Stem cell behavior and biomaterials
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CHAPTER 6

EVALUATION OF A NEW BIPHASIC CALCIUM PHOSPHATE FOR MAXILLARY SINUS FLOOR ELEVATION: MICRO-CT AND HISTOMORPHOMETRICAL ANALYSIS

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Submitted
ABSTRACT
Synthetic biphasic calcium phosphate (BCP) with a hydroxyapatite/β-tricalcium phosphate (HA/β-TCP) ratio of 60/40 (BCP60/40) is successfully used as alternative for autologous bone in patients undergoing maxillary sinus floor elevation (MSFE) for dental implant placement. A high percentage of HA in BCP60/40 may hamper efficient scaffold remodeling, but whether BCP with a lower HA/β-TCP ratio of 20/80 (BCP20/80) is more desirable is still unclear. Osteogenesis and neovascularization are pivotal in effective bone regeneration. We aimed to investigate whether differences exist in osteogenic and/or vasculogenic potential of BCP60/40 and BCP20/80 in patients undergoing MSFE. 20 patients undergoing MSFE were treated with BCP60/40 (n=10) or BCP20/80 (n=10). Bone, graft, and osteoid volumes, number of osteoclasts and blood vessels were determined by micro-Computed Tomography and histomorphometrical analysis of biopsies of the augmented region that was taken 6.5 months postoperatively prior to dental implant placement. Bone and osteoid volumes were higher at the most cranial side of the BCP20/80 biopsies compared to the BCP60/40 biopsies. Graft volumes, number of osteoclasts and blood vessels were similar in both groups. BCP20/80 showed enhanced osteogenic potential in patients undergoing MSFE compared to BCP60/40, due to either a faster bone remodeling rate, or an earlier start of bone maturation in BCP20/80 treated patients. Therefore, BCP20/80 might perform better as a scaffold for bone augmentation in the MSFE model than BCP60/40.

Key words: Bone substitutes, Sinus floor elevation, Morphometric analysis, Guided tissue regeneration, Bone regeneration, Clinical research, Clinical trials
INTRODUCTION

Maxillary sinus floor elevation (MSFE) is a frequently performed surgical procedure to restore insufficient jaw bone height in the posterior maxilla allowing dental implant placement.\textsuperscript{1,2} Autologous bone is the golden standard for clinical bone augmentation in MSFE,\textsuperscript{3} because it has osteoconductive as well as osteoinductive properties, contains osteogenic cells, and does not evoke immunogenic responses. Drawbacks of using autologous bone are for example limited availability of bone grafts, and morbidity at the donor site.\textsuperscript{4} An alternative to the golden standard is biphasic calcium phosphate (BCP) containing hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP). BCP is used as bone substitute material for dental and orthopedic applications. The chemical composition of BCP resembles the inorganic part of the natural bone matrix.\textsuperscript{5} HA is rigid, brittle, and hardly resorbed after application in MSFE, while β-TCP degrades faster and has a different resorption pattern.\textsuperscript{6} Furthermore, mesenchymal stromal cells on β-TCP form more bone than mesenchymal stromal cells on HA,\textsuperscript{7,8} which can be caused by differences in spatial distribution of bone forming cells on HA and β-TCP.\textsuperscript{8} For efficient scaffold remodeling, there should be a proper balance between the resorption time of the scaffold and the timing of new bone formation. BCP with a HA/β-TCP ratio of 60/40 (BCP60/40) represents the slowest resorbing variant of BCP currently used in the clinic, while a bone substitute with 100% β-TCP has the shortest resorption time and may lose its scaffolding properties too early. Several studies have evaluated and compared bone substitutes with different HA/β-TCP ratios against autologous bone or each other.\textsuperscript{3,9-13}

Up to now, two animal studies have been published comparing BCP60/40, BCP with a HA/β-TCP ratio of 20/80 (BCP20/80), and other bone substitutes with autologous bone.\textsuperscript{14,15} One comparative study on BCP with different HA/β-TCP ratios in mandibular bone defects in minipigs showed that BCP20/80 results in similar bone formation as autologous bone after 52 weeks.\textsuperscript{14} This study also showed that BCP60/40 results in similar bone formation as deproteinized bovine bone mineral.\textsuperscript{14} Another comparative study on BCP with different HA/TCP ratios in a calvarial bone defect in rabbits demonstrated that BCP60/40 and BCP20/80 provided more newly formed bone and increased bone density compared to pure β-TCP after 8 weeks.\textsuperscript{15} The same study also showed that BCP60/40 and BCP20/80 exhibit similar bone healing and biodegradation patterns after this period.\textsuperscript{15} BCP60/40 is successfully used as alternative for autologous bone in patients undergoing MSFE for dental implant placement, however, the high percentage of HA in BCP60/40 may hamper efficient scaffold remodeling. Whether BCP20/80 is more desirable to use as an alternative for autologous bone in patients undergoing MSFE for dental implant placement compared to BCP60/40 is still unclear since BCP20/80 has not been tested before in a clinical human model.

