and simultaneously pulling the device itself backward. Collection of 2–3 cc of bone was feasible with a mean surgical time of 10 min for this harvesting purpose. The collected bone was preserved in a sterile environment until grafting. The graft was mixed 1 : 1 with Bio-Oss® (Geistlich Pharma AG, Wolhusen, Switzerland) or BoneCeramic® (Straumann AL, Basel, Switzerland). For simplicity, in the paper, we will refer the groups as Bio-Oss or BoneCeramic, although grafting has been done with 50% autologous bone −50% biomaterial. All right sinus cavities were grafted with Bio-Oss and left sinus cavities with BoneCeramic. The use of dentures was not permitted until these dentures had been adjusted and refitted at least 2 weeks after surgery.

Second surgical stage: flapless dental implant placement

After 4–5 or 6–8 months of healing, implant sites were prepared in a second surgical stage with a trephine bur of 2.5 mm in diameter to obtain bone biopsies at the same sites where implants had to be placed. Every attempt was taken to keep the bone biopsy inside the trephine bur. Clinical and radiographic evaluation was done. Panoramic radiographs were used to assess new bone formation. At this time, a total of 20 implants were placed in a flapless procedure under local anesthesia. In all patients, biopsies of the grafted area were obtained ($n = 20$) through this surgical approach before implant insertion. The final peak of the insertion torque (IT) of each implant was measured when the implant was fully seated. Implant stability quotient (ISQ) readings were also obtained for each implant at placement time using the Osstell® ISQ system (Ostell, Göteborg, Sweden).

Third surgical stage: second surgery of implants

Four months after implant placement, all implants were exposed and definitive abutments were connected. Data were collected at the time of bone augmentation and at 4–5 and 6–8 months after sinus graft procedure.

Clinical and radiographic examination

Radiologic evaluation included preoperative Ortopantomography and maxillary CTScan. All the panorex were done with the same ortopantomograph (Orthopantomograph 200 D, Instrumentarium) and magnification was corrected (1 : 1). The radiographic records consisted of ortopantomography taken before bone graft surgery and at 4–5 or 6–8 months (at the moment of implant placement).

MicroCT analysis

The purpose of microCT analyses was to evaluate the 3D architecture parameters of the grafted bone structure and the volumetric Bone Mineral Density (vBMD). The analyses were performed blinded for the two commercial biomaterials used, Bio-Oss and BoneCeramic. The specimens were examined using a microCT machine (Skyscan 1172, Skyscan, Aartselaar, Belgium). Specimens were placed into 200 µl sterile microtubes, containing RNAlater® and in Parafilm® (American National Can™, Chicago IL, USA) to avoid degradation of samples and movement, respectively, on a sample holder in a vertical position to ensure parallel scanning conditions. The resolution was set at 7.8 µm voxel resolution X-ray tube current 100 µA and voltage 100 kV with a 0.5 mm aluminum filter. Specimens were rotated through 360 around the long axis (z-axis) of the sample. Three absorption images were recorded every 0.4 degree of rotation. The beam hardening was set to 20%, smoothing to 1 and ring artifact reduction to 6 during the reconstruction of the axial images (Nrecon v.1.4.4, Skyscan, Aartselaar, Belgium). Post alignment was optimized automatically.

After reconstruction, the same volume of interest (VOI) was applied as inside the reconstructed images of the two phantom rods. The same VOI was chosen for all the samples: a cylinder of 2.6 mm in diameter and a height of 1 mm. A 3D morphometric analysis was conducted with the CTan software (Skyscan, Aartselaar, Belgium) to determine the architecture of the newly formed bone in the grafted area. The grayscale threshold was set between 100 and 255. The 3D images were prepared in a volume rendering program (CTvox v.2.10, Skyscan, Aartselaar, Belgium) and further enhanced in an image editor (Adobe Photoshop CS3, Adobe, USA). Calcium hydroxyapatite phantom cylinders of a density of 0.25 and 0.75 g/cm3 were used for the BMD calibration and analyzed exactly in the same way as the bone samples. A calibration of the standard unit of X-ray CT density (Hounsfield unit, HU) was done, followed by the conversion from HU to BMD. vBMD was obtained for the grafted bone contained for each biopsy. The following 3D morphometric parameters were measured: Bone volume/total volume (BV/TV, ratio of the segmented bone volume to the total volume of the VOI), Bone surface (BS, surface of the region segmented as bone), Bone specific surface (BS/BV, ratio of the segmented bone surface to the segmented bone volume), Bone surface density (BS/TV, ratio of the segmented bone surface to the VOI), Trabecular thickness (Tb.Th, mean thickness of trabeculae, assessed using direct 3D methods), Trabecular separation (Tb.Sp, mean distance between trabeculae, assessed using direct 3D methods), Trabecular number (Tb.N, measure of the average number of trabeculae per unit length), Degree of anisotropy (DA, 1 = isotropic, >1 = anisotropic by definition; DA = length of longest divided by shortest mean intercept length vector).

