High-Temperature Sintering of Xenogeneic Bone Substitutes Leads to Increased Multinucleated Giant Cell Formation: In Vivo and Preliminary Clinical Results

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The present preclinical and clinical study assessed the inflammatory response to a high-temperature–treated xenogeneic material (Bego-Oss) and the effects of this material on the occurrence of multinucleated giant cells, implantation bed vascularization, and regenerative potential. After evaluation of the material characteristics via scanning electron microscopy, subcutaneous implantation in CD-1 mice was used to assess the inflammatory response to the material for up to 60 days. The clinical aspects of this study involved the use of human bone specimens 6 months after sinus augmentation. Established histologic and histomorphometric analysis methods were applied. After implantation, the material was well integrated into both species without any adverse reactions. Material-induced multinucleated giant cells were observed in both species and were associated with enhanced vascularization. These results revealed the high heat treatment led to an increase in the inflammatory tissue response to the biomaterial, and a combined increase in multinucleated giant cell formation. Further clarification of the differentiation of the multinucleated giant cells toward so-called osteoclast-like cells or foreign-body giant cells is needed to relate these cells to the physicochemical composition of the material.

Key Words: xenograft, bone substitutes, angiogenesis, animal study, biomaterials

INTRODUCTION

Tooth loss is often associated with concomitant alveolar bone resorption. Therefore, many patients in need of implant rehabilitation will also require bone augmentation with the aid of bone grafts or their substitutes. Such procedures have become a major part of implant placement procedures. In recent years, autogenous bone grafts have been applied as the gold standard of bone replacement in implant surgery and other orofacial surgical procedures. The use of autogenous bone grafts is based on the assumption that intraoperatively obtained autologous tissue exhibits ideal properties for bone tissue regeneration via the processes of osteoinduction, osteogenesis, osteoconduction, and osteointegration. However, because of the manifoldly limited availability and various complications and side effects associated with the application of autogenous bone grafts, attention is now shifting to bone substitute materials. These bone substitutes primarily include allo-, auto-, and xenogeneic cortical or cancellous bone grafts, synthetic materials based on hydroxyapatite (HA), and α- and β-tricalcium phosphate. In contrast to autologous bone grafts, these bone substitutes are readily available in large quantities and do not require a second surgical procedure for their harvest. However, the nonosteoinductivity of these bone substitutes may be associated with limitations.

For decades, xenogeneic bone substitute materials based on bovine bone tissue have been particularly important in clinical applications in the fields of medicine and dentistry for the treatment of various bone tissue defects. Thus, these types of substitute materials have been applied because their hard
bone matrix possesses a scaffold-like structure with physicochemical characteristics that are similar or identical to those of human bone. This allows the promotion of bone growth primarily because osteoconduction occurs simultaneously as the incorporation or biodegradation of these materials proceeds.7–9 Despite the skepticism associated with the use of animal-derived tissue and the purification procedures to remove immunologically active contents, such as cells and pathogens, such bone substitutes have proved to be mostly biocompatible and stable and also exhibit a regenerative capacity comparable to that of autologous bone transplants.10–12 However, tissue reactions to xenografts and the associated regenerative mechanisms have yet to be clarified. Various studies have demonstrated these types of substitutes remain present in augmentation/defect sites years after their implantation, which indicates tissue reactions that are not oriented toward the degradation of the materials (a situation similar to that of some synthetic bone substitutes) or toward the process of incorporation (a situation that has been described for the application of autologous bone).13–17 In this context, it is notable that the integration of the materials into the newly formed bone tissue within a defect area without significant degradation will not lead to complete regeneration of the lost tissue (ie, the condition of restitutio ad integrum will not be achieved).

The different physical and chemical methods used to purify animal donor tissue are thought to have a particular influence on the tissue reactions to these types of materials. Thus, heat treatment with different sintering temperatures has been outlined as a crucial manufacturing step that affects the material characteristics and the subsequent reactions to these materials.18,19 High processing temperatures are thought to be necessary for the complete deactivation of pathogens to prevent cross-infections.19 Indeed, Goller et al19 postulated that densification and mechanical properties steadily improve with increasing sintering temperature and that sintering at the higher temperatures of 1200°C and 1300°C yields significantly denser and stronger materials.