A novel bone substitute should offer a framework for regenerating and healing of the host bone as well as formation of blood vessels. Osteogenesis and angiogenesis are tightly coupled processes, and vascular development needs to be induced before osteogenesis can
take place. A comparative study of HA, TCP, and BCP60/40 in subcutaneous tissue under the thin skin muscle of the subscapular region in rats for up to 30 days showed that BCP60/40 results in similar vascularization as the TCP-group after 15 days, whereas the vascularization in the BCP60/40-group is comparable as the HA-group at the end of the study. Whether BCP20/80 enhances vascularization compared to BCP60/40 is still unclear.

Since implantation of BCP with different HA/TCP ratios in patients undergoing MSFE for dental implant placement influences the in vivo tissue reaction, we aimed to investigate whether differences exist in osteogenic and/or angiogenic potential of BCP60/40 or BCP20/80 in patients undergoing MSFE. We hypothesized that the use of BCP20/80 in human MSFE results in an increased bone volume and/or improved bone structure and/or enhanced vascularization compared to the widely used BCP60/40. In this study we report the first comparison of BCP60/40 and BCP20/80 for osteogenic and vasculogenic potential in patients undergoing MSFE, and demonstrate that BCP20/80 showed enhanced osteogenic potential compared to BCP60/40.

MATERIALS AND METHODS

Biphasic calcium phosphate scaffolds
Two types of calcium phosphate scaffolds were used: (1) Straumann® BoneCeramic 60/40 (Institut Straumann AG, Basel, Switzerland), a porous BCP scaffold composed of 60% HA and 40% ß-TCP (BCP60/40), and (2) Straumann® BoneCeramic 20/80 (Institut Straumann AG, Basel, Switzerland), a porous BCP scaffold composed of 20% HA and 80% ß-TCP (BCP20/80). To properly compare material composition without the risk of topographical influences overruling the compositional effects due to differences in manufacturing techniques, the two BCPs were produced by the same company, and had similar pore size (500-1000 µm), microporosity (2%), and porosity (90%).

Patient selection
Twenty patients were included in this study, who were partially edentulous in the posterior maxilla and required dental implants for prosthetic rehabilitation. All patients had an adequate alveolar bone height at the posterior maxilla of at least 3.1 mm (Table 1). Therefore, a preoperative panoramic radiograph was made and carefully examined for contour lines of the maxillary sinus floor and to determine the preoperative bone height at the planned dental implant positions. The average age of the patients was 53±14 years (mean±SD). Four females (54±21 years; mean±SD) and six males (54±10) undergoing MSFE were treated with BCP60/40, and seven females (46±10) and three males (66±9) undergoing MSFE were treated with BCP20/80 (Table 1). The patients included in this study were either non-smokers or moderate smokers smoking less than 10 cigarettes per day. Patients who required horizontal
bone augmentation, as well as patients with specific conditions – such as systemic diseases, drug abuse, heavy smokers, other semi-invasive dental treatments, and/or pregnancy - were excluded from participation in this study. All procedures were performed by one oral surgeon either in the Alrijne Hospital in Leiderdorp, or in the VU University Medical Center, Amsterdam, The Netherlands.

**Table 1.** Patient data. Gender, age, pre-implant bone height, and dental implant position in patients undergoing maxillary sinus floor elevation procedure treated with BCP60/40 or BCP20/80. BCP60/40, biphasic calcium phosphate with a hydroxyapatite/β-tricalcium phosphate ration of 60/40; BCP20/80, biphasic calcium phosphate with a hydroxyapatite/β-tricalcium phosphate ration of 20/80.

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Maxillary sinus floor elevation

All 20 patients underwent a unilateral MSFE procedure as previously described. Briefly, a preoperative clinical photograph was taken (Fig. 1A), and a lateral bony window was prepared and turned inward and upward, the generated cavity within the maxillary sinus was filled with either BCP60/40 or BCP20/80, and the wound was closed with Gore-Tex sutures (W.L. Gore and Associates, Newark, DE, USA) which were removed after 10-14 days. All patients received antibiotic prophylaxis, consisting of 500 mg amoxicillin, 4 times daily starting one day preoperatively and continuing 7 days postoperatively. After a healing period of 5 months post-MSFE, prior to dental implant placement, a panoramic radiograph was made to determine the increase in vertical bone plus bone substitute height at the planned dental implant positions (Fig. 1B).