Total RNA isolation

Frozen bone biopsies were cut with a bone saw to take 1 mm of grafted bone from each sample (Fig. 1). Then, samples were pulverized and used immediately for total RNA isolation using a monophasic solution of phenol and guanidine isothiocyanate (Tripure, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Total RNA was quantitated at 260 nm using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

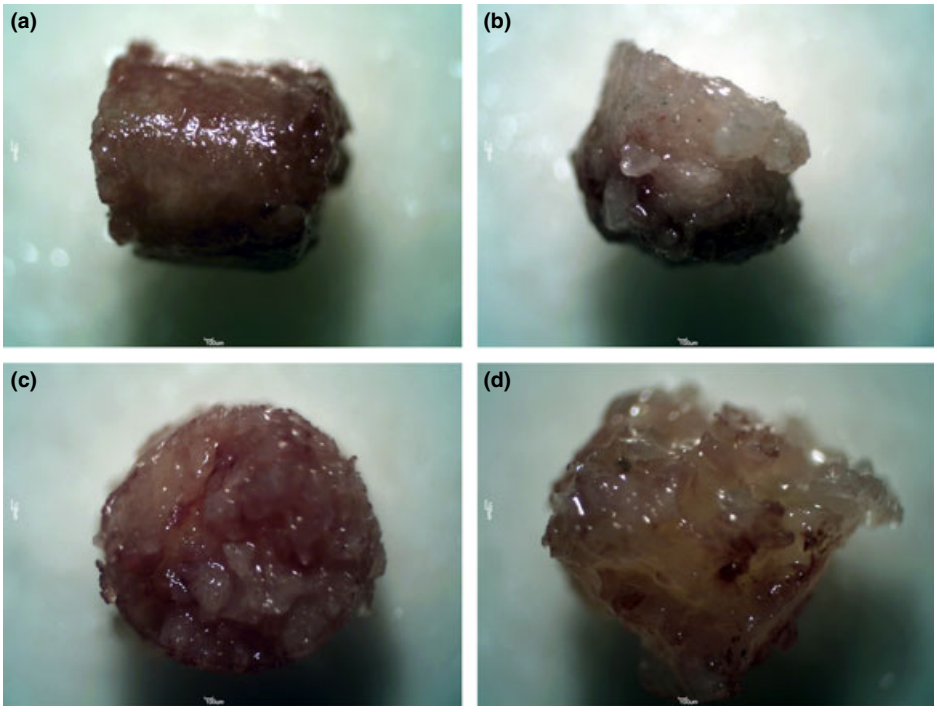

Fig. 1. Macroscopic pictures of the grafted area of bone biopsies used for micro-CT and biological analysis. (a) Bio-Oss at 4–5 months, (b) BoneCeramic at 4–5 months, (c) Bio-Oss at 6–8 months, and (d) BoneCeramic at 6–8 months.

Table 1. **Alveolar bone height before and after maxillary sinus floor elevation (MSFE) procedure and primary implant stability measurements: insertion torque value (ITV) and Osstell implant stability quotient (ISQ) value**

	4–5 months		6–8 months	
Variable	Bio-Oss (n = 6)	BoneCeramic (n = 4)	Bio-Oss (n = 6)	BoneCeramic (n = 4)
Bone height before MSFE (mm)	2.50 (2.00–4.70)	4.25 (1.00–6.00)	2.00 (1.00–4.50)	2.00 (0.000–5.00)
Bone height after MSFE (mm)	17.4 (12.7–21.3)	18.9 (13.2–21.8)	16.7 (13.3–17.5)	15.5 (12.1–17.5)
ITV (Ncm)	35.0 (35.0–50.0)	35.0 (35.0–50.0)	35.0 (35.0–35.0)	35.0 (35.0–35.0)
ISQ	70.0 (55.0–79.0)	67.0 (64.0–69.0)	70.0 (61.0–73.0)	68.0 (64.0–72.0)

The number of biopsies in each group is shown. Median (min–max) values are shown.

