Quantification of bone tissue regeneration employing β -tricalcium phosphate by threedimensional non-invasive synchrotron micro-tomography – a comparative examination with histomorphometry

Michael Stiller, DDS, MD, Dr. med. Dr. med. dent., Privatdozent¹ Alexander Rack, MS, Dr. ing.² Simon Zabler, MS, Dr. rer. nat.⁴ Jürgen Goebbels, MS, Dr. rer. nat.⁵ Olaf Dalügge, DDS³ Christine Knabe, DDS, Dr. med. dent., Privatdozentin³

¹Department of Maxillofacial and Facial-Plastic Surgery, Division of Oral Medicine,
 Radiology and Surgery, Charite – University Medical Center Berlin, Campus Benjamin
 Franklin, Aßmannshauser Str. 4-6, 14197 Berlin, Germany
 ²Forschungszentrum Karlsruhe, ANKA – Institute for Synchrotron Radiation, Germany
 ³Department of Experimental Dentistry, University Medical Center Berlin, Campus Benjamin
 Franklin, Aßmannshauser Str. 4-6, 14197 Berlin, Germany
 ⁴Hahn-Meitner-Institut / Helmholtz Centre Berlin, Department Materials Research, Germany
 ⁵Bundesanstalt für Materialforschung und –prüfung, Department Radiology, Germany

Corresponding author:

Privatdozent Dr. med. Dr. med. dent. Michael Stiller
Dept. of Maxillofacial and Facial-Plastic Surgery,
Division of Oral Medicine, Radiology and Surgery
Charite – University Medical Center Berlin
Aßmannshauser Str. 4-6
14197 Berlin, Germany
Fax: +49-30-450-562-922

Tel: +49-30-450-562-692

E-mail stiller@implant-consult.de

Abstract

Purpose

This methodical study presents a novel approach to evaluate the validity of two-dimensional histomorphometric measurements of a bone biopsy specimen after sinus floor elevation by means of high contrast, high resolution, three-dimensional and non-destructive synchrotron micro-tomography (SCT). The aim of this methodical description is to demonstrate the potential of this new approach for the evaluation of bone biopsy samples.

Materials and Methods

Unilateral sinus grafting was carried out exemplarily in two patients using a combination of β -tricalcium phosphate (β -TCP) and autogenous bone chips. For the first patient a β -TCP with 35% porosity and in the second with 60% porosity was used. At implant placement, 6 months after sinus grafting, a cylindrical specimen was biopsied from the augmented area. Subsequent to the histological embedding in resin the specimens were imaged using a SCT facility resulting in three-dimensional (3-D) images with approximately 4 µm spatial resolution (1.5 µm pixel size) for each patient's specimen. Subsequent to the SCT acquisition, tissue sections were prepared for histomorphometric analysis.

Results

Bone area fractions determined by two-dimensional (2-D) quantitative histomorphometry and by analysis of the corresponding 2-D slice from the SCT volume data were similar. For the first biopsy specimen (β -TCP with 35% porosity), the bone area fractions were 53.3% and 54.9% as derived by histomorphometry and by analyzing a SCT slice, respectively. For the second biopsy specimen (β -TCP with 60% porosity) the bone area fractions were 38.8% and 39% respectively. Although the agreement between the 2-D methods was excellent, the area fractions were somewhat higher than the volume fractions computed by 3-D image analysis on the entire SCT volume data set. The volume fractions were 48.8% (first biopsy specimen) and 36.3% (second biopsy specimen).

Conclusion

Although the agreement between the 2-D methods is excellent in terms of computing the area fractions, the structural 3-D insight which can be derived from classical 2-D methods, including histomorphometric analysis is considerably limited. This fact is emphasized by the discrepancy between the measured areas and volume fractions.

Key words sinus floor augmentation histomorphometry synchrotron micro-tomography multi-dimensional microscopy histovolumetry

1 Introduction

The evaluation of biomaterials with differing biodegradability and the characterization of the biological response to regenerative procedures which are subject to multifactorial influences require non-destructive diagnostic tools to elucidate the dynamics of these processes in a three-dimensional (3-D) manner. Conventional hard tissue histologic and histomorphometric methods with their complex sectioning procedures have the disadvantage of either damaging the 3-D structure of the tissue sample under investigation, i.e. the bone biopsies, or revealing only limited information regarding the dynamics of regenerative processes in highly complex anatomic structures [1]. In hard tissue histology it is not possible to produce serial sections which are suitable for 3-D reconstruction due to the loss of a tissue layer of approximately 50 µm in thickness during the sectioning process when using a diamond sawing microtome.