Dental implant surgery

Six and a half ± 0.9 months (mean±SD) after the MSFE procedure, dental implant surgery was performed under local anesthesia. A crestal incision was made with mesial and distal buccal vertical release incisions. A full-thickness mucoperiosteal flap was raised to expose the underlying alveolar ridge, which was inspected for sufficient bone volume for the intended dental implant placement. Then implant bed preparations were made and biopsies were collected with a hollow Straumann® trephine drill (Institute Straumann AG) with an outer diameter of 3.5 mm and an inner diameter of 2.5 mm, and using sterile saline for copious irrigation. Straumann® dental implants (Institute Straumann AG) with a diameter of 4.1 mm, a length of 10 or 12 mm, and a sand-blasted, large-grit, acid-etched surface were placed in the augmented maxillary sinus. Dental implants were placed in a single-stage surgical procedure, mounted with healing abutments (radiograph in Fig. 1C), and sutured with Gore-Tex sutures. Immediately after dental implant placement, another panoramic radiograph was made to check dental implant placement. The panoramic radiographs taken pre-MSFE, as well as before dental implant placement, were used for morphometric measurements to determine the increase in vertical bone plus bone substitute height at the planned implant positions, using digital software. Calculations were performed with the use of a conversion factor (1.25 x) that adjusted for magnification of the panoramic radiograph. Sutures were removed after 10-14 days. Patients were instructed to avoid loading of the dental implants during the post-implant surgical osseointegration time. After a 3-months osseointegration period, the superstructures were manufactured and placed.

Biopsy analysis

The bone biopsies taken during dental implant surgery with a hollow Straumann® trephine drill were fixated in 4% phosphate-buffered formaldehyde solution (Klinipath BV, Duiven, The Netherlands) for at least 24 h, removed from the drill, transferred to 70% ethanol, and stored
A new BCP for maxillary sinus floor elevation

until use for micro-CT analysis and histomorphometric analysis, as described below. For each patient, one biopsy was selected in the middle of the grafted area to exclude the effect of surrounding bone containing mechanically loaded dental elements and bone near the nasal wall of the maxillary sinus.

Figure 1. Clinical photographs of a maxillary sinus floor elevation procedure via a lateral approach allowing dental implant placement, and their corresponding radiographs to evaluate maxillary sinus and alveolar bone height. (A) preoperative photograph of the maxillary sinus of the patient, (B) maxillary sinus filled with graft material, (C) dental implant placement after 6.5 months.

Micro-CT analysis

Three-dimensional biopsy reconstructions of biopsies kept in 70% ethanol were obtained with a high-resolution micro-CT system PX5-925EA (μCT 40; Scanco Medical AG, Bassersdorf, Switzerland). To this end, we fixed biopsies in synthetic foam, placed vertically in a custom-made polyetherimide holder, and scanned them at an isotropic voxel size of 10 µm, source voltage of 70 kV, and current of 113 µA. Gray values, depending on radiolucency of the scanned material, were converted into corresponding values of degree of mineralization by the analysis software (Scanco Medical AG). The distinction between newly formed bone and graft material was made by using the highest value of the degree of mineralization in the pre-existing sinus floor bone as threshold value. A distinction could be made between the original non-grafted native bone of the residual maxillary sinus floor and the graft material, because the mineralization degree of the graft material was significantly higher than was the mineralization degree of bone. The degree of mineralization was expressed in mg HA/cm³. A low threshold between 650 and 1300 mg HA/cm³ to distinguish bone tissue from connective
tissue and bone marrow, and a high threshold between 1300 and 2500 mg HA/cm³ was defined to distinguish graft material from bone tissue. Using this simple thresholding resulted initially in the detection of a thin layer of non-existing “bone” covering the graft material throughout the grafted area of the sinus. Therefore, a new and so-called “onion-peeling” algorithm (evaluation program no.12; Scanco Medical AG) was used to discriminate between the newly formed bone deposited on the graft material itself. This method peels off voxels from the thin layer of “bone” (as measured with the simple thresholding method) and removes this layer when calcium phosphate is detected within a predefined extent of space. The digital images of the scanned biopsies were analyzed, starting from the caudal side of the biopsy, and continuing toward the cranial side (Fig. 2). Regions of interest (ROIs) of 1 mm² were defined, and to obtain more insight into where the bone was formed within the grafted area, ROIs were numbered in a consecutive sequence starting from the residual sinus floor up to the most cranial part of the biopsy. The transition zone (tz) indicates the first ROI where substantial graft material (> 1% graft volume per total volume) was observed when analyzing the biopsy from the caudal to cranial side. Native bone (nb) is located caudally from the tz. Depending on the length of the biopsy, we divided the ROIs after the tz in region I, region II, and region III. The number of ROIs in region I was 2, in region II it ranged from 0 to 3, and in region III from 0 to 2 (Fig. 2C). These ROIs were analyzed separately by micro-CT and histomorphometry (Fig. 2). For nb, tz, and each ROI, bone volume over total tissue volume (BV/TV) and graft volume over total tissue volume (GV/TV) were calculated, and compared between the BCP60/40 group and BCP20/80 group.

