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Synthetic bone substitute material comparable with xenogeneic material for bone tissue regeneration in oral cancer patients: First and preliminary histological, histomorphometrical and clinical results

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Shahram Ghanaati[1,2], Mike Barbeck[1,2], Jonas Lorenz[1,2], Stefan Stuebinger[3], Oliver Seitz[1], Constantin Landes[1], Adorján F. Kovács[4], Charles J. Kirkpatrick[2], Robert A. Sader[1]

[1]Department for Oral, Cranio-Maxillofacial and Facial Plastic Surgery, Medical Center of the Goethe University Frankfurt, Frankfurt am Main, Germany
[2]REPAIR-Lab, Institute of Pathology, University Medical Center, Johannes Gutenberg University, Mainz, Germany, [3]Center for Applied Biotechnology and Molecular Medicine, Zurich, Switzerland, [4]Private Practice, Nauheim, Germany

Address for correspondence:
Dr. Shahram Ghanaati, Department for Oral, Cranio-Maxillofacial and Facial Plastic Surgery, Medical Center of the Goethe University Frankfurt, Frankfurt am Main, Germany
E-Mail: shahram.ghanaati@kgu.de

ABSTRACT

Background: The present study was first to evaluate the material-specific cellular tissue response of patients with head and neck cancer to a nanocrystalline hydroxyapatite bone substitute NanoBone (NB) in comparison with a deproteinized bovine bone matrix Bio-Oss (BO) after implantation into the sinus cavity. **Materials and Methods:** Eight patients with tumor resection for oral cancer and severely resorbed maxillary bone received materials according to a split mouth design for 6 months. Bone cores were harvested prior to implantation and analyzed histologically and histomorphometrically. Implant survival was followed-up to 2 years after placement. **Results:** Histologically, NB underwent a higher vascularization and induced significantly more tartrate-resistant acid phosphatase-positive (TRAP-positive) multinucleated giant cells when compared with BO, which induced mainly mononuclear cells. No significant difference was observed in the extent of new bone formation between both groups. The clinical follow-up showed undisturbed healing of all implants in the BO-group, whereas the loss of one implant was observed in the NB-group. **Conclusions:** Within its limits, the present study showed for the first time that both material classes evaluated, despite their induction of different cellular tissue reactions, may be useful as augmentation materials for dental and maxillofacial surgical applications, particularly in patients who previously had oral cancer.

Keywords: Bio-oss, bone matrix, bone substitutes, foreign body reaction, hydroxyapatite, NanoBone, oral cancer, sinus floor augmentation

INTRODUCTION

Patients with atrophic upper alveolar bone who have been successfully treated for oral cancer should experience rapid oral rehabilitation, especially because their overall life expectancy is clearly reduced relative to the healthy population.[1] Rapid oral rehabilitation would increase their quality-of-life, given that mastication and articulation are both impaired by tumor resection-related tissue loss and scar formation. Until now, many clinicians have been hesitant to use materials other than autologous bone in these patients. This reluctance to use other materials results from the known osteoinductive, osteogenic and osteoconductive characteristics of autologous bone transplants.[2] In addition, there is a concern that alloplastic materials may not be adequately integrated within the tissue of former oral cancer patients because of their potentially impaired (bone) metabolism.

However, especially in patients who previously had oral cancer, additional elective interventions, like harvesting of autologous bone grafts from the iliac crest to augment the atrophic alveolar crest, create additional burdens as the procedure can increase donor site morbidity. In some cases, this technique requires general anesthesia and can be accompanied by complications for patients.[3]

To the best of our knowledge, no study to date has investigated the fate of synthetic bone substitute materials for bone regeneration within the sinus cavity of patients who previously suffered from oral cancers and were successfully treated. Recently, our workgroup has thoroughly investigated the tissue reactions and clinical outcome of a fully synthetic nanocrystalline hydroxyapatite (HA) bone substitute material (NanoBone® [NB], Artoss, Germany) embedded in a matrix of structured silica gel in numerous studies in animals and humans.[4-7] In an animal experimental trial, NB was implanted subcutaneously in Wistar rats to assess the vascularization and biodegradation of the biomaterial histologically and histomorphometrically. Although, there was no sign of new bone formation in this ectopic tissue, the graft material showed lower vessel density and vascularization than other synthetic bone substitute materials. In addition, relatively few tartrate-resistant acid phosphatase TRAP-positive and TRAP-negative multi-nucleated giant cells and macrophages were present on the surface of the material within the implantation bed and mononuclear cells such as macrophages were only minimally involved in the degradation of the material.[4] These findings revealed the dominance of multinucleated cells in the degradation of the evaluated materials. The absence of osteoinductivity and the above mentioned inflammatory cell response pattern, i.e., the primary role of multi-nucleated giant cells in the degradation of the materials, were also observed in muscle tissue of larger animals, like goats.[5]

In a further clinical trial of sinus augmentation in humans, NB showed undisturbed integration within the designated sinus area after 6 months. New bone formation was observed in all parts of the biopsy. Similar to the previous finding in animal soft-tissues, multi-nucleated giant cells were detected.[6] This study was, however, not able to determine whether the bone formation observed in all parts of the biopsy was related to an osteoinductive or an osteoconductive process. Accordingly, another clinical investigation was conducted to analyze the potential osteoinductive property of NB through histological observation of samples obtained 3 and 6 months after sinus augmentation.[7] New bone formation was observed to occur from the residual bone of the sinus cavity. Continuous bone formation was seen within all parts of bone biopsies performed 6 months after bone substitute implantation, whereas new bone formation was only detected in the caudal 2/3 parts of the biopsies obtained 3 months after material augmentation.[7] The overall assessment of the animal and clinical studies discussed above led to the conclusion that NB induces a similar cellular inflammatory pattern in the three different species investigated while contributing to new bone formation through osteoconductive rather than osteoinductive effects. Adverse tissue reactions, i.e., exaggerated inflammation or implant rejection, to NB were not observed in any of the studies. Therefore, this material is suitable for application in healthy human tissue.

The aim of the present study was to use NB as a potential alternative to autologous bone grafting also in patients who previously had oral cancer. A clinical split-mouth trial was performed, in which the deproteinized bovine bone substitute Bio-Oss® (BO, Geistlich, Wolhusen, Suisse), which is structurally and chemically similar to human extracellular bone matrix, was used as a control for NB. BO is obtained from two different bone types, i.e., cortical and cancellous bone of bovine origin and has been described in numerous investigations as a highly biocompatible, reliable and safe bone substitute for sinus and ridge augmentation and repair of periodontal defects.[8-11] Furthermore, different studies have demonstrated the integration of the xenogeneic bone substitute within its implant bed, which indicates that this material does not undergo notable cellular degradation.[9] In the present study, the impact of the different biological characteristics and physicochemical structures of the synthetic and biologic bone substitutes on bone formation in the augmented sinus cavity of former cancer patients was assessed on a histological, histomorphometrical and clinical level after 6 months and 2 years.

MATERIALS AND METHODS

Study design

The study was approved by the ethics commission of the University of Frankfurt am Main and conducted according to the fifth revision of the World Medical Association Declaration of 2000 in Helsinki. All patients gave informed consent prior to the sinus augmentation procedure.

In the present study, the two bone graft materials (BO and NB) implanted in the maxillary sinus of patients with cancer history was histologically examined 6 months after augmentation. Furthermore, dental implants placed in the augmented region were followed-up clinically and radiologically after 2 years.