Real-time RT-PCR

The same amount of total RNA (150 ng) from each sample was reverse transcribed to cDNA using High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA) according to the protocol of the supplier. Each cDNA was diluted and aliquots were stored at −20°C until the PCR reactions were carried out.

Real-time PCR was performed using the LightCycler FastStart DNA Master PLUS SYBR Green I (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. Real-time PCR was done for three reference genes: 18S ribosomal RNA (18S rRNA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin (ACTBL2), and nine target genes related to bone formation (collagen type I (COL1A1), alkaline phosphatase (ALP), runt-related transcription factor 2, (RUNX2), and osteocalcin (OC)), bone resorption (vacuolar type proton ATPase (H^+-ATPase), tartrate-resistant acid phosphatase (TRAP)), and inflammation (interleukin-6 (IL6), interleukin-10 (IL10) and tumor necrosis factor-α (TNFA)). A negative control without cDNA template was run in each assay. Each reaction contained 500 nM of the corresponding oligonucleotide primers (18s rRNA-F: 5′-GTAACCCGTTGAACCCCATT-3′; 18s rRNA-R: 5′- CCATCCAATCGGTAGTAGCG-3′; h- ACTBL2-F: 5′-AAGGGACTTCCTGTAACAATGCA-3′; h- ACTBL2-R: 5′-CTGGAACGGTGAAGGTGACA-3′; h GAPDH-F: 5′-TGCACCACC-AACTGCTTAGC-3′; hGAPDH-R: 5′-GGCATGGACTGTGGTCATGAG-3′; h-COL1A1-F: 5-CCTGACGCACGGCCAAGAGG-3′; h-COL1A1- R: 5′- GGCAGGGCTCGGGTTTCCAC-3′; h-ALP-F: 5′-CCGCTATCCTGGCTCCGTGC-3′; h-ALP- R: 5′-GGTGGGCTGGCAGTGGTCAG-3′; h-RUNX2-F: 5′- CTGTGCTCGGTGCTGCCCTC-3′; h-RUNX2- R: 5′- CGTTACCCGC-CATGACAGTA-3′; h-OC-F: 5′-GAAGCCCAGCGGTGCA-3′; h-OC-R: 5′- CACTACCTCGCTGCCCTCC-3′; h- H^+-ATPase -F: 5′- GGTGATGTCACAGCAG-AAGTTATG -3′; h- H^+-ATPase -R: 5′-TGCTCAATTCAGTGCCAAAGGAGT-3′; h-TRAP-F: 5′- CATGACCACCTTGGCAATGTCTC-3′; h-TRAP-R: 5′- CTGT-GGGATCTTGAAGTGCAGG-3′; h-IL6-F: 5′-AGGAGACTTGCCTGGTGAAA-3′; h-IL6-R: 5′- GCATTTGTGGTTGGGTCAG-3′; h-IL10-F: 5′-TTATCTTGTCTCTGGGCTTGG-3′; h-IL10-R: 5′-ATGAAGTGGTTGGGGAATGA-3′; h- TNFA-F: 5′- CTATCTGGGAGGGGTCTTCC-3′; h- TNFA-R: 5′-GGGGGTAATAAAGGGATTGG-3′), 5 μl of LightCycler FastStart DNA Master PLUS SYBR Green I (Roche Diagnostics, Mannheim, Germany), and 3 μl of cDNA in a final volume of 10 μl.

The amplification program consisted of a preincubation step for denaturation of the template cDNA (5 min 95°C), followed by 45 cycles consisting of a denaturation step (10s 95°C), an annealing step (10s 60°C) and an extension step (10s 72°C) for all, except for h-ALP and h-Osteocalcin that was 45 cycles consisting of a denaturation step (10s 95°C), an annealing step (5s 68°C) and an extension step (12s 72°C). After each cycle, fluorescence was measured at 72°C. A negative control without a cDNA template was run in each assay.

To allow relative quantitation after PCR, standard curves were constructed from the standard reactions for each target and housekeeping genes by crossing points (Cp) values, i.e., the cycle number at which the fluorescence signal exceeds background, versus log cDNA dilution. The crossing point readings for each of the unknown samples were used to calculate the amount of either the target or reference gene relative to a standard curve. Normalized mRNA levels were calculated as the ratio of relative concentration for the target genes relative to that for the geometric mean among the three reference genes (18S rRNA, GAPDH, and ACTBL2).