**Histology and histomorphometrical analysis**

After micro-CT scanning and dehydration in ascending alcohol series, the bone specimens were embedded without prior decalcification in low-temperature polymerizing methylmethacrylate (MMA; Merck Schuchardt OHG, Hohenbrunn, Germany). Longitudinal sections of 5 µm thickness were prepared with a Jung K microtome (Reichert Jung, Heidelberg, Germany). Midsagittal histological sections of each biopsy were stained with Goldner’s Trichome to distinguish mineralized bone tissue (green) and unmineralized osteoid (red). The histological sections were also divided in ROIs of 1 mm² for histomorphometric analysis. Identity of the histological sections were blinded during analysis to avoid evaluation bias. Depending on the length of the biopsy, the number of ROIs ranged from 1 to 10. For each separate area of interest, we performed the histomorphometric measurements with a computer using an electronical stage table and a Leica DC 200 digital camera. The computer software used was Leica QWin® (Leica Microsystems Image Solutions, Rijswijk, The Netherlands). Digital images of the sections were acquired at 100x magnification. A demarcation line was indicated between the “native bone” and the regenerated “grafted sinus floor”. Consecutive ROIs of 1 mm² were defined and numbered throughout the whole
biopsy as described under “Micro-CT analysis” (Fig. 2). For nb, tz, and each ROI, osteoconduction (mm bone ingrowth in graft area; tz is not included), bone volume over total tissue volume (BV/TV), graft volume over total tissue volume (GV/TV), and osteoid volume over total tissue volume (OV/TV) were calculated as previously described, and compared between the BCP60/40 group and BCP20/80 group.

Tartrate-resistant acid phosphatase (TRAcP) staining was used to visualize bone resorbing multinuclear cells (osteoclasts) within the biopsy sections. These sections were selected adjacent to biopsy sections that were stained with Goldner’s Trichrome method. TRAcP staining was performed according to a standardized protocol. The number of TRAcP osteoclasts in each section was measured at 200x magnification with the same computer software and microscope used for quantification of osteoconduction, BV/TV, GV/TV, and OV/TV in the Goldner-stained sections. Each tissue section used for TRAcP staining of osteoclasts was divided in consecutive optical areas of 1 mm² and numbered throughout the whole biopsy as described under “Micro-CT analysis” (Fig. 2), overlapping with the optical areas in the Goldner’s Trichome-stained sections as closely as possible. Within each area all red-colored multinuclear cells were identified as TRAcP-positive osteoclasts, and for each section the total number of TRAcP-positive osteoclasts was calculated, and compared between the BCP60/40 group and BCP20/80 group.