The deproteinized bovine bone material Bio-Oss (Geistlich Biomaterials, Wolhusen, Switzerland) is currently one of the most researched and widely used xenogenic biomaterials available. Bio-Oss is derived from the mineral remains of bovine bone. The purification methods for the removal of organic components include a low-temperature treatment at 300°C.29,30 In addition, the secure application of this bone substitute material is ensured by the careful selection of donor animals (health certificate, BSE-free) and quality and security controls based on the DIN and ISO norms.29 The structural characteristics of the bone substitute granules are stated to be “natural” in terms of the (ultra-)structure of the calcified matrix; that is, the macro- and the microporous morphology (the interconnecting pore system) and the surface nanostructure should be preserved.29

**Scanning electron microscopy**

Scanning electron microscopy was performed to examine the microstructure and the surface characteristics of the analyzed xenogenic bone substitute at the University of Bremen, Advanced Ceramics Group, Germany. A Camscan Series 2 scanning electron microscope with an accelerating voltage of 20 kV was used. Prior to microscopic SEM imaging, all samples were sputtered with gold (K550, Emitech).

**Preclinical in vivo study**

The described in vivo experiments were previously authorized by the Committee on the Use of Live Animals in Teaching and Research of the State of Rhineland-Palatinate, Germany. The animals were housed at the in vivo Laboratory Animal Unit of the Institute of Pathology. The animals were maintained under standard conditions (ie, ad libitum water, artificial light, and...
regular rat pellets; Laboratory Rodent Chow, Altromin, Germany).

**Experimental study design, subcutaneous implantation, and explantation procedures**

To conduct the in vivo study of the early (3, 10, and 15 days) and late (30 and 60 days) tissue reactions to the bone substitute, 40 female CD-1 mice between 6 and 8 weeks of age (Charles River Laboratories, Sulzfeld, Germany) were randomly allocated to 2 study groups. Twenty experimental animals were in the first study group, and 4 animals (n = 4) for each of the above-described study time points received the bone substitute materials by subcutaneous implantation (described below). The second study group also included 20 animals that served as the control group. Four of these animals (n = 4) received the surgical procedure without material insertion at each of the study time points.

The subcutaneous implantation procedure was conducted as previously described. Briefly, the experimental animals were first sedated using an intraperitoneal injection (10 mL ketamine [50 mg/mL] with 1.6 mL xylazine [2%]) followed by shaving and disinfection of the rostral subscapular implant region. The implantation procedure began with a horizontal incision to the subcutaneous tissue and the blunt formation of a subcutaneous pocket using surgical scissors. The bone substitute material was subsequently inserted into the tissue pocket, and the wounds were sutured with 6.0 Prolene (Ethicon, Somerville, NJ). After implantation, the experimental animals were laid on a warming plate and monitored until they recovered from anesthesia. The animals were then immediately relocated to their cages. Pre- and postoperative care of the animals was performed daily for the entire time span of the study.

After the respective time spans, the experimental animals were euthanized via an overdose of the above-described anesthetics, and the implanted bone substitute materials were explanted with the surrounding tissue. In the control group, the areas around the incisions were explanted. The tissue explants were then subjected to the histologic procedures described below.

**Clinical study**

For histologic analysis of this portion of the study, 3 bone specimens were collected 6 months after sinus augmentation according to previously described methods. Briefly, 2 patients with reduced dentition in the molar regions of their upper jaws and severe resorbed maxillary bones received sinus floor augmentation after providing informed consent. The implantations were performed at the private practice of one of the coauthors (M.S.). The implantation procedure was conducted via a crestal incision followed by the creation of a vestibular-based mucoperiosteal flap and a subsequent antrostomy of the maxillary sinus wall. In the implantation process, the exposed Schneiderian membrane was elevated, and the subantral space was enlarged to receive the investigated xenogeneic bone substitute material Bego-Oss mixed with blood obtained from the surgical site. After augmentation, the sinus window was covered with a native collagen membrane (Biogide, Geistlich, Wolhusen, Suisse), and primary wound closure was achieved. After a mean healing period of 6 months, bone biopsies of the augmented regions were simultaneously extracted with implant insertion, and the biopsies were subjected to histologic and histomorphometric analyses. In both surgical operations, intravenous Augmentin administration began intraoperatively and continued orally for 10 days postoperatively. Chlorhexidine (0.2%) was used as mouth rinse, and 400 mg of ibuprofen was also prescribed. The patients were instructed to avoid mechanical trauma to the wound, and the sutures were removed 2 weeks after surgery.