The maxillary sinus is a complex anatomical structure to be investigated, since it contains the alveolar crest, the oral mucosa as well as the respiratory epithelium of the nasal system. Since this region is of particular importance in oral implantology the human maxillary sinus is one of the most intensely studied regions with regard to placing dental implants [2]. The resorptive processes following tooth loss often lead to bone deficiencies in the posterior maxilla. As a result sinus floor augmentation has become a frequently performed and well-established surgical procedure for increasing the bone volume of the posterior maxilla [3]. Using biodegradable bone substitutes simplifies sinus floor elevation procedures by avoiding second site surgery for bone harvesting [4-14]. In recent years the use of tricalcium phosphate particles (β -TCP) as alloplastic bone graft material for sinus grafting procedures has received increasing attention in implant dentistry [15-18]. More recently, the use of β -TCP particles with increased porosity has been promoted in order to increase the biodegradability [19]. These particles exhibit a material structure with micro-, meso- and macropores, which is designed to enhance the degradation process.

In a previous study we examined the effect of two different β -TCP particulate bone grafting materials with varying porosity on the bone formation and on the osteoblastic phenotype expression six months after sinus floor elevation [20]. Bone area fractions and particle area fractions were determined histomorphometrically at two sites in each biopsy specimen: apically close to the Schneiderian membrane (sinus membrane) and also in the center of each cylindrical specimen. Histomorphometric analysis was carried out on a pair of sections which were 150 µm apart. A square area of 6.25 mm² was defined and measured semiautomatically using a light microscope and AnalySIS software (Olympus Inc, Japan). However, the disadvantage of this measuring procedure is that the evaluation of regenerated bone volume is based on 2-D measurements of only a selected area within the sample [20]. Furthermore, evaluation of particle degradation is based on the assumption that tissue regeneration proceeds uniformly throughout the volume of the biopsy specimen. Therefore usually multiple sections are analyzed histomorphometrically and the results are averaged, in order to improve the accuracy of these histomorphometrical measurements.

Significantly more detailed information regarding the 3-D structure of the entire regenerated bone volume can be obtained by non-destructive X-ray based volume imaging methods. Conventional computed tomography (CT) as used in medical imaging or micro-tomography (μ -CT) employing laboratory X-ray sources do not reach the spatial resolution and contrast which is common to histologic images so as to facilitate an accurate distinction between regenerated bone and residual bone substitute material [21, 55, 56]. Specially the high signal-to-noise ratio provided by volume images acquired via synchrotron micro-tomography is required for a quantitative 3-D image analysis subsequent to the data reconstruction.

In this study 3-D synchrotron micro-tomography (SCT) was applied in order to acquire additional information regarding the newly formed bone and the residual β -TCP particles in human biopsy specimens sampled six months after sinus floor elevation. 3-D μ -

CT employing synchrotron radiation is the most sophisticated development of X-ray computed tomography / micro-tomography, yielding higher resolution and higher contrast [22, 37, 39]. Synchrotron light sources feature a photon flux several orders of magnitude higher compared to laboratory X-ray sources used for the established micro-imaging methods in 2-D and 3-D [23-26]. Furthermore, synchrotron light sources produce a beam which propagates nearly parallel [27]. Hence, one can overcome limitations due to the finite X-ray source size by extending the distance sample-source up to more than 100 m. Given the high photon flux, the beam can be monochromatized, resulting in an increase of the density of contrast (down to a few per cent) and the suppression of so-called beam-hardening artifacts. Utilizing volume images obtained with monochromatic synchrotron radiation, the different material phases, e.g. bone and ceramic particles within a biopsy specimen, may be distinguished by differences in density [28-37].

Algorithms derived from stochastic geometry are then applied to classify each voxel (voxel is an acronym for "volume pixel") inside the volume image according to the material phase it contains. The accuracy of the results is mainly driven by the spatial resolution of the images: material phases can only be distinguished locally up to a precision given by the voxel size. Segmented images, where the material phase of each voxel is already identified, can be used for high quality 3-D visualization. They also provide the basis for the quantitative analysis of the bone tissue and residual grafting materials in the biopsy specimens. This analysis can then be used to compare in a quantitative manner the accuracy of the SCT 3-D image to conventional histomorphometric 2-D measurements of regenerated bone and degraded β -TCP particles in these biopsies as the standard measuring procedure (gold standard) [30-32].