The number of blood vessels was used to determine the vasculogenic potential within the biopsy sections. Blood vessels were counted at 200x and/or 400x magnification with the computer software and microscope used for quantification of BV and OV in the Goldner’s Trichome-stained sections. The size of the blood vessels was calculated as the total blood vessel area in µm². Depending on the size, blood vessels were divided in small (0-400 µm²), medium (401-1200 µm²), or large (>1200 µm²), and compared between the BCP60/40 group and BCP20/80 group.
Methods of micro-computed tomography and histomorphometrical analysis of bone biopsies. Bone biopsies are removed from the hollow burr and analyzed by using (A) micro-computed tomography, and (B) histomorphometry. (C) Schematic diagram for micro-computed tomography and histomorphometrical analysis. Biopsies were analyzed separately, starting from the caudal side of the biopsy, and continuing toward the cranial side. Regions of interest (ROIs) of 1 mm² were defined, and to obtain more insight into where the bone was formed within the grafted area, ROIs were numbered in a consecutive sequence starting from the sinus floor up to the most cranial part of the biopsy. The transition zone (tz) indicates the first ROI where substantial graft material (> 1% graft volume per total volume) was observed when analyzing the biopsy from the caudal to cranial side. Native bone (nb) is located before the tz (ROI 1). Depending on the length of the biopsy, we divided the ROIs after the tz in region I, region II, and region III. The number of ROIs in region I was 1 to 2, in region II it ranged from 0 to 3, and in region III from 0 to 2. For nb, tz, and each ROI, bone volume over total tissue volume (BV/TV) and graft volume over total tissue volume (GV/TV) were calculated, and compared between the BCP60/40 group and BCP20/80 group.
**Statistical analysis**
Data were obtained from 20 patients. Data are presented as mean±SD. Differences in osteogenic and/or vasculogenic potential in patients undergoing MSFE treated with BCP60/40 or BCP20/80 were tested with Mann-Whitney U-test. Differences were considered significant p<0.025. Statistical analysis was performed using IBM® SPSS® Statistics version 21 software package (SPSS Inc., Chicago, IL, USA) and GraphPad Prism® 5.0 (GraphPad Software Inc., La Jolla, USA).

**Study approval**
The use of calcium phosphate ceramics (allograft bone substitutes) for MSFE is an accepted grafting method. Since the study involved CE-marked devices (calcium phosphates) being used for their intended purpose, to be used as carrier material for bone augmentation in MSFE procedures, no specific regulatory approval from a medical ethical committee was required. The study was performed in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from all patients before the study-related procedures were undertaken.

**RESULTS**

**Clinical evaluation and implant survival**
During and after the MSFE procedure no adverse events and/or complications were reported. The augmentation height increased after the MSFE procedure, and the increase was similar in the BCP60/40 group (6.1±1.7 mm (mean±SD)) and the BCP20/80 group (6.5±1.6 mm). Wound healing went uneventful, and no implants were lost after implantation.

**Micro-CT analysis**
Micro-CT analysis was performed to determine bone volume over total tissue volume (BV/TV; Fig. 3A), and graft volume over total tissue volume (GV/TV; Fig. 3B). Bone volume decreased from the sinus floor towards the cranial side of the biopsies, and increased at the most cranial side. The percentages of BV/TV in the grafted area were similar for all regions in the BCP60/40 group and BCP20/80 group (Fig. 3B). In region I, BV/TV in the BCP60/40 group was 12.5±8.9% (mean±SD), and in the BCP20/80 group 20.4±16.3%. The percentage of BV/TV in region II was 1.7±3.5% (mean±SD) for the BCP60/40 group, and 2.5±3.4% for the BCP20/80 group. In region III, BV/TV in the BCP60/40 group was 8.1±12.0% (mean±SD), and 14.2±11.6% in the BCP20/80 group. No differences were observed in percentage of graft volume between both BCP groups. GV/TV remained constant throughout the more cranial side of the biopsies from both BCP groups (BCP60/40; region I: 12.6±4.9% (mean±SD), region
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Il: 11.6±7.5%, region III: 12.1±7.6% and BCP20/80; region I: 13.4±7.8%, region II: 14.1±3.9%, region III: 11.4±5.5%; Fig. 3B).

Figure 3. Micro-computed tomography analysis of biopsies taken after maxillary sinus floor elevation with BCP60/40 or BCP20/80. (A) Bone volume over total tissue volume (BV/TV), and (B) graft volume over total tissue volume (GV/TV) as assessed by micro-computed tomography analysis retrieved after maxillary sinus floor elevation with BCP60/40 or BCP20/80. BV/TV and GV/TV were assessed for native bone, transition zone, region I, region II, and region III. Values are mean±SD (n = 7-10). *Significantly different from BCP60/40, p < 0.05. BCP60/40, biphasic calcium phosphate with a hydroxyapatite/β-tricalcium phosphate ratio of 60/40; BCP20/80, biphasic calcium phosphate with a hydroxyapatite/β-tricalcium phosphate ratio of 20/80; BV, bone volume; TV, total volume; GV, graft volume; nb, native bone; tz, transition zone; R1, region I; RII, region II; RIII, region III.