Eight partly or completely edentulous patients (five women, three men) from the Department for Oral, Cranio-Maxillofacial and Facial Plastic Surgery, Frankfurt am Main, underwent a sinus elevation procedure.

Patients were computer generated and randomly assigned to one of the two biomaterial groups. According to the CONSORT statement of 2010, investigators of the histological, histomorphometrical and the clinical analysis were aware of the allocated arms, whereas the outcome assessors and data analysts were kept blinded to the allocation.

Patients who were included in this study had to be free of tumor recurrence for at least 8 months with a reduced dentition in the molar region of the upper jaw on both sides and a reduced bone amount in these regions. Further, the implant site had to be free form infection and included patients had an adequate oral hygiene.

Exclusion criteria were medical and general contraindications for a surgical procedure, chronic alcohol abuse, chronic liver- or kidney-disease, metabolic diseases (e.g., diabetes mellitus or

osteoporosis), bisphosphonate therapy or heavy smoking habits of more than 20 cigarettes per day.

The average age of the study group was 69.5 years (ranges from 57 to 80 years). According to the TNM- classification of malignant tumors, one patient underwent only tumor resection, whereas the remaining seven patients underwent intra-arterial chemotherapy with cisplatin prior to surgery to reduce the extent of the tumor. Of these seven patients, neck dissection was unavoidable in six because of their TNM classification. Two of the six patients received post-operative radiotherapy. A detailed overview of tumor localization, TNM classification and therapy is given in Table 1.

Basic anamnestic data including medical history and smoking habits were collected prior to tumor surgery and the present sinus augmentation study. All included participants were in relatively good general health and free of any sinus pathology.

Sinus augmentation was performed at an average of 22.1 months (minimum 8 months, maximum 32 months) after initial treatment of oral malignancy [Table 2]. Tumor clearance was confirmed clinically and radiographically at follow-up consultations. The height of the alveolar crest in the prospective implant site was reduced in all participants (less than 5 mm, with a mean of 3.06 ± 1.02 mm). Placement of dental implants followed on average 6 months (ranges from 5 months to 7 months) after the augmentation procedure [Table 2].

Surgical procedure

In all 8 patients, the augmentation procedure was conducted under general anesthesia. A standard access through crestal incision was performed by the surgeon to develop a vestibular-based mucoperiosteal flap according to previously described methods.[5,6] Using the Piezosurgery® device (Mectron, Carasco, Italy), antrostomy was performed to expose the Schneiderian membrane. No perforation of the sinus membrane occurred and no sinus pathology was observed. In one case, shallow Underwood septal formations presented to the surgeon, but did not interfere with the surgical procedure.

Through elevation of the sinus membrane, the subantral space was enlarged to receive the synthetic NB or the xenogeneic BO. Prior to the surgical intervention, a randomization process was used to decide, which bone substitute was used on each side. After the recipient area was prepared, both bone substitutes were mixed with blood obtained from the surgical site and a dense package was inserted in the generated cavity. Additional autogenous bone blocks or chips were not necessary. The surgical site was covered with a native collagen membrane (Biogide®, Gestlich, Wolhusen, Suisse). Primary wound closure was achieved with absorbable tension-free single sutures.

In a second surgical procedure, dental implants (CAMLOG© ScrewLine, Camlog Biotechnologies, Basle, Switzerland) were placed on average after 6 months (ranges from 5 to 7 months). Implant dimensions were determined prior to surgery based on radiography of the designated implant site. As patients in our study were partly or completely edentulous, implants were also inserted in non-grafted regions. In total, 58 dental implants were placed in the upper and lower jaws, of which 24 dental implants were inserted into the grafted maxilla regions. A detailed itemization

Table 1: All patients enrolled in the present study with location and classification of their malignancy and the individual therapy

Patient	Age	Malignancy	Localisation	TNM- classification	Therapy
1	62	SCC	Gingiva region 35-43	ypT2N0R0	i.a. Cisplatin; tumor resection; neck dissection
2	80	SCC	Palatum durum and molle region 27, Gingiva region 36-38	pT2G1R0	Tumor resection
3	69	SCC	Anterior floor of mouth	ypT2N1R0	i.a. Cisplatin; tumor resection; neck dissection; radiotherapy
4	64	SCC	Left palatum molle	ypT2N2cM0	i.a. Cisplatin; tumor resection; neck dissection
5	57	SCC	Right side lateral floor of mouth and right side retromolar space lower jaw	ypT2N2bR0	i.a. Cisplatin; tumor resection; neck dissection; radiotherapy
6	76	SCC	Right side retromolar space lower jaw	ypT4N0R0	i.a. Cisplatin; tumor resection; neck dissection
7	75	SCC	Left side lateral floor of mouth	ypT1N0M0	i.a. Cisplatin; tumor resection
8	73	SCC	Gingiva region 34-38	ypT0N2bM0	i.a. Cisplatin; tumor resection, neck dissection

SCC = Squamous cell carcinoma; i.a.=Intra-arterial

Table 2: An overview of the temporal progress from the first diagnosis of malignancy to the date of augmentation and implantation and the representative mean times

Patient	First diagnosis	Mean time	Date of augmentation	Mean time	Date of implantation
1	Feb 05	32 m	Oct 07	6 m	Apr 08
2	Sep 06	8 m	May 07	6 m	Nov 07
3	Apr 05	24 m	Apr 07	5 m	Sep 07
4	Mar 05	23 m	Apr 07	7 m	Nov 07
5	Apr 05	24 m	Apr 07	6 m	Oct 07
6	Dec 04	28 m	Apr 07	7 m	Nov 07
7	Oct 06	12 m	Oct 07	--	refused implantation
8	Apr 05	26 m	Jun 07	6 m	Dec 07

m=Month

Table 3: A detailed list of the number and sites of placed implants in the augmented and non-augmented regions

Patient	Number of implants and sites (upper jaw)	Number of implants and sites (lower jaw)	Implants placed in BO augmentation	Implants placed in NB augmentation
1	6: 13,15,16,23,25,26	2:31,41	2	2
2	6: 13,15,16,21,23,26	4: 33,32,43,42,	1	1
3	6: 13,15,16,23,25,26	0	2	2
4	6: 13,14,16,23,24,26	4: 33,32,43,42	1	1
5	6: 11,15,16,24,25,26	4: 31,32,41,42	2	2
6	6: 14,15,16,24,25,26	2:31,32	2	2
7	refused implantation	refused implantation	refused implantation	refused implantation
8	6: 13,15,17,23,25,26	0	2	2
Total	42 implants	16 implants	12	12

of implant numbers and sites are shown in Table 3.

In five edentulous patients, two biopsies were taken from each augmented region. One biopsy of each site was taken from two other patients with remaining teeth in the molar region. One patient refused implantation as he did not desire further surgery. Using trephine burrs (3 mm), 24 cylinder-shaped bone biopsies were obtained from the augmented sinus regions through a crestal approach prior to implantation.

The second procedure was performed on average 6 months after implant placement. Implant exposure was achieved using the roll flap technique and a healing abutment was subsequently incorporated. For prosthetic rehabilitation, removable dentures were prepared 3 weeks after implant exposure.