**Histologic study**

Both the explants from the preclinical analysis of the CD-1 mice and the bone cores from the clinical sinus elevation procedures were fixed in 4% formalin solution for 24 hours. Histologic procedures were then conducted as previously described. Briefly, the fixed animal tissue explants, which included the implanted bone substitute materials and the surrounding tissue or the areas around the control incisions, were sectioned into 3 segments of identical size. These segments contained the left, center, and right margin of the implantation beds of the biomaterials. Next, the segments were inserted into biopsyprocessing/embedding cassettes (HISTOSETTE, Sigma-Aldrich, St Louis, Mo). In addition, the specimens from the sinus augmentation sites’ prior implant insertion were placed into the embedding cassettes in toto for further histological preparation. The tissue samples from both study portions were then dehydrated using a series of increasing alcohol concentrations and a final xylene exposure followed by paraffin embedding. Subsequently, 3- to 5-μm-thick sections were prepared on a rotation microtome (Leica, Wetzlar, Germany).

For the animal tissue explants, 5 consecutive slides were stained with hematoxylin and eosin (H&E), azan, and tartrate-resistant acid phosphatase (TRAP) and were prepared for immunohistochemical detection of CD31 (murine vessels) for further quantitative and qualitative analyses of the tissue reactions to the bone substitute materials according to previously described methods.

For the human tissue samples, 6 different slides were prepared for the following stains: H&E, azan, sirius red, TRAP, and immunohistochemical staining for human CD31 (vessels). The procedures were performed to elucidate the tissue reactions to the bone substitute and its regenerative potential for sinus augmentation, as previously described.

**Histologic analyses**

The slides from both portions of the study were qualitatively analyzed via light microscopy with a focus on the outcome of the tissue-biomaterial interactions within the implantation beds and their surrounding tissue. These examinations were performed by 2 investigators (S.G. and M.B.) using an Eclipse 80i histological microscope (Nikon, Tokyo, Japan) as previously described. Special attention was given to the evaluation of the following parameters within the framework of the early and the late tissue response that were related to the implants: fibrosis, hemorrhage, necrosis, vascularization and the presence of neutrophils, lymphocytes, plasma cells, macrophages, and...
MNGCs and their subforms (i.e., TRAP-positive and TRAP-negative MNGCs). For presentation of the results, microphotographs were acquired with a Nikon DS-Fi1 digital camera and a DS-L2 digital sight control unit (both from Nikon) connected to the aforementioned microscope.

**Histomorphometric analysis**

The histomorphometric analyses were conducted on the slides that were stained with CD31 (murine/human) and the TRAP-stained slides from both portions of the study. The slides were initially digitized using previously described digitization methods. In brief, the complete biomaterial cross sections and their peri-implant tissues were assembled into so-called “total scans” with the aid of a specialized microscope consisting of an Eclipse Ni histological microscope, a DS-Fi1 digital camera (both from Nikon), and an automatic scanning table (Märzhäuser EK 14 mot, Wetzlar, Germany) connected to a computer system running the NIS-Elements software (V4.20 AR, Nikon). The combined image files were composed of up to 120 single histologic images at ×100 magnification and a resolution of 2500 × 1200 pixels. These image files were histomorphometrically analyzed for tissue reactions to the bone substitute materials using the NIS-Elements software.

The vascularization patterns of the implantation beds of the bone substitute (in vessel density, vessels/mm²), the percentage vascularization, and the numbers of material-associated MNGCs and giant cell subpopulations (i.e., TRAP-positive and TRAP-negative MNGCs cells/mm²) were measured with established methods to examine the compatibility of the tissue reactions to the biomaterials. The CD31-marked vessels within the implantation beds of the bone substitute granules in the slides from both portions of the study were manually marked using the area tool of the NIS-Elements software, which enabled the calculation of the area and the diameter of each vessel. In combination with the measurements of the total implant regions, these values permitted the calculation of vessel density (number of counted vessels/millimeter of the implant area) and percentage vascularization (percentage of the vascularized implant area based on the sum of the vessel area and the total implant area). The MNGCs and their subforms were manually counted using the count tool of the NIS-Elements software and their numbers related to the implant area (giant cells/mm²).

For the bone cores from the clinical portion of the study, the measurement functions of NIS-Elements were also used to analyze the tissue fractions (i.e., the areas of the newly formed bone tissue, the areas of the remaining bone substitute, and the amounts of connective tissue). Thus, the total areas of the 3 fractions within the cross sections were also manually marked, and the sums of the areas were related to the total area of the sections (area fraction/total area). Finally, the percentages of the different parts were calculated and subjected to statistical analysis.