Histology and histomorphometrical analysis
Histomorphometrical analysis was performed to determine osteoconduction (mm bone ingrowth in graft area; tz is not included), bone volume over total tissue volume (BV/TV), graft volume over total tissue volume (GV/TV), osteoid volume over total tissue volume (OV/TV), the number of TRACP-positive stained osteoclasts, and the number of blood vessels. The bone ingrowth, or so-called osteoconduction, from the native bone towards the cranial side of the biopsy was similar in both BCP groups (BCP60/40: 1.5±1.2 mm (mean±SD); BCP20/80: 1.8±1.8 mm; Fig. 4A). This indicates that the rate of osteoconduction was 0.2 mm per month for the BCP60/40 group, and 0.3 mm per month for the BCP20/80 group.
Histomorphometrical analysis of biopsies taken after maxillary sinus floor elevation with BCP60/40 or BCP20/80. (A) Bone ingrowth, so-called osteoconduction, in mm was determined in biopsies taken after maxillary sinus floor with BCP60/40 or BCP20/80 from the maxillary sinus floor towards the cranial side of the biopsies. (B) Bone volume over total tissue volume (BV/TV), graft volume over total tissue volume (GV/TV), and (C) osteoid volume over total tissue volume (OV/TV) as assessed by histomorphometrical analysis retrieved after maxillary sinus floor elevation with BCP60/40 or BCP20/80. BV/TV, GV/TV, and OV/TV were assessed for native bone, transition zone, region I, region II, and region III. Values are mean±SD (n = 6-10). *Significantly different from BCP60/40, p < 0.05. BCP60/40, biphasic calcium phosphate with a hydroxyapatite/β-tricalcium phosphate ratio of 60/40; BCP20/80, biphasic calcium phosphate with a hydroxyapatite/β-tricalcium phosphate ratio of 20/80; BV, bone volume; TV, total volume; GV, graft volume; OV, osteoid volume; nb, native bone; tz, transition zone; Rl, region I; RII, region II; RIII, region III.

Similar to the bone volume results obtained using micro-CT analysis, histomorphometrical analysis revealed that bone volume decreased from the sinus floor towards the cranial side of the biopsies, and increased again at the most cranial side. The percentage of BV/TV in the grafted area was similar in the BCP60/40 group and BCP20/80 group for region I (BCP60/40: 10.0±9.4% [mean±SD], BCP20/80: 14.7±13.0%), and for region II (BCP60/40: 0±0%; BCP20/80: 2.2±4.8%; Fig. 4B). At the most cranial side of the biopsy, region III, more bone formation
(p=0.022) was found in the BCP20/80 group (10.1±8.4% (mean±SD)) than in the BCP60/40 group (0.4±0.7%; Fig. 4B). Similar to the graft volume results obtained using micro-CT, no differences were observed in percentage of graft volume between BCP60/40 and BCP20/80 groups. GV/TV remained constant throughout the more cranial side of the biopsies from both BCP groups for region I (BCP60/40: 28.1±11.8% (mean±SD); BCP20/80: 31.5±4.8%); region II (BCP60/40: 35.7±9.7%; BCP20/80: 34.4±11.0%), and region III (BCP60/40: 35.0±14.4%; BCP20/80: 30.7±13.3%; Fig. 4C). Osteoid volume showed a similar trend as bone volume measurements (Fig. 4B,D). The osteoid volume decreased from the sinus floor towards the cranial side of the biopsies, and increased at the most cranial side. OV/TV was higher (p=0.014) in region III in the BCP20/80 group (0.8±0.8% (mean±SD)) than in the BCP60/40 group (0±0.1%; Fig. 4D).

Clusters of TRAcP-positive osteoclasts were found at the site of newly formed bone as well as native bone (Fig. 5A). The number of TRAcP-positive osteoclasts from sinus floor towards the cranial side of the biopsies was similar in the BCP60/40 group and BCP20/80 group (Fig. 5B). In region I, the number of TRAcP-positive osteoclasts in the BCP60/40 group was 6.2±8.4 (mean±SD), and in the BCP20/80 group 1.2±1.7. The number of TRAcP-positive osteoclasts in region II was 5.7±6.6 for the BCP60/40 group, and 1.8±2.5 for the BCP20/80 group. In region III, the number of TRAcP-positive osteoclasts in the BCP60/40 group was 4.3±6.2, and 1.2±2.2 in the BCP20/80 group.