In summary, the first aim of our study was to evaluate the diagnostic potential of SCT for quantitatively evaluating bone biopsies with a particular focus on the differentiation between regenerated bone and residual bone substitute material. The second aim was to demonstrate the accuracy of the histovolumetric assessment of the bone regeneration process on the basis of 3-D visualization and analysis of SCT images. Achieving the second aim would demonstrate limitations of conventional histomorphological techniques.

Materials and methods

Patient selection

The present study included two exemplarily selected patients who underwent sinus floor augmentation using β -TCP with different porosities. The selected patients were partially edentulous in the post canine region. In both patients augmentation of the sinus floor was required in order to facilitate dental implant placement in the posterior maxilla. After routine oral and physical examinations, the patients were selected and sinus floor augmentation procedures were planned. Both patients had good oral health, without active periodontitis and were non-smokers. Patients were fully informed about procedures, including the surgery, bone substitute materials and implants. They were asked for their cooperation during the treatment and research and gave their informed consent. In addition, the study was performed in accordance with ethic protocols approved at the Charite University Medical Center.

Sinus floor augmentation surgery

In both patients sinus floor elevation was carried out under local anaesthesia. Preoperative Xray examination was performed to evaluate sinus pathologies and anatomical variations, e.g. antral septae. A staged surgical approach (first sinus floor elevation followed by implantation after 6 months) was used as the height of the residual alveolar process was 1 mm in the posterior maxillary region. The space created between the maxillary alveolar process and the elevated Schneiderian membrane was filled using a combination (4:1 ratio) of β -TCP particles and autogenous bone chips. Postoperative X-rays were taken in order to determine the augmented hard tissue volume immediately after sinus floor elevation and to check for volume alterations after completion of the six-month healing period, i.e. prior to implant placement.

For the first patient (TCP), the β -TCP particles used were globular particles of 1000-2000 μ m grain size with 35% porosity (Cerasorb, Curasan AG, Kleinostheim, Germany). In contrast, the second patient (TCP-P) received β -TCP with a grain size of 1000-2000 μ m and 60% porosity (Ceros, Mathys AG, Bettlach, Switzerland). Autogenous bone chips were harvested in both patients from the tuber maxillae. The β -TCP grafting material was mixed with venous blood prior to delivery into the open sinus cavity. In order to avoid infections, both patients received clindamycin 1200 mg (Clindamycin ratiopharm 600 mg, Ratiopharm GmbH & Co., Ulm, Germany) daily for 7 days. This was in addition to an intravenous injection of 250 mg prednisolone (Solu-Decortin H 250, Merck KGaA, Darmstadt, Germany) in combination with daily oral administration of 800-1200 mg of ibuprofen (IBU ratiopharm 400 akut, Ratiopharm GmbH & Co., Ulm, Germany) to reduce pain and swelling.

Dental implant surgery and biopsy

After 6 months of healing the patients received implants. Dental implant placement and biopsy sampling were carried out under local anesthesia. In both patients biopsy specimen were performed at the dental implant sites with a depth of approximately 12 mm using a trephine burr (3.5 mm outer diameter and 2.75 mm inner diameter) with saline irrigation. In each patient one biopsy was harvested at one of the sites, where a dental implant was to be placed. The site in which the height of the original residual alveolar process (prior to augmentation) was approximately 1 mm was chosen for biopsy. The biopsy specimens from both patients were 2.5 mm in diameter and approximately 5 mm in length. These specimens were used for histological, histomorphometric and SCT investigations. The specimens contained both the grafted area and the previously existing area of the sinus floor, which was

approximately 1 mm in height. The residual native crest was not included in the histological, histomorphometric and SCT analysis.

Preparation of biopsy specimens for synchrotron micro-tomography and histomorphometry