In both surgical operations, Augmentin was started intraoperatively via intravenous application and continued orally for 10 days post-operatively. Chlorhexidine 0.2% as a mouth rinse and 400 mg of ibuprofen were also prescribed.

Bone grafting substitutes

NB®

NB® is a fully synthetic bone substitute material that is composed of HA crystallites with an average size of 60 nm. According to previous investigations, the HA granules are embedded in a matrix of structured silica gel.[12,13] In the manufacturing process, sintering of the nanocrystalline HA can be avoided by using the sol-gel technique with temperatures below 700°C. Pores with sizes varying from 5 to 50 nm were detected within the silica gel. The large internal surface area of the nanoporously structured bone substitute (up to 84 m^2/g) is the result of numerous open links of the silica gel, which interacts in loose connections with the HA crystals. The macroscopic structure of the bone substitute can be described as a fir cone with an average length of 2 mm, an average diameter of 0.6 mm and a porosity of 60-80%.

BO®

BO® is a xenogeneic graft material that consists of deproteinized bone mineral of bovine origin. The material characteristics of BO, including the processing and preparation of the graft material, were previously described.[8] With regard to the risk of disease transmission, organic components are removed during manufacturing by a chemical extraction process. Granules have a diameter varying from 0.25 to 1.0 mm and are sintered at 600°C, which results in a porosity of 70-75% with pore sizes ranging from a few nanometers to 1,500 nm. BO seems to be chemically and physically similar to human extracellular bone matrix and has been widely reported to constitute an effective bone graft matrix.[8-10]

Scanning electron microscopy

The materials were examined using an AMRAY 1810 T scanning electron microscope (SEM). The inner surface was measured by the Brunauer-Emmet-Teller method using an ASAP 2000 surface and porosity analyzer (Micromeritics, Norcross, GA, USA). Nitrogen 5.0 was used as the measuring gas and the samples were degassed for 16 h under vacuum.

Tissue preparation and histology for human bone biopsies

In total, 24 biopsies were harvested from 24 implants placed in the augmented maxillary sinus for analysis of bone-biomaterial interactions. All biopsies were analyzed histologically as previously described.[4-7] All biopsies were harvested 6 months after implantation, simultaneously with the placement of the implants. The explants were then fixed in 4% neutral buffered formalin for 24 h and decalcified in 10% Tris-buffered ethylenediaminetetraacetic acid (Carl Roth, Karlsruhe, Germany) at 37°C for 10 days. Subsequent dehydration was achieved in a series of increasing alcohol concentrations followed by xylol. All biopsied tissues were subsequently embedded in paraffin and sections with a thickness of 3-5 µm were cut in the longitudinal plane along the sagittal axis using a microtome (Leica, Wetzlar, Germany). To analyze the central portion of the bone cores by light microscopy, sections on eight consecutive slides were prepared from each biopsy. The slides were stained as follows: The first slide was stained with hematoxylin and eosin (H and E), the second was stained with Masson-Goldner's trichrome and counterstained with Weigert's iron hematoxylin and the third slide was stained with FastGarnet GBC Base solutuin and counterstained with Mayer's hematoxylin**.** According to a previously described method, the fourth slide was used to identify osteoclast-like cells by histochemical staining with TRAP.[4,14]

Furthermore, the consecutive fifth and sixth slides were subjected to immunohistochemical detection of vessels using a human CD31 antibody as well as detection of the TRAP5 enzyme. In the staining pre-treatment, the slides were deparaffinized in four portions of xylene and hydrated in two portions of a decreasing series of ethanol followed-by two portions of demineralized water. Heat-induced epitope retrieval for 30 min followed

processing steps described above. For blocking of endogenous peroxidase, a solution of 3% H_2O_2 in demineralized water was applied for 30 min. Subsequently, staining was performed with primary antibodies against the TRAP5 enzyme (LIFESPAN Biosciences, LS-C18195/11703) with a dilution of 1:40 and against CD31 (DAKO, M0823, clone C70A) with a dilution of 1:50 for 30 min in a humid chamber. The antibodies were then detected using the Dako REAL™ EnVision™ detection system Peroxidase/3,3′-Diaminobenzidine, Rabbit/Mouse-Kit (Dako, Glostrup, Denmark) and visualized by DAB. Counterstaining with Mayer's hemalaun was then applied. After dehydration by increasing ethanol concentrations up to 100% and a final treatment with xylene, the slides were coated with mounting medium and covered with coverslips. The seventh and eighth slides of every biopsy were used as negative controls for immunohistochemical staining in the absence of the primary antibody.

Qualitative/histological analysis

Histopathological evaluation was conducted as previously described by the authors SG and MB in accordance with established and published methods.[4,6,15,16] The implantation bed and peri-implant tissue were examined by evaluating the tissue-bone substitute interaction. Characteristics such as signs of fibrosis, necrosis and hemorrhage; the occurrence of neutrophils, plasma cells, lymphocytes, multinucleated giant cells, macrophages and TRAP-positive osteoclast-like cells as well as the level of vascularization were evaluated to describe the inflammatory response induced by the biomaterials. Microphotographs were obtained using a Nikon DS-Fi1 digital camera and a digital sight control unit (Nikon, Tokyo, Japan).

Quantitative/histomorphometrical analysis

The histomorphometrical analysis was performed following a standardized study protocol and using a research scanning microscope in combination with NIS-Elements software (Nikon, Tokyo, Japan).[7,15] Briefly, images of the total implantation beds ("total scans"), i.e., of the bone substitutes and their corresponding peri-implant sinus tissue, were digitized using a DS-Fi1/Digital camera connected to an Eclipse 80i histological microscope (Nikon, Tokyo, Japan) that was equipped with an automatic scanning table (Prior, USA). The resulting image data had a × 100 magnification. To analyze the vascularization and giant cell activity of the implant area, the CD31- and TRAP-stained slides were digitized for histomorphometrical measurements.

The vascularization within the implantation beds was analyzed separately by marking the vessels with the NIS-elements "Annotations and Measurements" tool. The software allows measurement of the area (in µm²) and mean vessel diameter (in µm). Vessel density was calculated based on the total number of vessels with respect to the total area of the specific implantation bed (vessels/mm²). The percent vessel area was calculated by summing the area of all vessels in the total implantation area. For each slide, the number of vessels/mm² and the total vessel area were determined.

The corresponding TRAP-stained slide was digitized and used to count the number of giant cells, i.e., TRAP-negative and TRAP-positive multi-nucleated giant cells, using the NIS-Elements "count" tool. These cells were counted manually and the number of each cell fraction was calculated with respect to the total implantation area of the slides (cell number/mm²).

Implant 2-year follow-up examination

To determine the clinical success of the applied graft materials, the state of the dental implants inserted in the augmented maxilla was evaluated clinically and radiographically 2 years after implant placement.

Seven of eight patients with augmentation of the sinus area received dental implants in a second surgery 6 months after augmentation, whereas one patient (no. 7) refused the placement of implants in both jaws for personal reasons. Another patient (no. 3) dropped out of the follow-up group after 1 year, as she preferred postoperative care close to her home. At this point in time, the implants were stable with no signs of peri-implant infection or inflammation. Patient no. 4 died of pneumonia. Thus, the patient population for the 2-year follow-up examination consisted of five patients who received implants in the augmented area. Overall, 42 implants were available for examination, of which 18 were placed in the augmented area and 24 in natural bone.