**Statistical analysis**

The data from the histomorphometric measurements of both portions of the study were compared across the study groups with analyses of variance followed by Fisher least significant difference post hoc assessments that were performed with GraphPad Prism software (Prism 6 V6.01, GraphPad Software Inc, La Jolla, Calif). Inter- (*) and intraindividual (±) significant differences were deemed significant when the P values were less than 0.05 (*/±P < .05), and highly significant when the P values were less than .01 (**/±P < .01) and .001 (***/±P < .001). Finally, the data were presented graphically as the mean values ± standard deviations.

**RESULTS**

**Structural analysis of the bone substitute material**

Initially, the material structures were examined via SEM. This analysis revealed granule structures with fragments of calcified extracellular matrix of the cancellous bone tissue that had trabecular structures containing tissue-specific macropores of different sizes (Figure 1). Broken edges were frequently observed in the granule-like material fragments (Figure 1a through c). Closer inspection of the material surfaces revealed relatively smooth surface structures combined with the presence of micropores and relatively high numbers of dustlike particles located across the entire particle surface (Figure 1c and d).

**Preclinical in vivo study in CD-1 mice**

All animals survived the experiments, and no signs of any macroscopically detectable wound disorders were observed throughout the study period. In addition, over the observed time period, no mobility disorders were detectable.

**Histopathologic Assessment of the Mouse Tissue Reactions to the Bone Substitute**

The histopathologic analysis of the tissue reactions to the bovine-based bone substitute material Bego-Oss revealed that the granules of this material were embedded within a fibrin-like matrix on day 3 after implantation (Figure 2a). Only low numbers of mononuclear cells were detectable within the intergranular spaces and on the surface of the material granules (Figure 2a). These cells were mainly identifiable as monocytes, but some single granulocytes were also present (Figure 2a). At this time point, no signs of the ingrowth of complex tissue structures or vessels were observed within the implantation beds of the bone substitute granules, but some vessels were identified in the peri-implant tissue (Figure 2a).

At day 10 after implantation, the granules were embedded within a cell- and fiber-rich connective tissue (Figure 2b). At this time point, in addition to mononuclear cells, MNGCs cells were distributed within the intergranular connective tissue and on the material surface (Figure 2b). Furthermore, moderate numbers of microvessels were found within the implantation beds (Figure 2b).

At day 15 following implantation, the tissue that surround ed the bone substitute granules underwent further vascularization, and the numbers of MNGCs on the surfaces of the granules also increased (Figure 2c). These conditions continued until day 60 (Figure 2d and e).

Histological analysis of the TRAP expression of the material-associated giant cell activity revealed that beginning on day 10 after implantation and continuing until the end of the study,
the TRAP-positive MNGCs represented the major fraction of the detected MNGCs. Two representative slides from day 15 are shown in Figure 3.

Histomorphometric Analysis of Vascularization

The histomorphometric analysis of the vascularization parameters within the implantation beds of the bone substitute granules revealed that no vessels were located within the intergranular spaces at day 3 after implantation (Figure 4a and b). Furthermore, these analyses revealed that a greater number of vessels and a higher percentage of vascularization were found in the control group (7.94 ± 1.94 vessels/mm² and 0.06% ± 0.01%) at this time point, but that these differences were not significant (Figure 4a and b).
Beginning on day 10 after implantation, the analysis of the vessel density revealed relatively low numbers of vessels within the implantation beds of the xenogeneic bone substitute (13.99 ± 2.08 vessels/mm²), and these numbers did not significantly differ from those of the control group (5.13 ± 0.98 vessels/mm²), but did differ from the values observed on day 3 (***P < .001; Fig. 4a). At this time point, the values of the control group did not differ from that of the former study time point (Figure.
4a). The vessel density values remained stable until day 15 after implantation in the Bego-Oss study group (15.19 ± 2.24 vessels/mm²); thus, no significant differences were found compared with the values measured for the former study time point (Figure 4a). In addition, no significant differences were found compared with the values of the control group (4.95 ± 1.69 vessels/mm²), which also did not significantly differ from the values of the former study time point (Figure 4a). At day 30 after implantation, an increase in the vessel density was observed in the Bego-Oss study group (43.28 ± 6.02 vessels/mm²). Thus, intra-individual differences compared with day 15 after implantation were found (**P < .001; Figure 4A). Furthermore, highly significant differences were found in the comparison with the values of the control group (5.03 ± 0.91 vessels/mm²), while these control group values were not significantly different from the values at the former time point (Figure 4a). No further changes in vessel density were found at day 60 after implantation in the bone substitute study group (40.28 ± 6.23 vessels/mm²), or the control group (6.69 ± 1.89 vessels/mm²); thus, no significant intra-individual differences were found but highly significant differences between the 2 groups were apparent (**P < .001; Figure 4a).