The bone specimens were processed using a novel technique which facilitated performing immunohistochemical analysis on undecalcified hard tissue sections as described previously [38]. The tissue samples were immediately fixed in an ethanol-based fixative Neofix^R (Merck KGaA, Darmstadt, Germany) at room temperature (20-22°C) for 5 days. This was followed by dehydratation and infiltration. Next the specimens were embedded in a resin, which was composed of pure metylmetacrylate and n-butyl-metracrylate to which benzoyl peroxide (BPO catalyst, Merck KGaA, Darmstadt, Germany) and polyethylene glycol 400 (Merck KGaA, Darmstadt, Germany) and 1.5 ml N,N-dimethyl-p-toluidine (Merck KGaA, Darmstadt, Germany) were added. Samples were polymerized in polyethylene vials at 4°C for 4-7 days. This resin was selected because it maintained the antigenicity of the tissue and at the same time also provided adequate material properties for cutting 50 µm thick sections with a sawing microtome. After polymerization the blocks were removed from the vials and excess resin was trimmed away. Both resin blocks, including whole biopsy cylinders, underwent SCT scans prior to histological sectioning and histomorphometrical evaluation. In summary, Fig. 1 sketches the process of comparing conventional histomorphometric investigations measuring the regenerated bone and degraded β -TCP particles with the SCT-based evaluation.

Synchrotron micro-tomography

Experiments were carried out at the BAM*line*, a beamline at the Berlin Electron Storage Ring Company for Synchrotron Radiation (BESSY) in Berlin, Germany. The BAM*line* is the first hard X-ray beamline at the third generation synchrotron light source BESSY [40, 41]. Its SCT facility is operated by the Bundesanstalt für Materialforschung- und prüfung (Federal Institute for Materials Research and Testing, Berlin, Germany) in cooperation with the Hahn-Meitner-Institut / Helmholtz Centre Berlin (Department Materials Research – SF3, Berlin, Germany) [40-43].

The experimental equipment used includes: a sample manipulator for the alignment, the required rotation stage for the tomography scan, and a high resolution imaging X-ray detector. Detector concepts dedicated to SCT are commonly based on projecting the luminescence image of a scintillator magnified via microscope light optics onto a CCD chip. These detectors are used to obtain radiographic projection images from third generation synchrotron light sources with a very good signal-to-noise ratio, spatial resolution up to the submicrometer range, and exposure times of only a few seconds or less [39]. SCT can be viewed as a modern 3-D microscope offering a choice of different contrast mechanisms in a non-destructive manner [22].

The optimal X-ray energy for the tomographic scans was determined experimentally to be approximately 20 keV (monochromatic): assuming that the best contrast is given when the condition $\mu \cdot D = 2$ (μ : linear X-ray attenuation coefficient, D: sample diameter) is fulfilled which equals to an overall X-ray transmission T by the sample of T = 13.5% [57]. As scintillating screen a 50 µm thick cadmium tungstate (CdWO₄) single crystal glued on a 0.5 mm thick yttrium-aluminum garnet substrate (Y₃Al₅O₁₂) was chosen. Its luminescence image is projected onto the CCD chip of a Princeton Instruments camera VersArray:2048B with 9x magnification optics (leading to an effective pixel size of 1.5 µm and a real spatial resolution of around 4 µm). The choice of the pixel size was determined by the dimensions of the sample (2.5 mm diameter) and the finite size of the CCD chip. In contrast to medical computed tomography, the sample in SCT is rotated while the light source is fixed in position (Fig. 2). The sample movements were performed with high precision linear and rotation

stages manufactured by Micos GmbH (Eschbach, Germany) [43]. In our case 1500 projection images were recorded for each CT scan of one of the bone specimens. For the reconstruction the common filtered back projection algorithm [22] was applied utilizing a software package which was developed by the SciSoft group of the European Synchrotron Radiation Facility (ESRF) [44].

Synchrotron micro-tomography - image processing

The absorption contrast in standard micro-tomography is given by the local X-ray attenuation coefficient which mainly depends on the local density of the material and the atomic number of its elements. The dependence on the X-ray energy is omitted in our case since a monochromatic beam is used [22, 23, 35-37]. The resulting values from the volume reconstruction are translated into grey-scales and the whole volume data set is then rendered in three dimensions (Fig. 3 and 6). For the rendering process of the volume images, a commercial software package by Volume Graphics GmbH (Heidelberg, Germany) was used [45].

In order to derive quantitative results from the tomographic images, first the spatial information of the different material phases (bone and ceramic particles, Fig. 4, 5 and Fig. 7, 8) was separated into Boolean images which contain only the morphological information of one phase. The algorithm used is based on a threshold hysteresis in combination with region growing: an initial range of grey values is used to define for example what certainly is bone information within the image. A second, broader range of grey values defines what might be the bone information. The algorithm extends then in successive stages already identified bone information with image pixels which are a) direct neighbors of already identified bone information and b) contain grey values within the second range. The algorithm terminates when no remaining pixels are present which fulfill the two conditions [46]. A manual optimization of the threshold parameters is required, done by visual inspection of selected

slides in gray-scales and Boolean. The results are volume images in which the very pixels associated with the bone information are marked as "1" and the remaining background is set to "0". Simple counting of ones and zeroes delivers the spatial bone density. Further algorithms can be applied on the Boolean images to reduce noise and obtain more detailed information regarding for example the orientations of objects or spatial correlations [46, 47, 59].