All patients were examined clinically by the authors SG and JL. General health as well as extra- and intra-oral constitution was observed before the implant was assessed.

With regard to tumor anamnesis and the specific requests of the patients, standardized hard and soft-tissue indices such as the gingival bleeding index were considered to be not convincing, as the quality and quantity of hard and soft-tissue were restricted by resective tumor surgery and radiation. Therefore, parameters for implant success were determined according to indices previously described by Neukam and Esser[17] [Table 4].

These indices include survival of the implant; absence of pain, discomfort or dysaesthesia; manually detectable implant mobility; and peri-implant infection or putrid secretion. Additional radiographic analysis was performed using orthopantomogram (OPG) or computed tomography, in comparison with images already recorded for the postoperative tumor staging procedure.

Analysis of radiographic images can verify clinical parameters [Tables 1-4] and allow potential peri-implant osteolysis to be detected.

Statistical analysis

Quantitative data are shown as the mean ± standard deviation (SD) and a one-way univariate analysis of variance accompanied by LSD- (least significant difference) *post-hoc* assessment was used to compare groups using SPSS 16.0.1 software (SPSS Inc., Chicago, IL, USA). Differences were considered significant (*) at

Table 4: Success metrics according to Neukam *et al*.[17]

1.	No persisting subjective discomfort as pain, foreign substance feeling or dysaesthesia
2.	No peri-implant infection with putrid secretion
3.	No mobility of the implant
4.	No peri-implant osteolysis

$P < 0.05$ and highly significant (**) at $P < 0.01$. SigmaPlot 11.0 software (SigmaPlot, Systat Software Inc., Erkrath, Germany) was applied for calculating graphs.

RESULTS

Qualitative SEM results

In this first analysis step the overall morphology and surface morphology of both materials was evaluated using SEM at two magnifications (×25 and ×2000) to visualize the physical material characteristics.

NB®

The NB granules showed the described fir cone-like structure with areas of thin and thick diameter [Figure1:a1]. Furthermore, small and large granules were observed. The surface analysis showed a relatively smooth shape and no pores were observable at high magnification [Figure1:a2].

BO®

In contrast to the synthetic bone substitute, the bovine material showed a trabecular-like structure with invaginations and pores of different sizes [Figure 1:b1]. Furthermore, rills that were lined up with the direction of the trabecular were visible. The analysis at high magnification revealed a rough and irregular surface with apparent fiber-like structures [Figure 1:b2].

Qualitative histology of the analyzed extracted bone cores

NB®

Six months after augmentation of the sinus cavity, the nanocrystalline bone substitute NB was well-integrated within the surrounding tissue covered by newly generated trabecular bone tissue (BT) [Figure 2:a1 and 2:a2]. The intergranular spaces were filled by cellular granulation tissue with fibroblasts, lymphocytes, monocytes and macrophages as well as a high number of blood vessels [Figure 2:a1]. Parts of the surface area that were not covered by bone were encased by multi-nucleated giant cells [Figure 2:a1, 2:a2 and 2:a3]. Analysis of the multi-nucleated giant cells through histochemical TRAP staining revealed that these cells could be divided into osteoclast-like TRAP-positive cells and TRAP-negative multi-nucleated giant cells [Figure 3:a1]. The TRAP-positive multi-nucleated giant cells were visibly dominant and covered almost the entire surface of the NB granules throughout the total implantation bed. In addition, immunohistochemical TRAP5 enzyme detection revealed TRAP-5-positive multi-nucleated giant cells on the surface of granules that were not covered by new bone [Figure 3:a2].

BO®

The bovine bone substitute BO was well-integrated within the surrounding host tissue. The granules were also embedded in newly formed trabecular BT, whereas the hard tissue occupied the major part of the bone substitute surfaces [Figure 2:b1]. In contrast to the HA-based material, the granule surface area that was not hedged by bone showed low adherence of multi-nucleated giant cells and was mainly covered by mononuclear cells [Figure 2:b1 and 2:b2]. Furthermore, the intergranular connective tissue showed a low level of granulation tissue with a lower amount of cells in combination with a comparably low fraction of matrix fibers and vessels. Cellular elements such as fibroblasts, monocytes and macrophages as well as a very low number of lymphocytes were observable within the surrounding tissue [Figure 2:b1 and 2:b2]. Signs of osteogenesis were often visible at the surfaces of the material, where active osteoblasts were located [Figure 2:b3].

TRAP staining confirmed that only a small proportion of the BO surface area was covered by multi-nucleated giant cells. Most of these cells were TRAP-negative [Figure 3:b1]. These few multi-nucleated giant cells showed signs of TRAP-5 expression [Figure 3:b2].

Comparative histomorphometrical analysis of the fractions within the extracted bone cores

Tissue distribution

The histomorphometrical analysis of the extracted bone cores focused on the distribution of connective tissue, new BT and remaining bone substitute. The samples of the study group augmented with NB showed values of 53.87 ± 5.12% for connective tissue, 21.85 ± 5.96% for newly formed bone and 24.28 ± 3.26% for the remaining bone substitute [Figure 4]. The values in the BO group were 34.14 ± 4.45% for connective tissue, 25.73 ± 7.94% for newly formed bone and 40.13 ± 3.53% for the remaining bone substitute [Figure 4]. Further statistical analysis revealed a significantly higher fraction of the remaining bone substitute in the BO group (**$P > 0.01$) than in the NB group. The fraction of connective tissue was significantly lower in the BO group than in the NB group (***$P > 0.001$). No significant differences were observed for BT formation between the two groups [Figure 4]. In addition, significantly more BO than newly formed BT was found in the implantation beds of the BO group (*$P > 0.05$) [Figure 4].

Vascularization of the implantation bed

Vessel density

This was analyzed by relating the number of vessels within the implantation bed to an area of 1 mm^2 (number of vessels/mm^2) of the implantation bed. The vessel density was significantly higher in biopsies from NB augmentation sites than in biopsies from BO augmentation sites (**$P > 0.01$) [Figure 5a]. Accordingly, 13.32 ± 2.64 vessels/mm^2 were observed within the implantation bed of NB biopsies compared with 6.17 ± 1.38 vessels/mm^2 in BO biopsies [Figure 5a].

Percent vascularization

A significantly higher percent vascularization was observed for the implantation of NB relative to that of BO (***$P > 0.001$) (2.66 ± 0.78 vs. 0.86 ± 0.07%) [Figure 5b].