Statistics

Grouped data are presented as median (min–max) values. The Wilcoxon test was done to assume parametric or non-parametric distributions for the normality tests. Differences between Bio-Oss and BoneCeramic were assessed by Wilcoxon test for Ratio or by paired t-test. SPSS® program for Windows, version 17.0 (SPSS, Chicago, IL, USA) was used. In four patients, two different biopsies with the same biomaterial from one site were combined and paired versus one biopsy with the other biomaterial in the other site. Results were considered statistically significant at the P-values ≤ 0.05.

Results

Clinical evaluation, implant stability, and survival

All MSFE procedures were performed with only two perforations group I of the Schneiderian membrane according to the classification of Hernández-Alfaro et al. (Hernandez-Alfaro et al. 2008) and healing of the sites proceeded uneventfully. At the time of the re-entry, an adequate bone volume had been formed and primary stability of the

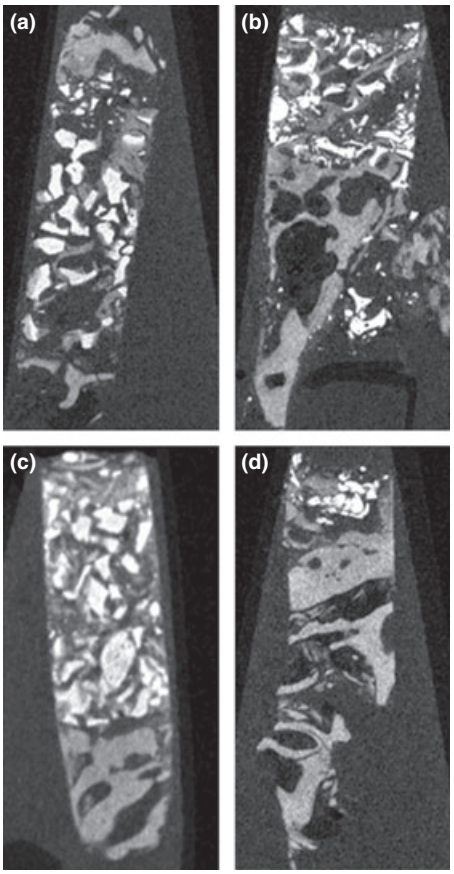

Fig. 2. Micro-CT slice of bone biopsies showing native and grafted bone for the different groups included in the study. (a) Bio-Oss at 4–5 months, (b) BoneCeramic at 4–5 months, (c) Bio-Oss at 6–8 months, and (d) BoneCeramic at 6–8 months.