Histomorphometry

After completion of the SCT analysis the resin blocks which contained the biopsies were glued to acrylic slides (Plexiglas GS 209, Röhm GmbH & Co. KG, Darmstadt, Germany) using an epoxy resin based two-component adhesive (UHU GmbH & Co. KG, Bühl, Germany). 50-µm-sections were cut using a Leitz 1600 sawing microtome (Leitz, Wetzlar, Germany). These sections were then grounded and polished with 1200 and 4000 grit silicone carbide paper (EXAKT 400CS grinding system, EXAKT Apparatebau GmbH & Co. KG, Norderstedt, Germany). Prior to immunohistochemical staining, deacrylation of sections was performed by immersion in toluene, xylene and acetone. Subsequently they were rinsed in distilled water and placed in Tris-buffer (pH 7.4). Immunohistochemical staining was the performed as described previously [20] and Mayer's haematoxylin was used as a counterstain.

Histomorphometric analysis was performed on a pair of sections which were 150 μ m apart. The sections were measured semiautomatically using a light microscope (Olympus, Hamburg, Germany) in combination with a digital camera (Colourview III) and SIS AnalysisTM software (Olympus, Hamburg, Germany). Furthermore, a square area of 6.25 mm² was defined in the longitudinal plane for each specimen. In both of these biopsy areas the surface area that consisted of bone and the area that consisted of graft material were measured in mm². Additionally the area fraction of bone as well as the area fraction of grafted material was analyzed as percentage of the total area.

Results

Clinical Results

For both patients a perforation of the Schneiderian membrane, a frequent intraoperative complication that often results in an incomplete bone regeneration, did not occur. No postoperative complications occurred after sinus augmentation as well. Normal wound healing was observed after both the first (sinus floor grafting) and the second surgical procedure (implant placement surgery).

Patient 1 (TCP particles with 35% porosity)

Synchrotron micro-tomography - visualization

The analysis performed begins with the raw volume image as delivered by the reconstruction algorithm. First all voxels in the grey-scale image of the biopsy specimen which belong to the material phase "bone" were separated into a Boolean image. This separated image was the basis for a high quality 3-D visualization and a later quantitative analysis of the bone biopsy specimen (Fig. 3). The 3-D visualization of the tomographic volume image clearly shows the regenerated bone volume which formed in conjunction with degradation of the TCP particles. Minimal remains of degraded ceramic particles were partially embedded in newly formed bone which was predominantly lamellar bone with different degrees of mineralization – higher degrees in the central areas (light red colored) and lower degrees in the marginal areas of the regenerated bone areas (dark red colored).

Comparative measurements of regenerated bone area: histomorphometry vs. 2-D analysis of a SCT slice

In the biopsy specimen the β -TCP grafting material was present as achromatic rounded or scalloped granules depending on the phase of resorption. Granules were embedded or

surrounded in newly formed bone, which was predominantly lamellar bone as shown in Fig. 4 (left side). First, the plane in SCT volume image corresponding to the plane of the histological section was identified by manual scanning (Fig. 4, right side). Here, we used the fact that tomographic data acquisition started at 0 degree with the later histologic section of specimen orientated perpendicular to the incoming synchrotron beam. Thus, only meridional and sagittal manual scanning of the volume data sets was required to find the corresponding slices.

Then the histomorphometric 2-D image analysis of the histological section with the corresponding slice of tomographic data set was compared. The 2-D histomorphometric image analysis was then performed on both sets of data. The area fractions of newly formed bone as well as of grafted material were analyzed as percentage of the total of a predefined sample area. Histomorphometric semiautomatical assessment using a light microscope, digital camera and SIS AnalysisTM software shows a bone area fraction of 53.3%. For the quantitative analysis of the corresponding tomographic image we used the Boolean image of the "bone" phase (Fig. 5 - left, white areas).

Comparing 2-D-image analysis of a corresponding slice of this tomographic data set according to Boolean image showed a bone area fraction of 54.9% in this predefined sample area. The absolute difference of only 1.6% shows a good correspondence of the histomorphometric assessment using a digital camera and SIS-Analysis software and the analysis of corresponding tomographic slice of the SCT data set.