Total amount of multi-nucleated giant cells within the implantation bed

The number of these cells within all parts of the implantation bed was related to 1 mm^2 of the total area (number of cells/mm^2). A significantly higher amount of multi-nucleated giant cells was observed in the implantation bed of NB-implanted sites than in the implantation bed of BO-implanted sites (***$P > 0.001$) (50.40 ± 7.16 vs. 16.37 ± 1.72

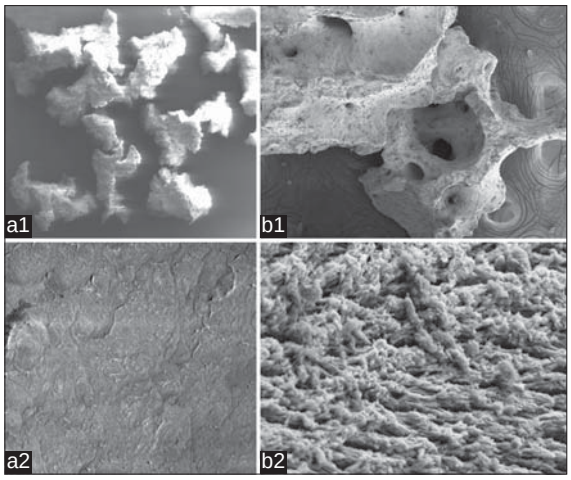

Figure 1: Scanning electron microscope images of the analyzed bone substitute materials. a1 and a2 display the shape and the surface structure of the synthetic bone substitute NanoBone®, whereas b1 and b2 show these characteristics for the xenogenic material Bio-Oss®. Note the fir cone-like shape and the smooth surface pattern of NB in contrast to the trabecular-like structure of BO, which has a rough surface texture (upper row: ×25 magnification, scale bar = 1 mm; lower row: ×2000 magnification, scale bar = 20 μm)

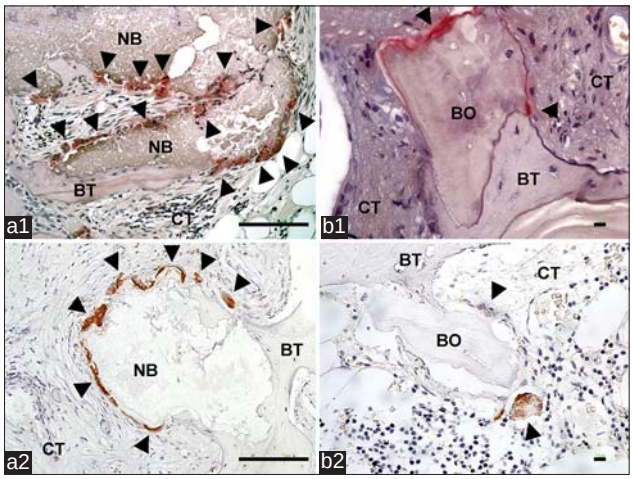

Figure 3: The histochemical and immunohistochemical detection of tartrate-resistant acid phosphatase, which is mainly expressed by surface-adherent multi-nucleated giant cells (arrow heads). Although the surfaces of the NanoBone® granules (NB) that point at the peri-implant connective tissue were covered completely by TRAP-positive giant cells (a1), these areas only sporadically contained these cells in the case of the BioOss® (BO) granules (b1) (BT = bone tissue) (a: ×100 magnification, scale bar = 100 μm; b: ×200 magnification, scale bar = 10 μm). In addition, the immunohistochemical TRAP-staining (a2, b2) supported the above-mentioned findings (a2: ×100 magnification, scale bar = 100 μm; b2: ×200 magnification, scale bar = 10 μm)

cells/mm²) [Figure 6a].

Biomaterial-associated TRAP-positive and TRAP-negative multi-nucleated giant cells

The distribution of biomaterial-associated TRAP-positive and TRAP-negative multi-nucleated giant cells for both materials was quantitatively analyzed by relating the number of cells to 1 mm² (number of cells/mm²) of the implantation bed. The

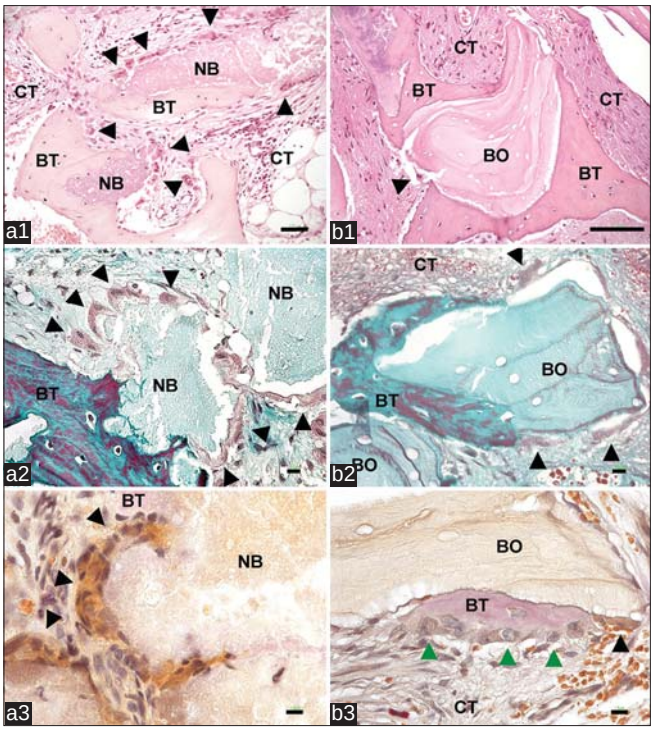

Figure 2: The tissue reaction to the two analyzed bone substitute materials. Within the NB-implantation bed the material-tissue interface that was not covered by bone was almost completely populated by multi-nucleated giant cells (arrow heads) (a1-a3); (a1: H and E staining, ×100 magnification, scale bar = 100 μm); (a2: Masson-Goldner staining, ×400 magnification, scale bar = 10 μm); (a3: Fast garnet GBC staining, ×600 magnification, scale bar = 10 μm). The granules of BioOss® (BO) were integrated within connective tissue and showed a comparable less expressed granulation tissue (b1). Granule surfaces were mainly covered by BT and only small surface areas were bordered by connective tissue (b1 and b2). Within these areas, the material-tissue interfaces contained mostly mononuclear cells and only a few multi-nucleated giant cells (arrow heads) were detected (b1 and b2). Signs of osteogenesis were often visible at the surfaces of the material, where active osteoblasts were located (green arrow heads) (b3). (b1: H and E staining, ×200 magnification, scale bar = 100 μm) (b2: Masson-Goldner staining, ×400 magnification, scale bar = 10 μm); (b3: Fast Garnet GBC staining, ×600 magnification, scale bar = 10 μm)

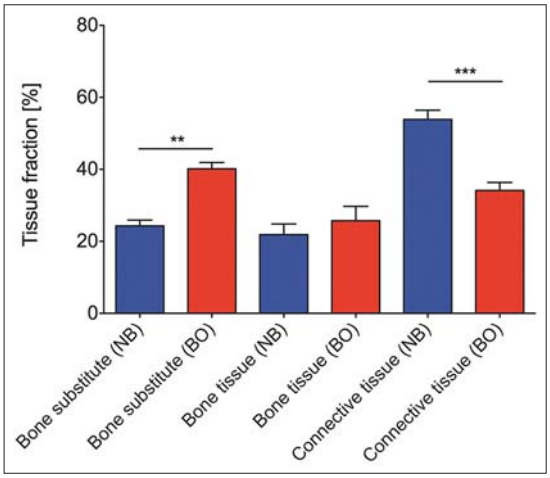

Figure 4: The histomorphometrical analysis of the tissue distribution within the implantation beds of the two analyzed bone substitute materials, i.e., the measurements of the contained new bone tissue, connective tissue and remaining amount of the materials (**/*** = statistical significance)