The number of blood vessels was counted to study blood vessel formation in biopsies taken after maxillary sinus floor elevation with BCP60/40 or BCP20/80. Depending on the size, blood vessels were defined as small (0-400 µm²), medium (401-1200 µm²), or large (>1200 µm²) blood vessel. No differences in number of small, medium or large blood vessels could be observed in the BCP20/80 group compared to the BCP60/40 group (Table 2).
Figure 5. Osteoclast activity in biopsies taken after maxillary sinus floor elevation with BCP60/40 or BCP20/80. (A) To visualize and calculate the number of osteoclasts within the biopsies, consecutive sections were tartrate-resistant acid phosphatase (TRAcP)-stained (red) in biopsies taken after maxillary sinus floor elevation with BCP60/40 or BCP20/80. Number of TRAcP-positive osteoclasts was assessed for native bone, transition zone, region I, region II, and region III. Values are mean±SD (n = 7-10). *Significantly different from BCP60/40, $p < 0.05$. BCP60/40, BCP with a hydroxyapatite/ß-tricalcium phosphate of 60/40; BCP20/80, BCP with a hydroxyapatite/ß-tricalcium phosphate ratio of 20/80; TRAcP, tartrate-resistant acid phosphatase; BV, blood vessel. Black arrows: TRAcP-positive osteoclasts. Magnification: 200x.

Table 2. Histomorphometrical analysis of number of blood vessels (%) in biopsies taken after MSFE with BCP60/40 or BCP20/80. Values are mean (n = 6-7). *Significantly different from BCP60/40, $p < 0.05$. BCP60/40, biphasic calcium phosphate with a hydroxyapatite/ß-tricalcium phosphate ratio of 60/40; BCP20/80, biphasic calcium phosphate with a hydroxyapatite/ß-tricalcium phosphate ratio of 20/80; blv, blood vessel.

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DISCUSSION
This study aimed to investigate whether differences exist in osteogenic and/or vasculogenic potential of BCP60/40 and BCP20/80 in patients undergoing MSFE. We found that (i) the bone ingrowth (in mm) from the native bone towards the cranial side of the biopsy was similar in both BCP groups; (ii) BV/TV was higher in the BCP20/80 group than the BCP60/40 group at the most cranial side of the biopsy; (iii) GV/TV was similar in both BCP groups in all regions; and (iv) OV/TV was higher in the BCP20/80 group than in the BC60/40 group at the most cranial side of the biopsy; (v) the number of TRAcP-positive osteoclasts was similar in both BCP groups; (vi) no differences in number of small, medium, or large blood vessels could be observed in both BCP groups. Therefore, our results showed more bone formation and osteoid deposition in patients who underwent MSFE with BCP20/80 than patients with BCP60/40, demonstrating that BCP20/80 might perform better as a scaffold for bone augmentation in the MSFE model than BCP60/40.

In this study, BCP60/40 and BCP20/80 were manufactured by the same company to exclude possible differences as a result of manufacturing. BCP60/40 and BCP20/80 showed formation of new bone when placed in the maxillary sinus. In a bilateral sinus elevation procedure, the bone ingrowth speed in the BCP60/40 group was between 0.5 and 1.0 mm per month,\textsuperscript{21} while the rate of osteoconduction from the native bone towards the cranial side of the biopsy in this study was lower for both BCP groups. The difference in bone ingrowth speed might be explained by the different method used for numbering of the regions of interest or by the time point of dental implant placement. In the present study, dental implants were placed 6.5 months after the MSFE procedure, while in our previously published bilateral sinus elevation procedure study the dental implants were placed 3 to 8 months after the MSFE procedure.\textsuperscript{21} HA is rigid, brittle, and hardly resorbed, while ß-TCP degrades faster and has a different resorption pattern.\textsuperscript{6} The lower the HA/ß-TCP ratio, the more dissolution of BCP.\textsuperscript{22} Based on the lower HA/ß-TCP ratio in BCP20/80, a higher resorption rate in BCP20/80-grafted biopsies than in BCP60/40-grafted biopsies was expected. This would allow more space for osteoconduction with new bone ingrowth as well as blood vessel formation in the BCP20/80 group. The micro-CT and histomorphometrical analysis showed similar bone formation from the native bone towards the cranial side in the biopsies of both BCPs, but although micro-CT analysis showed a similar trend, only histomorphometrical analysis resulted in significant more bone formation in the BCP20/80 group at the most cranial side of the biopsy. The same pattern as found for bone formation was observed for osteoid deposition in the grafted areas. Active bone formation at the cranial side of the biopsies has also been observed in our clinical trial, where patients undergoing bilateral MSFE, were treated with ß-TCP or BCP60/40 with and without freshly isolated autologous stromal vascular fraction (SVF) of adipose tissue containing multipotent cells.\textsuperscript{23} In that study, more active bone formation was observed at the cranial side of the biopsies with SVF than without
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SVF, which might be due to transdifferentiation of adipose stem cells within the SVF towards bone-forming cells, or caused by increased adipose stem cell paracrine recruitment of progenitor cells from the lateral bony window capping the bone reconstruction compartment and/or the human Schneiderian membrane of the maxillary sinus. It has recently been shown that this membrane, which is lifted during MSFE to insert the graft material, contains a cell population with potential for osteogenic differentiation. Since in our study we did not include multipotent cells on the scaffolds, the observed high bone volumes in the most cranial side of the biopsies may be due to osteoconduction from the cranial side from this membrane or lifted lateral bony window. The noted divergence between the levels of osteoconduction between both BCP types, with the HA/ß-TCP ratio being the only parameter being different, suggests that a higher TCP content may positively contribute to the osteoconduction rate. This is in line with a previous study showing that TCP has a relatively late-occurring remodeling phase, supports cell ingrowth, and promotes osteogenic differentiation of osteoprogenitor cells. Moreover, the findings in our study support the suggestion to keep the lateral bony window of the maxillary sinus in situ.