The histomorphometric analysis of the vascularization percentages revealed that the Bego-Oss study group showed a higher vessel area on day 10 after implantation (0.61% ± 0.07%) compared with that of the study group (0.07% ± 0.02%). However, no significant inter- or intra-individual differences were found (Figure 4b). On day 15 after implantation, increases in the percentage vascularization were found in both the bone substitute study group (1.69% ± 0.41%) and the control group (0.24% ± 0.10%), and the statistical comparisons revealed highly significant inter-individual differences (**P < .001; Figure 4b). Furthermore, the increase in the Bego-Oss study group was also significantly greater than that of the former study time point (**P < .05), but no intra-individual differences were found in the control group at this time point (Figure 4b). On day 30 after implantation, an additional highly significant increase in the percentage vascularization was found in the Bego-Oss study group (**P < .01; 2.96% ± 0.39%), but no intra-individual differences were found in the comparison of the values of the control group (0.29% ± 0.06%) to those of day 15 (Figure 4b). However, highly significant differences were still present in the comparison of the 2 groups (**P < .001; Figure 4b).

The increases in the percentage vascularization were maintained in both groups on day 60 after implantation, but a highly significant intra-individual difference was present only in the Bego-Oss group (5.17% ± 0.79%; ***P < .001; Figure 4b). Thus, the percentage vascularization of the control group (0.41% ± 0.16%) was not significantly different when compared with day 30 after implantation (Figure 4b). A highly significant difference between the groups was also found at this final study time point (**P < .001; Figure 4b).

Histomorphometric Analysis of the Total MNGCs and Their TRAP-Negative and TRAP-Positive Subpopulations

The histomorphometric analysis of the numbers of material-induced MNGCs and their subforms (ie, TRAP-positive and TRAP-negative MNGCs) revealed that none of these cells were present in the implantation beds of the xenogenic bone substitute material Bego-Oss on day 3 after implantation (Figure 4c). Beginning on day 10 after implantation, biomaterial-associated MNGCs were found in moderate numbers (15.08 ± 3.09 MNGCs/mm²) and were associated with the material granules. Statistical analysis revealed a highly significant increase of the numbers of cells compared with day 3 after implantation (***P < .001; Figure 4c). Additionally, the measurements revealed that high numbers of this cell type expressed TRAP (9.89 ± 2.84 TRAP[+] MNGCs/mm²), and smaller numbers of TRAP-negative MNGCs were found (5.19 ± 0.32 TRAP[−] MNGCs/mm²; Figure 4c). However, no significant differences were found between the amounts of either subpopulation of MNGCs (Figure 4c).

On day 15 after implantation, further increases in the numbers of biomaterial-associated MNGCs were observed (23.59 ± 5.68 MNGCs/mm²), but these were not significantly different from the numbers of the former study time point (Figure 4c). At this time point, more TRAP-positive (16.14 ± 4.19 TRAP[+] MNGCs/mm²) than TRAP-negative MNGCs (7.45 ± 1.84 MNGCs/mm²) were observed within the implantation beds, but this difference was not significant (Figure 4c).

On day 30 after implantation, the numbers of material-induced MNGCs (22.02 ± 4.16 MNGCs/mm²) were at the same level as they were on day 15, and there were no significant intraindividual differences (Figure 4c). Furthermore, greater amounts of TRAP-positive than TRAP-negative (6.24 ± 0.92 TRAP[+] MNGCs/mm²) MNGCs (15.79 ± 4.40 TRAP[+] MNGCs/mm²) were found in the implantation beds of the xenogenic bone substitute also at this time point, but these values were not significantly different (Figure 4c).

On day 60 after implantation, the xenogenic bone substitute material Bego-Oss evoked a total number of MNGCs (21.18 ± 5.98 MNGCs/mm²) that was comparable to the numbers observed at the former 2 study time points, and these differences were not significant (Figure 4c). The measurements of the subforms of these cells revealed that, again, more of the multinucleated cells were also TRAP-positive at this final study time point (14.95 ± 4.59 TRAP[+] MNGCs/mm²) than were TRAP-negative (6.23 ± 2.31 MNGCs/mm²), but this difference remained nonsignificant (Figure 4c).