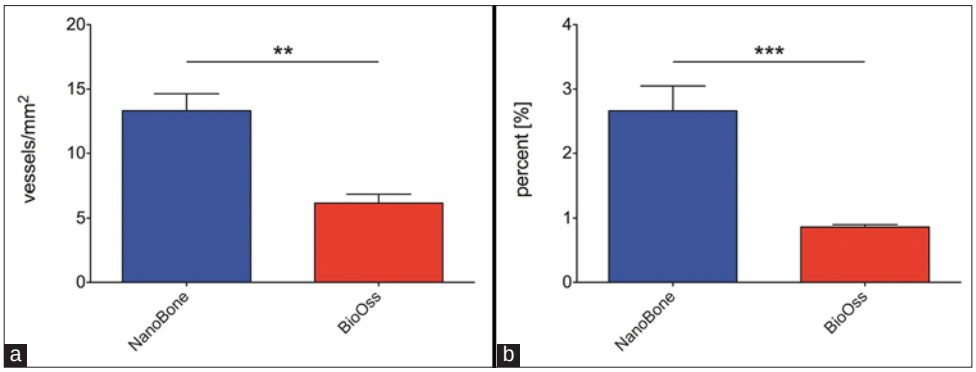

Figure 5: The histomorphometrical analysis of the vascularization of the implantation beds of the two analyzed bone substitutes. (a) Vessel density, (b) Percentage vascularization (**/*** = statistical significance)

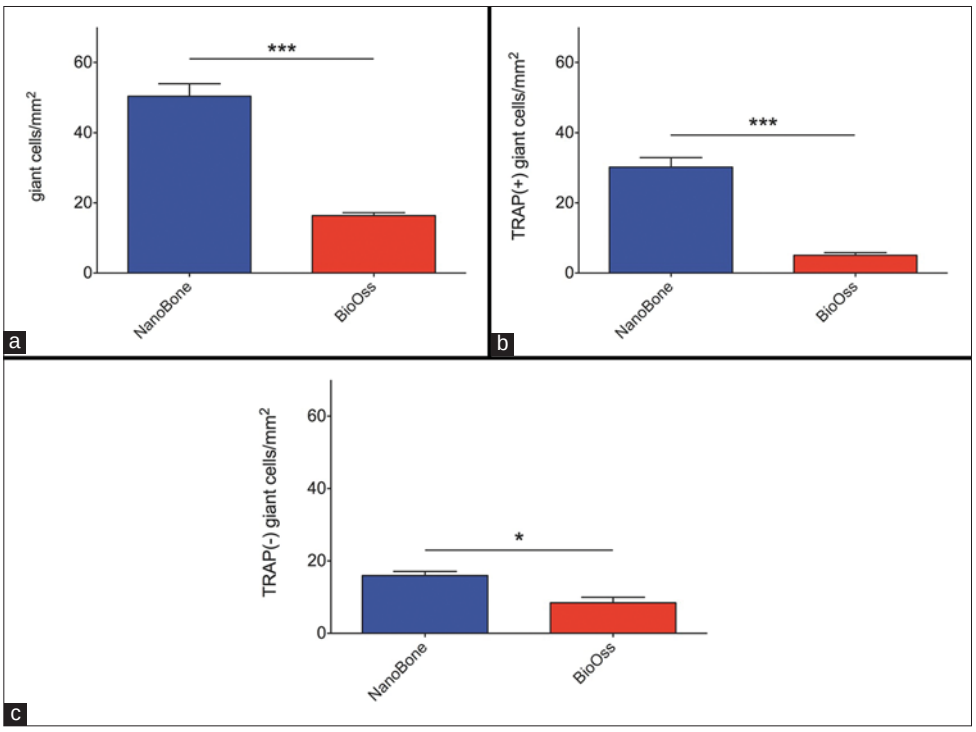

Figure 6: a) The histomorphometrical analysis of the total amount of material-adherent multinucleated giant cells, b) Associated with the analyzed bone substitute materials and both of its subforms: The tartrate-resistant acid phosphatase-positive, c) TRAP-negative giant cells. */**/*** = statistical significance

number of TRAP-positive multi-nucleated giant cells located on the bone substitute surfaces was significantly higher in biopsies from NB augmentation sites than in biopsies from BO augmentation sites (***$P > 0.001$) (30.23 ± 5.41 vs. 5.09 ± 1.45 cells/mm²) [Figure 6b]. Furthermore, a significant difference was also established for biomaterial-associated TRAP-negative multinucleated giant cells within the two implantation beds (*$P > 0.05$) (15.97 ± 2.28 vs. 8.45 ± 3.04 cells/mm²) [Figure 6c].

Clinical and radiological evaluation of the inserted implants after 2 years

Eight patients with tumor anamnesis received augmentation of the subantral space with BO and NB in a split-mouth trial. All patients were free of tumor recurrence by the time of the 2-year follow-up.

Five patients received 18 implants in the augmented area and could therefore be included in the follow-up examination as three patients dropped out of the clinical follow-up group after successful augmentation for different reasons [Section "Implant 2-year follow-up examination" and Table 5]. The distribution of inserted implants in sinuses augmented with BO and NB was 1:1. Accordingly, in each group, nine implants were followed-up 2 years after implantation.

The emphasis of this study was on the implants placed in the augmented region. Inserted implants were examined after 2 years based on the previously described methods of Neukam and Esser, i.e., evaluation of implant position/stability, subjective discomfort,

Table 5: The results of the clinical follow-up investigation regarding the failure of the implants in total and according to the different bone substitute materials, as well as the persisting subjective discomfort, peri-implant infection, implant mobility and peri-implant osteolysis (−=Non-existing, +=Existing)

Patient	Implant loss total	Implant loss BO augmentation	Implant loss NB augmentation	Persisting subjective discomfort	Peri-implant infection	Implant mobility	Peri-implant oseolysis
1	0	0	0	-	-	-	-
2	0	0	0	-	-	-	-
3	Follow-up refused	Follow-up refused	follow up refused	Follow-up refused	Follow-up refused	Follow-up refused	Follow-up refused
4	deceased	deceased	deceased	deceased	deceased	deceased	deceased
5	0	0	0	-	-	-	-
6	1	0	1	-	-	-	-
7	Refused implantation	Refused implantation	Refused implantation	Refused implantation	Refused implantation	Refused implantation	Refused implantation
8	0	0	0	-	-	-	-
Total	1	0	1	-	-	-	-

peri-implant infection, implant mobility and peri-implant osteolysis.[17]

Implants in situ

In the study group augmented with NB, eight of nine examined implants remained *in situ* and stable at the follow-up investigation 2 years after placement. One implant failed, which represents a failure rate of 11.1%. The failed implant was placed in a patient with remaining teeth in the posterior region because of peri-implantitis. All conservation techniques, such as surface cleaning and laser treatment, were attempted but were unsuccessful. The prosthetic denture was adapted and contributed to a functional and satisfying esthetic condition for the patient.

In the study group augmented with BO, all nine inserted implants remained stable at the follow-up investigation 2 years after placement. Thus, the survival rate for implants placed in areas augmented with BO was 100%.

Clinical appearance

Subjective discomfort, peri-implant infection or implant mobility were not detected for any of 17 remaining implants. At least one patient from the NB group mentioned discomfort with his denture after 2 years; however, this discomfort was not caused by the implants. The discomfort was immediately addressed, followed-by an evaluation of the extension and function of the denture.

Radiological assessment

OPG-based radiography after 2 years was performed by the authors (SG and JL) to evaluate potential peri-implant osteolysis as a sign of advanced peri-implantitis.

None of the in-place implants demonstrated obvious peri-implant osteolysis. All implants were in contact with the bone throughout the whole length, except for the first torsion of the implant. No vertical or apical bone defects were present.