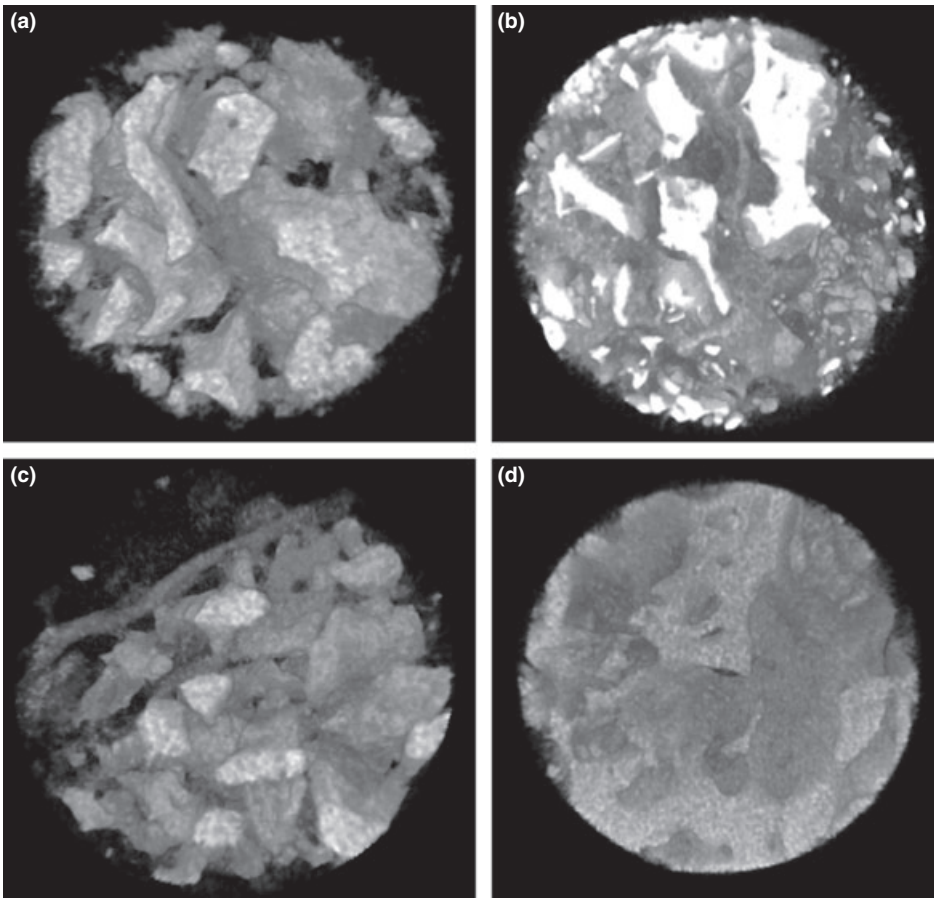

Fig. 3. Three-dimensional model of the volume of interest analyzed in the grafted area for the different groups included in the study. (a) Bio-Oss at 4–5 months, (b) BoneCeramic at 4–5 months, (c) Bio-Oss at 6–8 months, and (d) BoneCeramic at 6–8 months.

Table 2. **Bone morphometric parameters and BMD analyzed in the grafted area by μCT**

Variable	Abbreviation (units)	4–5 months		6–8 months	
		Bio-Oss	BoneCeramic	Bio-Oss	BoneCeramic
Bone volume fraction	BV/TV (%)	37.0 (22.8–56.8)	42.0 (14.4–66.5)	48.2 (21.6–90.0)	43.2 (13.5–67.4)
Bone volume	BV (mm^3)	2.10 (1.30–3.25)	2.40 (0.81–3.77)	2.75 (1.24–5.12)	2.47 (0.78–3.82)
Bone surface	BS (mm^2)	72.6 (57.4–81.4)	78.4 (70.6–94.9)	46.5 (28.2–64.6)	52.3 (35.6–70.0)
Bone specific surface	BS/BV (/mm)	32.8 (23.6–52.9)	32.8 (25.2–86.7)	23.6 (5.5–37.1)	23.9 (17.9–45.9)
Bone surface density	BS/TV (/mm)	12.7 (10.1–14.2)	13.8 (12.5–16.8)	8.17 (4.97–11.3)	9.20 (6.20–12.3)
Trabecular thickness	Tb.Th (mm)	0.166 (0.094–0.185)	0.115 (0.100–0.150)	0.234 (0.159–0.556)	0.185 (0.110–0.220)
Trabecular separation	Tb.Sp (mm)	0.156 (0.108–0.182)	0.130 (0.070–0.250)	0.186 (0.097–0.281)	0.195 (0.100–0.330)
Trabecular number	Tb.N (/mm)	2.45 (2.05–3.14)	3.64 (1.40–4.38)	1.82 (1.42–2.09)	2.12 (1.27–3.36)
Degree of anisotropy	DA	1.28 (1.19–1.40)	1.33 (1.07–1.53)	1.39 (1.19–2.06)	1.27 (1.12–1.49)
Bone mineral density	BMD (g/cm^3)	0.838 (0.570–0.880)	0.920 (0.450–0.970)	0.845 (0.700–0.910)	0.795 (0.580–1.02)

Median (min–max) values are shown.

implants was achieved in all cases independent of the biomaterial used and healing time (Table 1). Thus, no differences were observed in the maxillary bone height before and after MSFE, the torque values or the ISQ values either depending on time or on the biomaterial used. No clinical signs of inflammation were observed during the follow-up, and no implant failures were observed during a follow-up of at least 1 year for the different groups, resulting in a 100% of implant survival.

Bone morphometric parameters and BMD

The quantitation of bone morphometric parameters in the volume of interest (Figs 2 and 3) analyzed by micro-CT analysis is shown in Table 2. No significant differences in the bone morphometric parameters or BMD were observed in the biopsies studied 4–5 months after sinus elevation among the two biomaterials. However, a trend toward higher trabecular thickness was envisaged for the Bio-Oss group. In the samples obtained 6–8 months after sinus elevation, although no statistical significance was reached, higher bone volume fraction and also bone volume was observed for the Bio-Oss group, and this could be related to the tendency of a higher trabecular thickness observed in this group.