The results from the micro-CT and histomorphometrical analysis showed a similar trend in remaining graft volumes in both BCP groups, but a lower percentage of graft volume was observed by micro-CT analysis than our histomorphometrical analysis. A similar discrepancy was found in our previous studies, and can be explained by the fact that micro-CT analysis comprises three-dimensional measurements, while histomorphometrical analysis comprises two-dimensional measurements. During histological preparation, graft material is dissolved, which gives the false impression that the granules are solid while being porous. Despite the differences in data obtained by micro-CT and histomorphometrical analysis, they are complementing each other very well; micro-CT analysis is based on differences between structures and mineralized compounds, while histomorphometrical analysis provides information on cells and soft tissue organization and structure.

We found similar numbers of TRAcP-positive osteoclasts in the BCP60/40 group and the BCP20/80 group in all regions, but lower numbers of osteoclasts were observed in the BCP20/80 group than the BCP60/40 group for the grafted area. The micro-CT and histomorphometrical analysis showed more bone formation and subsequent bone remodeling in the BCP20/80 group, thus explaining the lower number of osteoclasts we found at the time point of our analysis. This suggests that cells in the BCP20/80 biopsies have a higher bone-forming capacity, and cells in the BCP60/40 biopsies have a higher bone-resorbing capacity.

Bone is highly vascularized, and vascular development needs to be induced prior to osteogenesis. Both BCP60/40 and BCP20/80 in patients undergoing MSFE apparently had sufficient and comparable angiogenic potential to form new bone. In contrast, in our clinical trial where 6/10 patients underwent bilateral MSFE and were treated with either ß-TCP or
BCP60/40 with and without freshly isolated autologous SVF of adipose tissue, bone formation could be linked to increased blood vessel formation (unpublished data).\textsuperscript{23,28} The SVF consists of a heterogeneous mixture of cells including endothelial cells and lineage-committed progenitor cells.\textsuperscript{29,30} This suggests that freshly isolated autologous SVF of adipose tissue may trigger increased blood vessel formation which will allow nutrient diffusion and waste removal crucial for cell recruitment, survival, proliferation, differentiation, and tissue remodeling. It seems that freshly isolated autologous SVF of adipose tissue increase nutrient delivery, enabling more cell penetration towards the interior of the grafted area, and thus faster tissue remodeling compared to our current study without cells. SVF fits in a so-called one-step surgical procedure.\textsuperscript{28} Our results suggest that SVF-seeded BCP20/80 might perform better than SVF-seeded BCP60/40 in the MSFE model during a one-step surgical procedure. Further preclinical \textit{in vivo} studies are needed before clinical implantation can be considered.

A limitation of the present study is that we compared two BCPs by using biopsies from unilateral sinus floor elevation in different patients. Since the anatomical structure of the maxillary sinus varies between patients, bilateral sinus floor elevation models would be more appropriate to compare two BCPs. In future studies, BCP60/40 and BCP20/80 should be compared in bilateral sinus floor elevation models to eliminate the variation in anatomical structure of the sinus between patients.

In summary, the use of BCP20/80 in human MSFE resulted in a higher bone and osteoid volume at the cranial side of the biopsies than BCP60/40, but no differences in vascularization could be observed. Therefore, we conclude that BCP20/80 showed enhanced osteogenic potential in patients undergoing MSFE compared to BCP60/40, due to a faster bone remodeling rate, or an earlier start of bone remodeling in BCP20/80 treated patients. This demonstrates that BCP20/80 might perform better as a scaffold for bone augmentation in the MSFE model than BCP60/40.
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