A detailed radiological investigation of the augmentation and implantation including three dimensional bone formation analysis and bone density measurement will follow in a separate publication of our work group.

Implants in non-augmented regions

All 24 implants inserted in non-augmented regions remained *in situ* and stable. No signs of peri-implant infection or osteolysis were observed.

DISCUSSION

Many xenogeneic and synthetic bone substitutes are commercially available with different physico-chemical properties, but little is known about their specific ranges of application. One point that has to be distinguished is the health condition of the patients. Bone metabolism and tissue regeneration potential can be disrupted by the presence of different diseases and different pharmaceutical agents. Therefore, understanding the tissue reaction to bone substitute materials is essential for their specific clinical application. Such an understanding requires the analysis of involved cells and the recognition of their contribution to the material-specific tissue reaction and should lead to the synthesis and the selection of appropriate grafting materials.

In the present study, the tissue reaction to a synthetic and a bovine-based bone substitute material was comparatively investigated after implantation into the sinus cavity of tumor patients with squamous cell carcinoma who had undergone radical tumor surgery accompanied by pre-operative intra-arterial chemotherapy and partially postoperative combined radio-chemotherapy. The elements and the extent of the tissue-material-interaction as well as the outcome of BT regeneration were assessed by means of established histological, histomorphometrical and clinical methodologies.[7,14,15] The focus of the present study was to outline the material integration pattern and to detect the cellular inflammatory pattern involved in biodegradation of the materials as well as the extent of implantation bed vascularization.

The qualitative and quantitative histological tissue analysis of the present study showed that NB induced the formation of a well-marked granulation tissue, which was accompanied by a significantly higher number of multinucleated giant cells, when compared with the mild inflammatory tissue reaction observed

within the implantation bed of BO. The multi-nucleated giant cells within the implantation bed of both materials could be divided into TRAP-positive and TRAP-negative cells and originated from the soft-tissue covering the implanted material, which is assumed to derive from the elevated sinus cavity membrane (Schneiderian membrane). In both experimental groups, TRAP-positive and TRAP-negative multi-nucleated giant cells were observed at the interface between the bone substitute material and the granulation tissue, whereas none of these cells was detectable on the surface of the new BT in the biopsies. This cell type is believed to constitute the final state of phagocytic cells such as macrophages and is known to have the capacity of releasing lytic enzymes such as TRAP into the subcellular compartment between the biomaterial and the cell membrane.[18]

Although the exact differentiation pattern of this cell type has not yet been clarified, the increased occurrence of these multi-nuclear cells in combination with a distinct granulation tissue and a higher level of inflammation suggest that the observed TRAP-negative and TRAP-positive multinucleated giant cells within the implantation bed may be classical "foreign body giant cells" involved in the degradation of both material groups. Thus, the presence of these cells may reflect the effort of the organism to degrade the bone substitute material. Consequently, the process within the implantation bed can be considered to be an inflammatory condition, in which the organism tries to eliminate the "foreign body". In case of BO, which is a naturally derived material, fewer multi-nucleated giant cells were observed, most likely because of structural and chemical mimicry of the autogenous organic bone matrix. Within the implantation bed of NB, which is a fully synthetic bone substitute material with physico-chemical characteristics different from those of the inorganic bone matrix, an increased number of multi-nucleated giant cells were observed.

In agreement with our findings, NB has induced a comparable tissue reaction in three different species: Rats (subcutaneous tissue),[4] goats (muscle tissue)[5] and humans (sinus cavity of healthy human patients).[6,7] In an additional *in vivo* study, a mononuclear cell response was observed after implantation of bovine-based collagen membranes,[16,19] whereas multi-nucleated giant cell formation was observed for silk fibroin-based micro-nets.[20]

The present study also found that the material's physico-chemical characteristics influenced the extent of the implantation bed vascularization, as NB induced a significantly higher vascularization relative to BO. These results suggest that in the case of NB, the organism requires a more extensive vascular network to support the biodegradation of the material. The implantation of BO, in contrast, induced less vascularization, which could be related with fewer requirements for biodegradation, i.e., increased biocompatibility. A well-vascularized connective tissue provides transport pathways for the mononuclear cells such as cells of the macrophage line and other inflammatory active cells, i.e., lymphocytes and granulocytes, into the implantation bed to support biodegradation. In the case of NB, mononuclear cell-based resorption and degradation seemed to be insufficient, thus requiring macrophages to fuse to multi-nucleated giant cells to provide enhanced degradation and resorption of the synthetic material.[21] Similar to macrophages, multi-nucleated giant cells are modulators of the tissue reaction to materials and are able to produce (vascular endothelial growth factor),[4,15,22] which plays a key role in angiogenesis and is thus involved in vessel sprouting in wound areas.[23]

Furthermore, the increased granulation tissue within the implantation bed of NB and the increased degradation of NB may result in the increase of connective tissue observed in this group when compared with the BO group. This connective tissue may be used by the organism to "fill the gaps" that remain after the implanted material is degraded. In the BO group, granules showed minimal biodegradation. These tissue reactions demonstrate clear differences for a synthetic versus a natural material.

The multi-nucleated giant cells within the sinus cavity may also be considered to be osteoclasts because of the surrounding bony microenvironment. These cells fulfill osteoclast-specific criteria as they possess multiple nuclei and can be detected on the surface of the bone substitute material. According to *in vitro* experiments and several *in vivo* bone model studies,[24-27] these cells contribute to the material's degradation in the framework of "creeping substitution," which forms the basis of new bone formation.[28] Under these circumstances, released calcium ions among other substances are assumed to trigger new bone formation.[29] In this study, the methods used for detection of multinucleated giant cells, i.e., the histochemical detection of the TRAP enzyme family as well as the immunohistochemical detection of the TRAP5 enzyme group, were not able to assign this cell type to the osteoclast family or to the foreign body giant cell type. To the best of our knowledge, there is no immunohistochemical antibody that allows the detection of TRAP5b, which is a specific marker molecule for osteoclasts.[30] The histochemical detection method used in this study stains both subforms of TRAP, i.e., the osteoclastic TRAP5b molecule as well as the TRAP5a molecule, which is thought to be related to inflammatory processes of mono- and multi-nuclear cells, such as macrophages and foreign body giant cells.[30] However, it may be possible to detect the differentiation of these cells toward foreign body giant cells or osteoclasts after application of frozen techniques for material processing. These techniques are currently under evaluation in our group. The differentiation pattern of the multinucleated giant cells adherent to bone substitute materials is of particular interest. Materials that induce a tissue reaction with involvement of osteoclasts may provide osteoinductive bone regeneration because osteoblastic differentiation and activity are known to be positively related to osteoclastic molecules such as BMPs, PDGF (Platelet-derived growth factor)-bb/PDGFR (Platelet-derived growth factor receptor)-β, Sema4D and EphrinB2.[31-35]

In this study, the extent of new BT regeneration within the implantation bed of the two materials was assessed through histological and histomorphometrical analysis. In both implantation beds, new bone formation within the augmentation site was homogenously distributed. Both materials showed osteoconductive properties as in both cases new bone was always found in direct contact with the bone substitute material

and no new bone was found randomly within the connective tissue. Interestingly, the amount of newly formed bone on NB augmentation (21.85 ± 5.96%) did not significantly differ from that on BO augmentation (25.73 ± 7.94%), although significant differences in the pattern of the inflammatory cell reaction, vascularization and the extent of connective tissue formation within the implantation bed were observed. This is an interesting observation. Hence, the amount of the new BT within the sinus cavity seemed to be independent of differences in the aforementioned parameters, such as the number of multi-nucleated giant cells and the amount of connective tissue and degree of implantation bed vascularization. Thus, new bone formation within the augmented sites seemed to be independent of the remaining bone substitute material as long as the material could structurally guide bone ingrowth without premature degradation. The amount of remaining bone substitute material was significantly higher for BO than for NB, which suggests that bone formation may be more stable with BO.