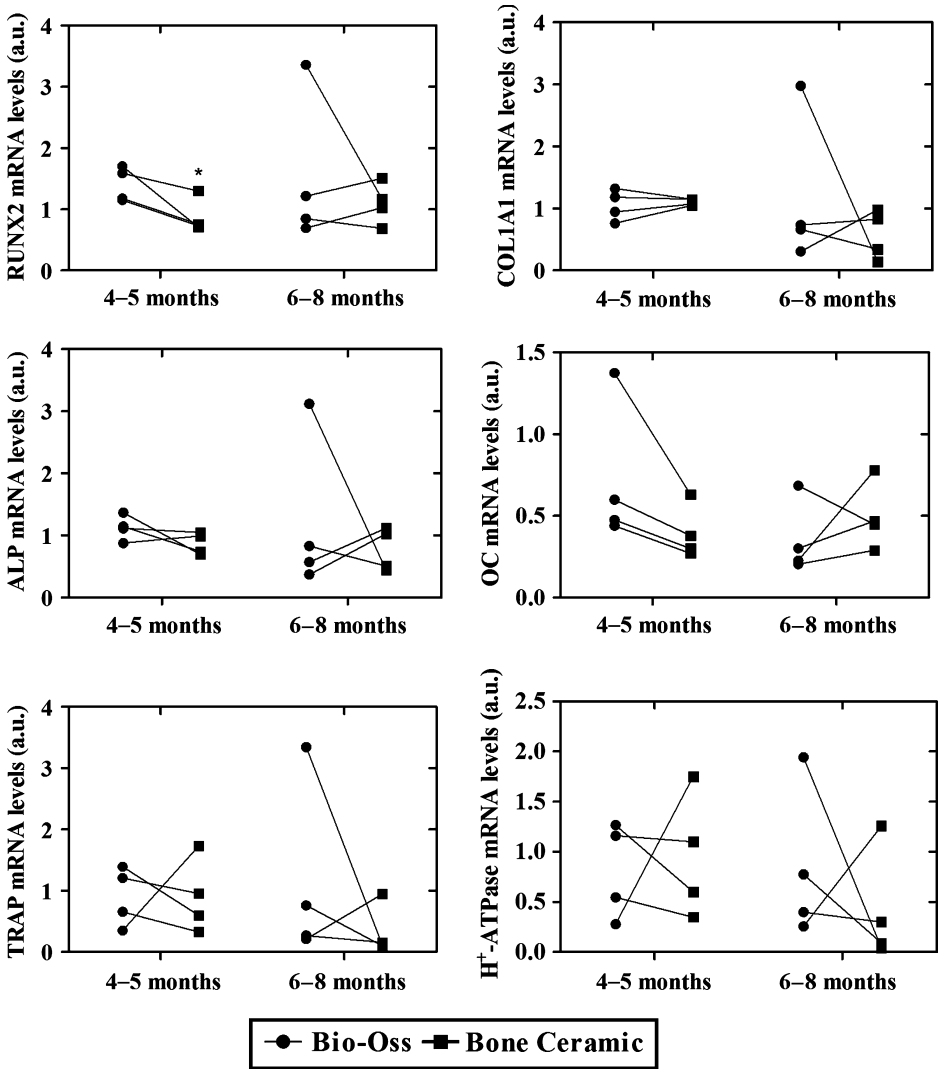

Fig. 4. Expression of bone markers. Data were normalized relative to reference genes (18S rRNA, GAPDH, and ACTBL2). Values represent means ± SEM. Paired *t*-test was performed to assess differences between Bio-Oss and BoneCeramic: **P* = 0.044.

Gene expression of bone and inflammation markers

The regulation of bone specific genes in the grafted bone after MSFE has yet to be addressed. Figure 4 shows relative mRNA levels of bone formation-related genes (COL1A1, RUNX2, ALP, OC) and of bone resorption genes (TRAP and H^+-ATPase). In the group studied 4–5 months after MSFE, a higher expression of the bone forming genes RUNX2 and OC was observed in the biopsies grafted with Bio-Oss compared with those grafted with BoneCeramic, although statistical significance was only reached for RUNX2. No differences were observed for the bone resorption markers TRAP and H^+-ATPase at this time point.

Gene-expression profiles of pro-inflammatory (TNFA, IL6) and anti-inflammatory (IL10) cytokines were also investigated (Fig. 5). No differences in gene expression were observed in samples studied 4–5 months after MSFE. In the 6–8 months group, higher expression levels were observed in the biopsies grafted with BoneCeramic compared with the ones grafted with Bio-Oss, although significance was not reached.