Preliminary clinical results

Histopathologic Assessment of the Tissue Reactions to the Bone Substitute

The histologic analysis of the tissue reactions to the xenogenic bone substitute material Bego-Oss within the bone cores revealed that material granules were detectable throughout the entire lengths of the cores (Figure 5a through c). Moreover, the newly formed bone tissue/matrix was also observable throughout the entire implantation area of the granules (Figure 5a). This bone tissue was primarily observed on the surfaces of the Bego-Oss granules, which were often completely embedded within the newly built bone matrix (Figure 5a). The surface areas of the material granules that were not covered by bone matrix were surrounded by cell- and vessel-rich connective/granulation tissue (Figure 5b). The same cell types (ie, monocytes, macrophages, granulocytes, lymphocytes, and
revealed a vessel density of 14.74 vessels/mm², which indicates an inflammatory state (Figure 5b). Monocytes and MNGCs were found directly attached to the granule surfaces that were covered by the connective tissue (Figure 5b and c). Further histologic analysis of the TRAP enzyme revealed that most of the material-associated MNGCs showed signs of TRAP expression (Figure 5c).

**Histomorphometric Analysis of the Tissue Fractions Within the Implantation Beds**

The histomorphometric measurements of the tissue fractions within the bone cores of the xenogenic bone substitute material revealed that the highest fraction was present within the connective tissue (44.84% ± 5.33%), and that significantly lower fractions of newly formed bone tissue (31.63% ± 5.69%, *P < .05) and remaining bone substitute (23.53% ± 3.77%; **P < .01) was found (Figure 6a).

**Histomorphometric Analysis of the Total MNGCs and Their TRAP-Negative and TRAP-Positive Subpopulations**

The histomorphometric analysis of the material-associated MNGC formation revealed a mean value of 37.08 MNGCs/mm² within the implantation areas of the Bego-Oss granules (Figure 6b). Thus, highly significantly greater amounts of TRAP-positive (26.81 ± 4.86 TRAP[+] MNGCs/mm²) than TRAP-negative (10.27 ± 1.87 TRAP[−] MNGCs/mm²) MNGCs were found within the implantation beds of the Bego-Oss, and both values were significantly different (*P < .05; Figure 6b).

**Histomorphometric Analysis of Implantation Bed Vascularization**

The histomorphometric analysis of implant bed vascularization revealed a vessel density of 14.74 ± 1.39 vessels/mm² and a percentage vascularization of 1.99% ± 0.54% in the bone cores that were treated with the bone substitute Bego-Oss (Figure 6c).

**DISCUSSION**

Xenogenic bone substitutes have been established as alternatives to autologous bone transplants and synthetic bone substitutes within the last decades because of their availability and proven regenerative potential.10–12 Thus, it is thought that xenogenic bone matrices should exhibit physicochemical characteristics that are comparable to their human equivalents.7–9 Accordingly, xenogenic bone materials might promote the process of material-mediated bone regeneration more successfully than synthetic materials.10,31,32 However, each xenogenic bone substitute requires a purification process to remove all of the immunogenic contents of the animal donor prior to their application.33,34 Finally, the remaining calcified extracellular matrix of the donor bone tissue is believed to initiate an osteoconductive bone repair process that is associated with its incorporation as a result of cellular degradation.7 Different purification methods for xenogenic bone substitute materials have been applied. These methods include various chemical and physical treatment steps.7 Heat treatment or “sintering” is one such physical procedure that is typically used in the preparation process. Sintering involves the application of different temperatures (ie, high- and low-grade temperatures) and has been shown to enable the purification of the xenogenic donor tissue.35 The heat treatment of bovine bone tissue with temperatures beginning at 700°C has been described to induce the loss of the bony microstructure and the densification of the bone microstructure while not changing the macrostructure. By contrast, no influence on the bone structure has been found at lower temperatures.19 Furthermore, high-grade heat treatment (approximately 1300°C) induces the generation of calcium phosphate phases, which are thought to be linked with the dehydroxylation of the matrix HA.35,36 Based on these findings, heat treatment of the xenogenic donor tissue at different temperatures presumably has a substantial influence on the tissue reactions and regenerative capacities of those materials.

To the best of our knowledge, no in vivo data regarding the influence of heat-related structural differences in xenogeneic bone substitute materials on the inflammatory tissue reactions were previously available. Therefore, the aim of this study was to analyze the influence of high-grade heat treatment of xenogenic bone tissue with temperatures greater than 1250°C, which produces the bone substitute Bego-Oss, on cell and tissue reactions and regenerative potential. This study series included an analysis of the material structure via SEM, preclinical studies of the tissue reactions using the subcutaneous implantation model in CD-1 mice with a follow-up of 60 days, and an analysis of clinical experiments in which the xenogenic material Bego-Oss was used for sinus augmentation prior to surgical placement of dental implants.