Based on the findings of this study and the previously published clinical studies of our group with NB,[6,7] we consider the reaction within an augmented sinus cavity as a combination of bone and soft-tissue responses to the augmented material. The primary ingrowth of soft-tissue occurs from the soft-tissue covering the material, i.e., the Schneiderian-membrane and is mediated by the migration of macrophages and lymphocytes as well as the ingrowth of extracellular matrix components and fibroblasts into the intergranular space. New bone formation, however, occurs from the BT, i.e., the residual bone tissue of the sinus floor. After implantation of bone substitute materials into the sinus cavity, soft-tissue penetrates into the implantation bed to enable the degradation of the material, which results in a competition between soft- and hard-tissue that will end in a balance between material degradation and BT regeneration or in premature material degradation with insufficient BT regeneration.

Recently, our group has established a soft-tissue model in the subcutaneous tissue of small animals to mimic soft-tissue penetration into the implantation bed. The ultimate goal was to comparatively study the pattern and the extent of the inflammatory response to several bone substitute materials in relation to their physico-chemical material characteristics and to enable a systematic comparison of the tissue responses to different classes of materials. According to this model, physico-chemical material changes in shape, porosity and morphology as well as different chemical compositions of a bone substitute can result in different mononuclear cellular tissue reactions, i.e., different extents of mono- and multi-nucleated cell formation, differing implantation bed vascularization and different patterns of intergranular connective tissue ingrowth.[14,15,36,37] The implantation of both materials investigated in this study in the subcutaneous tissue of Wistar rats[4] or CD-1 mice (unpublished data) resulted in histological findings similar to those obtained after implantation into the human sinus cavity. Thus, the subcutaneous implantation model seems valid as an initial step in biomaterial research.

In the follow-up period of 2 years in the present study, one inserted implant was lost in the NB group. These results should, however, not be over-interpreted when considering the impaired soft-tissue condition in the oral cavity of this specific patient population and the decrease in salivation after tumor therapy. The clinical results furthermore reveal that despite the different patterns of inflammatory reaction and higher amount of remaining BO granules, satisfactory mastication and denture status were achieved in both groups. Additionally, these results call into question the accuracy of the percentage of newly formed bone as a parameter to determine a material suitable for sinus cavity augmentation. Previously published human studies with these two bone substitute materials showed comparable values for new bone formation in patients with no cancer anamnesis 6 to 9 months after implantation.[7,37,38] In a clinical study, in which biopsies obtained 3 and 6 months after sinus augmentation with NB were analyzed, a new bone formation of 31.29 ± 2.29% was detected 6 months after augmentation.[7] Another human study, in which BO was implanted into the sinus cavity of humans, determined that the amount of new BT was 29.8 ± 2.6% 8 months after augmentation.[37] The amount of new BT was 14.7 ± 5.0% with BO in a study where biopsies were obtained 9 months after sinus augmentation in humans.[38] These findings suggest that regardless of the bone substitute material used, the bone formation rate in human sinus cavities is restricted to approximately 15-40% within an observation period of approximately 1 year. The data from the present study indicate that both bone substitute materials are able to contribute to new bone formation within the sinus cavity of former tumor patients to an extent similar to that observed in healthy populations.[7,37,38]

Thus, within the limits of this study and in this particular patient population, NB provides the same level of bone regeneration as the "natural" bovine bone substitute material BO. Compared with BO, NB, however, induced an increased extended foreign body reaction that resulted in a different pattern of inflammatory response and a higher vascularization rate. Whereas the synthetic HA-based bone substitute showed a distinct degradation behavior, the xenogenic material integrated into the newly formed tissue, which has also been described by different authors.[37,39,40] Thus, the bovine material has to be viewed as a permanent implant, whereas the synthetic material constitutes a degradable implant. These results raise the question of whether complete regeneration of new BT should be accompanied by complete degradation of the bone substitute material or by the integration of the material into the new BT. Tailoring of the physico-chemical characteristics of synthetic bone substitute materials to induce an inflammatory pattern similar to that observed for BO should also be investigated. It may be beneficial to produce bone substitute materials that induce a mononuclear cellular response in which the mononuclear cells do not fuse to become multinucleated giant cells and where the materials will not be extensively degraded. Such materials may provide a regeneration profile similar to that of BO. However, the present study has shown that for both types of material, clinical success was achieved in a complex clinical situation, namely cancer with chemo-/radiotherapy.

The results of this study are encouraging, as they show that degradable synthetic-based materials are useful even in former tumor patients with known impaired healing mechanisms.

Moreover, this patient population often requires further reconstruction of other bone segments, such as segments of the upper and lower jaw. Thus, the present data may be useful for the evaluation and use of synthetic-based materials and bovine-based materials for tissue reconstruction in former tumor patients. This study also provides evidence to support the use of synthetic materials for clinicians who have reservations regarding synthetic bone substitute materials because of their induction of a foreign body response.

Finally, the present study demonstrated that in both groups, biomaterial-related implant survival contributed to oral rehabilitation, which in turn contributed to improved mastication, articulation and consequently increased quality-of-life. These results confirm that despite different inflammatory pathways, both materials appeared to contribute to clinical implant stability in this patient population for at least 2 years. Patients with cancer of the oral cavity have a 5-year survival rate of 61%.[1] The use of the materials evaluated in this study may help this patient population to achieve a better quality-of-life, especially because the use of the above-mentioned materials reduces the need for additional surgical procedures.

The results of the presented study with a population of only eight patients should not be over-interpreted to suggest that all tumor patients should be rehabilitated with synthetic and xenogeneic bone graft materials without further considerations and specification of the correct indication.

These results, however, indicate that even in this patient population, both biomaterials support successful new bone formation, which is mandatory for successful placement of dental implants following sinus augmentation. Further investigations in larger populations of former tumor patients are necessary to evaluate implant performance and long-term stability in augmented regions treated with other synthetic or xenogeneic bone substitute materials. However, other bone substitute materials have to be carefully selected using a systematic *in vivo* approach, i.e., in different tissues and species as well as in healthy patients, prior to application to patients with a history of oral cancer.

CONCLUSION

The present study analyzed for the first time the material-specific tissue response of patients with oral cancer to a synthetic and an animal-derived bone substitute material. Histological analysis showed two different material-specific tissue reactions with different numbers of inflammatory cells and degree of vascularization. However, both materials supported new bone formation within the sinus cavity to a similar degree. Implants in both augmented regions underwent undisturbed healing, but one implant inserted within a site augmented using the synthetic material was lost during the observation period of 2 years.

The data of the present study are encouraging as they show that synthetic materials can be similar to bovine materials for use in dental and maxillofacial surgery applications, particularly in oral cancer patients.

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