Discussion

Despite the clinical success of Bio-Oss and BoneCeramic has often been corroborated by histologic and histomorphometric findings (Cordaro et al. 2008; Iezzi et al. 2012; Schmitt et al. 2013), this is the first study that evaluates the regulation of bone specific genes in the grafted bone after MSFE. The purpose of our evaluation is to increase the knowledge of the clinician that uses biomaterials in MSFE procedures in the biological events that occur at different healing times and in relation to the graft microstructural characteristics.

The microstructure of Bio-Oss and BoneCeramic particles probably enhance the effectiveness of these materials. Thus, their pore architectural characteristics and mechanical properties have been characterized in an earlier report, having Bio-Oss 42% of porosity and 130 μm of pore size and Bone-Ceramic® 66% porosity and 129 μm of pore size (Sabetrasekh et al. 2011). One of the key features of synthetic graft materials is the level of macro and microporosity that play an important role in vascularization of the bone graft, which, in turn, supports the proliferation and differentiation of osteoblasts and the ingrowth of new bone into the graft (Campion et al. 2011). In fact, previous studies comparing these two biomaterials have concluded that both are suitable for MSFE (Cord-

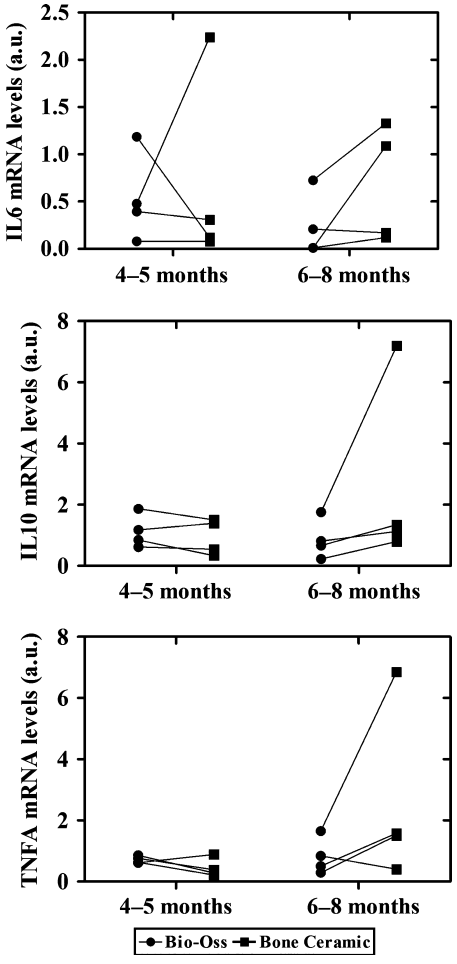

Fig. 5. Expression of inflammation markers. Data were normalized relative to reference genes (18S rRNA, GAPDH and ACTBL2). Values represent means ± SEM. Wilcoxon test was performed to assess differences between between Bio-Oss and BoneCeramic.

aro et al. 2008; Iezzi et al. 2012; Lindgren et al. 2010, 2012a,b; , Lindgren et al. 2009; Schmitt et al. 2013) and have proved that the main difference between both biomaterials is their resorption rate (Cordaro et al. 2008; Iezzi et al. 2012). Bio-Oss degrades very slowly, as particles of this bone graft can be found 4–10 years after sinus floor elevation (Piattelli et al. 1999). Other xenograft used in MSFE have shown a substantial resorption of the biomaterial after 9 months, with a gradual diffusion of calcium ions from the xenograft to the newly forming bone (Hurzeler et al. 1996; Merkx et al. 2003; Raghoebar et al. 2005; Ramirez-Fernandez et al. 2013a,b,a,b; Tong et al. 1998). In contrast, the b-TCP contained in BoneCeramic reabsorbs rapidly (Zijderveld et al. 2005). In our study, we show that both biomaterials are present in the biopsies after 4–5 months, while BoneCeramic could not be distinguished from the newly bone formed after 6–8 months on the micro-CT images. Thus, its higher resorption rate made some differences with Bio-Oss in their morphometric measurements, as shown in the tendency to lower percentage of bone volume. It must be noted that in our study, morphometric measurements and BMD have been done scoring both the bone graft and the new bone formed as a threshold in the microCT analysis could not be set to distinguish both BoneCeramic from the newly formed bone at 6–8 months. There is no evidence whether or not a long-lasting slowly resorbed bone graft is preferable to complete resorption of the bone graft. On one hand, a very low resorption rate may hamper replacement of the graft by new bone; on the other hand, a rapid degradation of the graft may not be concurrent to bone formation, leading to less bone production than graft resorption. In fact, a recent study shows a similar amount of newly formed bone and implant survival rates after 3 years regardless of the biomaterial used (Lindgren et al. 2012a,b).