Initially, the results of the structural analysis of the xenogenic bone substitute revealed that the material granules exhibited the regular macroscopic structure of spongy bone matrix with a trabecular organization and macropores. The investigations at higher magnification revealed a relatively smooth surface structure combined with micropores, and large amounts of dustlike particles were found across the entire surfaces of the particles.

The histologic results of the preclinical in vivo portion of the study revealed that the bone substitute granules were embedded within a granulation tissue beginning on day 10 and continuing until the end of the observation time at 60 days after implantation. Within this time span, this intergranular tissue contained different inflammatory cells, such as granulocytes, lymphocytes, and cells of the monocyte/macrophage line. This intergranular tissue also contained moderate numbers of MNGCs at the surfaces of the material granules. The histomorphometric measurements revealed that the total number of MNGCs was moderate, beginning on day 10 and continuing without significant change until the end of the observation time 60 days after implantation. However, the TRAP-positive fraction of these cells was still not significantly different from the TRAP-negative fraction. The level of vascularization was also high. Thus, the vessel density analysis revealed that similar moderate vessel numbers were found on days 10 and 15 after implantation and that, beginning at day 30 after implantation, a significant increase in vessel density was observed; this increase was also maintained without a significant difference on day 60 after implantation. The histomorphometric analysis of the percentages of vasculariza-
tion also revealed a constant increase in the vessel area over the time frame of the study. In addition, the studies revealed that both vascularization parameters of the experimental group were significantly higher than those of the control group beginning at day 15 or day 30 after implantation. These results indicate that the material-induced (inflammatory) tissue reactions manifested in the granulation tissue, particularly the progressive induction of MNGCs, had a (time-delayed) effect on the vascularization of the implant beds of the xenogeneic bone substitute. This effect is likely because this cell type is a syncytium of monocytes and macrophages that has been shown to exhibit a strong influence on the process of wound healing and tissue regeneration via the expression and secretion of different chemokines and cytokines. In particular, the expression of the angiogenic factor vascular endothelial growth factor (VEGF) by this cell type was demonstrated in a former study by our group in which VEGF was found to influence the vascularization of the implant beds of synthetic bone substitute materials.

The previously described tissue reaction pattern was additionally confirmed by the results of the clinical portion of the study, which also demonstrated the occurrence of material-associated granulation tissue within the bone cores 6 months after the application of the high-temperature–treated xenogeneic bone substitute material. This granulation tissue included the same cell types, moderate numbers of material-associated MNGCs, and a relatively high rate of vascularization within the implantation beds of Bego-Oss. Interestingly, the first observation of this study was that material-associated MNGCs were found at similar concentrations in both the subcutaneous connective tissues of the CD-1 mouse and the human tissues after sinus augmentation. This finding endorses the validity of the preclinical implantation model.

The comparison of the presented surface analysis results with previous data from another study of bovine-derived xenogeneic bone substitute material whose purification included a heat treatment at 300°C (Bio-Oss) reveals a condensed surface structure of the Bego-Oss, while the surface structure of the Bio-Oss possessed a rough and fibrillar surface texture. Thus, the data of Goller et al. that described an increase in the densification of the bone microstructure with an increase in the temperature of the heat treatment of bovine bone tissue is confirmed.

The comparison of the preclinical results of this study with existing data concerning Bio-Oss, a xenogeneic bone substitute that is treated with low-grade temperatures up to 300°C and has also been examined by our group using the same standardized implantation model and the same histologic and histomorphometric analysis methods, revealed higher numbers of material-associated MNGCs and greater vascularization in the case of the Bego-Oss. These data are further confirmed by a former clinical study by our group in which Bio-Oss was included, as the results of this study also revealed reduced numbers of MNGCs and reduced vascularization compared with the values for Bego-Oss after the clinical application presented in this study.

The histomorphometric analysis of the clinical portion of the study showed that the bone growth exhibited an increased fractional area of approximately 32% bone tissue, while this value for the bone substitute was approximately 24%. However, the results from similar studies in which the low-heat–treated xenogeneic bone substitute Bio-Oss was applied have also demonstrated its successful clinical application for sinus augmentation in different clinical trials. Thus, in these trials, bone growth values from 11.8% to 30% were reported, and the remaining areas of the material were between 28% and 40.5%.