Comparative measurements: 2-D methods vs. true 3-D volume image analysis by SCT

The 3-D image analysis after processing the tomographic volume data set revealed that 48.8% of the whole biopsy specimen volume consisted of newly formed bone 6 months after sinus floor augmentation. The value of the bone volume determined by processing the 3-D SCT data differed from the values for the bone area fraction, which were determined by conventional histomorphometry, by 8.45%.

Patient 2 (TCP-P particles with 60% porosity)

Synchrotron micro-tomography - visualization

The 3-D visualization of the entire biopsy cylinder specimen acquired using SCT is shown in Fig. 6. Six months after sinus floor augmentation the different degrees of mineralization of the newly formed bone are clearly visible in terms of different shades of red colour. The blue particles represent residual β -TCP particles. When comparing the image depicted in Fig. 3 (TCP) to the image shown in Fig. 6 (TCP-P) as well as Fig. 4 with Fig. 7 it becomes evident that the TCP-P bone biopsy specimen displays a tightly-meshed bone pattern with increased density. This is accompanied by a decrease in the volume of bone marrow spaces and the yet unmineralized fibrous matrix. Furthermore residual particles of the degrading ceramic grafting material were partially embedded in newly formed bone. Degradation of TCP-P had reached a more advanced stage compared to TCP.

Comparative measurements of regenerated bone area: histomorphometry vs. 2-D analysis of a SCT slice

Identically to patient 1, we first compare the semiautomatical measurement of regenerated bone area in per cent of the deacrylated section relative to the whole biopsy specimen area (measured using a light microscope, digital camera and SIS AnalysisTM software) with the 2D image analysis of the SCT data set's slice which corresponds to this histologic section. In this patient's biopsy specimen, in which the more porous β -TCP grafting material was used, a considerably greater fragmentation of β -TCP was noted as shown by SCT (see Fig. 7). However, still only a few remaining ceramic particles, which were well embedded in regenerated lamellar bone were detectable. Histomorphometric assessment using a light microscope and SIS AnalysisTM software showed a bone area fraction of 38.8%. To derive 2-D quantitative results from the corresponding tomographic image, the density of regenerated bone tissue was measured according to the slice of the Boolean volume image

which corresponded to the histological section (Fig. 8, white areas). Here an area bone fraction of 39.0% was found.

Comparing 2-D image analysis of a corresponding slice of this tomographic data set (relative to a predefined sample area) to the conventional histomorphometric evaluation revealed an absolute difference of only 0.2%. Once more a good correspondence of the histomorphometric assessment using a digital camera plus SIS-Analysis software and the 2-D image analysis of the corresponding SCT slice was demonstrated.

Comparative measurements: 2-D methods vs. true 3-D volume image analysis by SCT

Like for patient 1, 3-D bone volume measurement of the biopsy specimen were done with a true 3-D volume imaging procedure by SCT in per cent relative to biopsy specimen volume and compared with the histomorphometric imaged bone, measured relative to corresponding slice area. 3-D image analysis showed that 36.3% of the entire biopsy specimen volume consisted of regenerated bone six months after sinus floor augmentation. The difference between the bone volume determined by SCT and the corresponding bone area fraction using conventional histomorphometry of 6.45% was therefore less compared to the case in which the less porous β -TCP was used and in which a more cancellous bone regeneration pattern was observed.

Discussion

In this paper a novel approach to evaluate human bone biopsy specimens by means of synchrotron micro-tomography (SCT) is presented. Using SCT, bone regeneration subsequent to grafting the sinus floor with different types of porous TCP materials was analyzed. Evaluation of the amount of bone formed usually is based on histomorphological data obtained from one or several histological sections [16-18, 48-50]. However, conventional histological evaluation and corresponding histomorphometric measurements provide only 2-D

information. This diagnostic procedure is associated with the risk that the very sections, which are selected for performing histomorphometric measurements, do not appropriately represent the bone volume of the entire bone biopsy specimen. Furthermore, if the regenerative potential of neighboring tissues with different morphology (alveolar process, Schneiderian membrane and periosteum) on a defect or space to regenerate is not clearly verified or unknown, 3-D analyzing methods like high resolution SCT are indicated to explore the dynamic and spatial distribution of regenerative phenomena in such complex anatomic structures like the augmented human maxillary sinus.