Despite lack of differences in the clinical outcome, the different resorption behavior of the two selected bone graft might influence the healing process. Here, we have focused on gene-expression levels of bone formation, resorption and inflammation markers to give some insight into the biological events that occur during healing and graft consolidation before implant placement.

Bone formation requires the recruitment, migration, and differentiation of osteogenic cells. Osteoblast differentiation is mediated by the transcriptional factor runx2/Cbfa-1, a master regulator of osteogenic gene expression that is necessary for the osteoblast lineage commitment (Ducy et al. 1997) and, as well, regulates the expression of bone extracellular matrix protein genes that encode for bone sialoprotein, osteocalcin, and collagen type I (Harada et al. 1999). Our results suggest that Bio-Oss was more osteoinductive than BoneCeramic as observed in the increased RUNX2 mRNA levels, which might have led to the induction in the expression of osteocalcin, the more specific bone marker, which is synthesized and secreted exclusively by differentiated osteoblasts at the late stage of cell maturation (Boskey et al. 1998). In fact, osteocalcin has been found as the best predictive marker for osseointegration of titanium implants in an animal model, highlighting the importance of this marker in bone regeneration (Monjo et al. 2013). Thus, our molecular results indicate higher osteoblast differentiation in samples grafted with Bio-Oss after 4–5 months of healing; although these differences are not translated to differences in the bone morphometric parameters, the bone mineral density values, or in the height of the maxillary bone or primary implant stability evaluation, neither these differences are observed after 6–8 months of healing.

In addition to new bone formation, the bone graft has to be resorbed and gradually be replaced by new bone. Several phenotypical markers are characteristic of differentiated osteoclasts including TRAP and the vacuolar type proton ATPase that drives HCl secretion for dissolution of bone mineral (Roodman 1996). Similar expression levels were found for these two markers in both groups. However, although a trend to lower bone volume and bone volume/tissue fraction indicating a higher graft resorption for the BoneCeramic group was observed after 6–8 months of healing. These findings point that the increased graft resorption observed in the BoneCeramic group is mainly mediated by chemical dissolution and not by osteoclast activity, in agreement with earlier observations showing that solved b-TCP particles are phagocyted by multinucleated giant cells (Eggli et al. 1988; Jensen et al. 2006).

Few limitations of the present research should be noted: (i) the limited number of patients especially as they were split over two time points. However, as the study was designed as a split-mouth in which each patient acts as his/her own control, much of the inter-subject variability is removed, resulting in increased study power and reducing the sample size required compared with a parallel-group design (Pandis et al. 2013). (ii) The lack of histological analysis, as it would provide further information on the healing process, including the presence of inflammatory cells, graft and bone resorption, and new bone formation. While histological analyses have been extensively investigated by other authors (Caubet et al. 2011; Cordaro et al. 2008; Frenken et al. 2010; Hallman et al. 2002; Schmitt et al. 2013), this is the first report to study gene expression in biopsies from MSFE. As the molecular analysis implies the disruption of the samples, prior to RNA isolation, the biopsies were evaluated by micro-CT, a more sensitive method than conventional histomorphometry in the evaluation of 3D bone morphometric parameters (Muller et al. 1998). (iii) mRNA expression results might not necessarily correlate with protein levels, the major direct executors of life processes, which might reflect gene function more directly than mRNA. However, an overall positive correlation between mRNA and protein expression levels has been reported (Guo et al. 2008).

Conclusion

In conclusion, our results indicate that when combined with autologous graft in the short-time Bio-Oss is more osteoinductive than BoneCeramic and that the already reported enhanced resorption of BoneCeramic is not mediated by osteoclasts. However, these differences at the molecular level are not translated to important differences in the morphometric parameters and BMD of the grafted bone. Moreover, it seems that with this composite graft we have used (50% autologous graft/50% biomaterial), the bone healing time can be shortened to 4–5 months, compared with the classical 9–12 months waiting time you need with a 100% autologous graft as described by other authors (Hallman et al. 2002; Yildirim et al. 2001). Further investigation will be required to perhaps emphasize an ideal sinus graft for MSFE.

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