The extent of new bone formation within the implantation bed of Bego-Oss is comparable to that observed for Bio-Oss, while the amounts of the remaining bone substitute seemed to decrease in the case of Bego-Oss. Thus, the presented data support the conclusion that heat treatment at high temperatures, which has been shown to influence the microstructure of bovine donor tissue in terms of structural densification, results in greater material-induced inflammatory responses compared with xenogeneic bone substitute materials that are based on materials purified at low temperatures. The present findings are interesting particularly in terms of the different studies on the cellular biodegradation of Bio-Oss. In this context, many authors have found that Bio-Oss is integrated into the bone tissue and persists in the implantation beds for many years. Moreover, it is known that delays in the degradation process combined with persistence of the material within the bony implantation bed can prevent the regeneration of bone tissue defects to restitutio ad integrum.

Thus, the results of the present study raise the question of whether greater numbers of MNGCs are evidence for greater levels of cellular biodegradation of these types of biomaterials. Furthermore, it should be mentioned that the differentiation of this multinucleated cell type has yet to be completely clarified. This material-associated multinucleated cell type is called an “osteoclast-like cell type” based on its multinucleation, the expression of lytic enzymes such as TRAP, and its occurrence within the implantation beds of calcium phosphate–based bone substitute materials, which is comparable to that of bone matrix–resorbing osteoclasts. Thus, this cell type has been explicitly stated to be an osteoclastic cell type because bone matrix–related osteoclasts are also characterized by these aforementioned features. Multinucleated giant cells have also been found within the implantation beds of many biomaterials with chemical compositions that are different from those of calcium phosphates, including collagen-based membranes, silk fibroin–based materials, and many other synthetic biomaterials. Thus, recent results indicate that these cells could be considered to be of the (inflammatory) foreign body giant cell type, which is induced by “unnatural” synthetic materials, the expression of typical markers, and the involvement of similar cell compartments. However, based on the densification of the bone microstructure and the synthesis of calcium phosphate phases observed in xenogeneic materials treated at higher temperatures, it is conceivable that high-temperature heat treatment shifts the bone matrix toward “unnatural” material characteristics that are identified by the host as those of a foreign body. In this case, it is understandable that the extent of the formation of (inflammatory) MNGCs in this study is comparable to that which occurs in the implantation beds of HA-based synthetic materials. Thus, further long-term studies are necessary to elucidate whether the comparable
high numbers of MNGCs within the implantation beds of Bego-Oss will lead to substantial biodegradation of the used material or to material encapsulation and persistence. Either of these results will substantially contribute to our understanding of the progression of the observed MNGCs toward osteoclasts or foreign-body giant cells.

CONCLUSION

The present study focussed on the analysis of material structure via SEM and in vivo host tissue reactions to Bego-Oss, which is an exemplary xenogeneic bone substitute material sintered at a high temperature of approximately 1250°C. The in vivo study had 2 parts, namely, after subcutaneous implantation in CD-1 mice and from human sinus floor following sinus augmentation.

The results of the SEM analysis revealed densification of the material surface of the Bego-Oss that seemed to elicit a relatively strong (inflammatory) tissue reaction in both implantation beds that included relatively large numbers of MNGCs and strong implantation bed vascularization. The levels of tissue reaction found within the implantation beds of the Bego-Oss were higher than those that have previously been reported based on observations of the reactions to other xenogeneic bone substitutes sintered at lower temperatures. However, these levels compare favorably with those observed for synthetic bone substitutes.

Nevertheless, further studies will be needed over a longer time period to ascertain whether these large numbers of MNGCs in the implantation beds of the Bego-Oss will result in faster biodegradation of the biomaterial or its extended persistence. Furthermore, the differentiation of the MNGCs and their detailed roles in the process of material degradation require further clarification in additional studies to bring the functions of this cell type into line with the process of bone tissue regeneration via xenogenic bone substitutes.

ABBREVIATIONS

BSE: bovine spongiform encephalopathy
H&E: hematoxylin and eosin
HA: hydroxyapatite
MNGC: material-induced multinucleated giant cell
SEM: scanning electron microscopy
TRAP: tartrate-resistant acid phosphatase
VEGF: vascular endothelial growth factor

ACKNOWLEDGMENTS

The authors would like to thank Mrs Ulrike Hilbrig and Mr Mykhaylo Reshetnykov for their technical assistance.

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