In a previous study, we applied immunohistochemical and histomorphometric analysis for examining biopsy specimens which were sampled six months after grafting the sinus floor using two types of TCP granules with varying porosity. In this study we demonstrated that with both tricalcium phosphate grafting materials bone formation and matrix mineralization was still actively progressing six months after augmentation of the sinus floor [20]. Thus, when using this kind of methodology the histomorphometric data are thought to represent the total volume of newly formed bone, bone marrow and matrix mineralization only on slices 50 μ m apart along the axis of the biopsy cylinder. Perpendicular to the axis of the biopsy specimen assessment of spatial trabecular orientation and subsequent quantitative estimation of bone forming process seems difficult.

In our current SCT study different patterns of bone regeneration were observed in both patient biopsy specimens. When the less porous TCP was used, a more closely meshed lamellar bone was noted compared to the patient in which the more porous TCP (TCP-P) was used. According to Peters and Reif [19] this phenomenon seems to be due to the difference in resorption time or rather biodegradation, which is caused by the difference in meso- and macroporosity, interconnectivity, crystal size and grain size of the TCP particles. During chemical degradation and biological resorption of heterogeneous calcium phosphates a decomposition into small particles takes place. Depending on the phase purity a weaker microstructure caused by more soluble phases result. Obviously both TCP used in this study showed different mechanical properties, phase purity, solubility and porosity which caused the different pattern of bone regeneration.

Furthermore, our current study, which compared conventional histomorphometry and synchrotron micro-tomography, showed that SCT images can deliver additional important information regarding the degree of mineralization of the newly formed bone. This is due to the higher density contrast using monochromatic synchrotron radiation and the suppression of beam hardening artifacts. In general, SCT allows for a high resolution, 3-D imaging with different contrast modes depending on the chosen experimental parameters. Here, our setup was optimized for absorption contrast in order to distinguish between the newly formed bone and the degrading bioceramic particles. With respect to the histomorphometric evaluation of both biopsy specimens, the values which were obtained histomorphometrically for the amount of newly regenerated bone demonstrated a very good correspondence with values derived from Boolean images of the SCT scans, in which only the morphological information of one phase - "bone tissue" - was compared in two dimensions with the conventional histomorphometric evaluation using SIS AnalysisTM software. In the case of the tightly meshed regenerated bone pattern, which was observed after sinus floor augmentation with more porous β -TCP a true amount of 36.3% of the entire biopsy volume was recorded as regenerated bone by 3-D processing of the SCT data. The difference between measured true bone volume and the corresponding biopsy area using conventional histomorphometry was therefore 2% less compared to the case in which the less porous β -TCP was used and in which a more cancellous bone regeneration pattern was observed. In both cases, a discrepancy of 6.45% and 8.45%, respectively, was observed between the histomorphometric evaluation and the true volume measurement of regenerated bone using SCT imaging. These findings suggest that in both biopsy specimens quantification of regenerated bone by 2-D histomorphometry reveal more regenerated bone than in 3-D SCT. On the other hand 2-D histomorphometry

would also sometimes underestimate the regenerated bone. An explanation for this difference could have methodical based reasons. First one has to remark that the reduction of a complex 3-D problem like the amount of regenerated bone in a biopsy specimen to a 2-D setting results in a loss of information and therefore increases the measurement error. This is emphasized when one takes a look at Fig. 3 where the difference between a selected 2-D slice and the real volume image is depictured. Investigations previously done by comparing histologic sections with laboratory micro-tomography (µ-CT) already indicated our findings [56]. Namely in that study and even by using a μ -CT system with an order of magnitude less resolving power a discrepancy between quantitative results derived from histomorphometry and μ -CT of $\pm 10\%$ was already noted. Furthermore, SCT is a non-destructive technique whereas for histologic sample preparations it is know that already the cutting and preparation process damages and therefore changes significantly the specimen [55]. Additionally, in SCT a more precise determination of the specimen's volume is possible as it can be simply derived by the area (results can be compared with the known value of the trephine burr used to biopsy the specimen) of the specimen in an axial slice multiplied by the number of slices and the pixel size. In case of 2-D histomorphometry this value is not accessible and therefore typically determined locally by the border of the specimen as visible in the histologic image. In case of a cut not perpendicular to the central axis of the columnar shaped specimen this can lead to significant distortions and increase the measurement error as well.

The main advantage of SCT (in absorption contrast mode) with respect to conventional μ -CT is the much higher photon flux used, which enables one to work with a monochromatic beam. If the specimen is inhomogeneous and consists of different materials with different densities then the higher photon flux provides better statistics and therefore a higher contrast between the different phases within the sample [58]. Additionally, the choice of an energy at which the sample fulfills the already mentioned condition of optimal transmission (T = 13.5%) allows one to avoid star like artifacts, caused e.g. by highly dense

components or the so-called beam-hardening artifacts [53]. In view of the excellent bone microstucture visualization on a histologic scale in this study SCT seems to be a more reliable non-destructive X-ray imaging method than laboratory micro-tomography and microradiography as demonstrated in other methodological studies [1, 21, 51, 52, 55]. SCT can even offer a new dimension of nanostructural insight into hard tissues when X-ray optics are used [53]. However, in contrast to SCT, micro-tomography using laboratory sources cannot render volumetric information of a biopsy specimen but only structural characteristics of the cancellous bone such as trabecular thickness, number of trabeculae and separation [1, 26]. A comprehensive study on the visualization and quantification of bone formation around titanium implants using µ-CT demonstrated the huge differences of tomograms acquired using microfocus- and synchrotron X-ray sources [54]. This research group clearly demonstrated that only the visualization of the morphology of mineralized bone tissue by SCT shows an acceptable degree of agreement with the morphology which is visualized by classical histological micrographs. As shown in our study and in contrast to histomorphometry, SCT is an excellent tool to investigate the 3-D microarchitecture of biopsy specimens based on visualization of the bone regeneration pattern and spatial organization of hard tissue structures.

Due to the limited availability of synchrotron radiation beamtime, SCT is actually not the method of choice to examine a large number of samples for a statistical verification. But with regard to the presented SCT data it is important to compare histomorphometric data and SCT data evaluating a greater number of bone biopsy specimens for gathering the required volume information in order to verify whether our findings are consistent. Consequently, a subsequent larger study has been initiated by our research group.

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Captions to Figures

Fig 1

Conventional histomorphometric investigation process for measuring the amounts of regenerated bone and degrading β -TCP particles, and SCT image processing procedure.

Fig 2

Sketch of a typical set-up for synchrotron micro-tomography: the radiation coming from the synchrotron light source passes a monochromator and then the sample. The beam as transmitted by the sample is converted into a visible light image by a scintillating screen and recorded by a CCD chip optically coupled to the scintillator [39, 43]. A sample manipulator is used to align and rotate the specimen for the tomographic scans.

Fig 3

Patient 1: high quality 3-D visualization of bone biopsy (containing the of less porous TCP) image acquired by synchrotron micro-tomography, note dark red colored areas with lower and light red colored areas with higher degree of mineralization.

Fig 4

Patient 1: Identification process of the histologic section of the biopsy with the less porous β -TCP and of the corresponding plane of the SCT volume image by a manual scanning procedure.

Fig 5

Patient 1, left: 2-D Boolean image from SCT data set for quantitative analysis of the "bone" phase represented by white areas. Right: original SCT slice in gray-scales.

Fig 6

Patient 2: high quality 3-D visualization of the bone biopsy (containing the more porous TCP) image acquired by synchrotron micro-tomography, note different areas of red brightness representing different degrees of bone mineralization.

Fig 7

Patient 2: Identification process of the histologic section of the biopsy with more porous β -TCP (top) and of the corresponding plane of SCT volume image by a manual scanning procedure (bottom).

Fig 8

Patient 2, left: 2-D Boolean image from SCT data set for quantitative analysis of the "bone" phase represented by white areas. Right: original SCT slice in gray-scales.

Fig. 1

Resin embedded bone biopsy sampled after a staged sinus floor elevation procedure



Synchrotron micro-tomography (SCT) 3D-reconstruction of volume images and visualization



Sectioning of the resin embedded bone biopsy for histomorphometric evaluation



Semiautomatically measurement of the area fraction of newly formed bone relative to the whole biopsy area using a light microscope, digital camera and SIS AnalysisTM Software



Identifying the corresponding plane of a histologic section in the SCT-volume image by a manual scanning procedure



Comparing histomorphometric 2D image analysis of the histologic slice and the corresponding slice/area of the (Boolean) tomographic data set



3-D bone volume measurement of bone biopsy in percent relative to whole biopsy volume

Comparison of the histomorphometrically determined amount of newly formed bone after sinus floor augmentation

using a true 3-D volume imaging procedure by synchrotron micro-tomography



Fig.2



















Fig. 6



Fig. 7





Fig. 8