Evaluation of substitutes for bone: Comparison of microradiographic and histological assessments

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Abstract

We created defects of standard size in the frontal bones of adult pigs and filled them with four different materials. On six occasions (at 1, 2, 4, 8, 12, and 26 weeks), samples were harvested, and evaluated by computing microradiographic images. We examined the specimens histologically as controls.

After insertion of anorganic materials, microradiographic evaluation was easy and precise, and there were no significant differences between them and the histological controls (p = 0.2). A quantitative evaluation of chemically sterilised bone by computer was not possible for more than 4 weeks.

Keywords: Microradiographic evaluation; Bone regeneration; Anorganic bone matrix; Phycogenic hydroxyapatite; Chemically sterilised bone; Animal model; Prospective study

Introduction

Autogenous bone is still regarded as the gold standard for the repair of bony defects in the maxillofacial region.  However, the quantity of bone is limited and there is additional morbidity, as harvesting autogenous bone requires a second operation. To minimise these risks, substitute materials alone or in combination with autogenous bone may be used. The advantages of these materials are unlimited supply, easy sterilisation, and storage. Despite the increase in the number of products, no single ideal material has emerged.

To evaluate bony growth after the use of new materials for bony substitution small rodents are commonly used, but, for research into bony growth in the maxillofacial region, pigs are more suitable. Histological analysis is considered the gold standard for evaluation of the growth of bone in bony defects. However, it has limitations, one of which is that the histological section shows only one specific area of the cross-section, which does not necessarily reflect the entire defect. The diversity in histological scoring systems also makes comparison among studies difficult. Another option is microradiography, which provides a picture of bony growth with high-resolution radiographs. It detects bony boundaries with accuracy, obtains images from an entire defect, and makes it possible to measure areas of growth of bone.

Microradiography produces images of mineralised structures. Currently available substitutes for bone are made of many diverse materials. At present, no data are available from comparative in vivo studies about the influence of the composition of a substitute on the result of microradiography. Our aim, therefore, was to compare microradiography of four...
bone-graft materials: two anorganic bone matrices, a phycopgenic hydroxyapatite, and chemically sterilised bone (Fig. 1). The study was approved by the local animal ethics commit-
te of the government of Midfrankonia, Ansbach, Germany (approval 621-2532.31-5/00).

Material and methods

Materials for bone grafting

Anorganic bone matrix (ABM 1; OsteoGraf®/N-300; Dentsply Friadent, Mannheim, Germany) is deproteinised bovine bone (1100 °C; hydroxyapatite) with particles ranging from 0.25 to 0.42 mm (Friadent GmbH Mannheim, Germany; Produktinformation, 2003). The second anorganic bone matrix (ABM 2; Bio-Oss®; Geistlich Pharma AG, Wolhusen, Switzerland) is separated from all organic material by a stepwise annealing process (up to 300 °C), followed by chemical treatment (sodium hydroxide) that leaves a porous hydroxyapatite material. The porous phycopgenic hydroxyapatite derived from algae (PHA; FRIOS® Algipore®; Dentsply Friadent, Mannheim, Germany) is prepared by hydrothermal conversion of the calcium carbonate of algae in the presence of ammonium phosphate at about 700 °C. This process preserves the porosity of the algae. The material is in particulate form with particles ranging from 0.3 to 2 mm, and pores in the range of 5–10 μm.

Chemically-sterilised bone (CSB; Navigraft®; Tutodent, Neunkirchen, Germany) is a bovine graft treated by osmoly-
sis, sodium hydroxide, hydrogen peroxide, and acetone. The size of the granules is between 0.25 and 2 mm. The inter-
connecting porosity of the bone is still present (pore sizes of some 100 μm).

Animals

The pig’s morphological and anatomical characteristics allow the results obtained to be transferred to humans. In comparison with a dog, rates of regeneration of bone in adult pigs have greater correlation with those in humans (pigs 1.2–1.5 mm/day; dogs 1.5–2.0 mm/day; humans 1.0–1.5 mm/day). We studied 12 adult female pigs.

Test groups

Four test groups were formed and examined at six different times (1, 2, 4, 8, 12, and 26 weeks). Two animals were killed at each test period, each of which had nine bony defects. The first experimental groups consisted of four defects in each:
ABM 1, ABM 2, PHA, and CSB. There were two controls: one contained autogenous bone, and there was one empty defect.

Operations

The operations were similar in all animals, caused only minor pain, and did not impair the pigs’ ability to move. All operations were done under general anaesthesia with intubation. The animals were given streptomycin, 500 mg/day 1 h preoperatively and for 2 days postoperatively.

An incision was made in the skin and the periosteum of the skull, and nine identical bony defects were created with a 1 cm trephine drill (Roland Schmid, Fuerth, Germany). The size of the defects (10 mm in diameter, 10 mm deep) met the requirements for a critical size defect in pigs. Without a suitable augmentation material such defects are not completely regenerated with bone, but are partly filled with connective tissue. The forehead was chosen because this bone is of desmal origin, and does not depend on a central blood supply. The internal plate of the neurocranium was left intact. The bone harvested with the trephine drill was used to fill the defects. The individual defects were separated using a standard cutting system (Exakt). These were reduced to 150–180 μm with a grinding unit (Exakt Apparatebau GmbH, Norderstedt). The samples were radiographed in the Faxitron® cabinet radiographic unit using 11 kV tube voltage and 0.25 mA for 6 min, with virtually no gap between the specimen and the film, to attain as high an exposure as possible. The developed radiographs (Kodak, Stuttgart, Germany) were scanned with an AGFA scanner at 1200 dpi and 12-bit grey-scale, and stored in Tiff-format.

Necropsy and preparation of the specimens

Two animals were killed at each time period with an intravenous injection of 20% pentobarbitone solution. The skull caps were removed and immediately frozen at −80°C. Before the specimens were prepared, a radiograph was taken (40–45 kV, 0.25 mA, and 5 min) in a Faxitron® cabinet unit (Faxitron Cabinet X-ray Systems, Illinois, USA), to detect the defects. The individual defects were separated using a standard cutting system (Exakt Apparatebau GmbH, Norderstedt, Germany), and the specimens were fixed by immersion in 4% paraformaldehyde at 4°C to render the organic matrix insoluble. They were dehydrated in an ascending series of alcohol at room temperature in a dehydration unit (Shandon Citadel 1000, Shandon GmbH, Frankfurt, Germany). Xylol was used as an intermediary fixative. They were embedded in Technovit® 7200 (Heraeus Kulzer, Kulzer Division, Werheim, Germany) and polymerised under a blue light for 8 min. The stained sections were examined under a light microscope (Axioskop, Zeiss, Jena, Germany) and the data were fed into a computer through the attached video camera. The KS 300 software (Zeiss, Jena, Germany) was used.

Histomorphometry

The specimens, which had been further reduced to 30 μm and polished, were first stirred continuously for 5 min in 10% hydrogen peroxide, then rinsed under cold running water, dried, and stained for 15 min in toluidine blue O solution. Any excess stain was removed by rinsing the specimens under running water. They were allowed to dry briefly, then sections were coated with Technovit® 9100® (Heraeus Kulzer, Kulzer Division, Werheim, Germany) and polymerised under a blue light for 8 min. The stained sections were examined under a light microscope (Axioskop, Zeiss, Jena, Germany) and the whole area of the defect could be evaluated, specific regions of interest (ROI) had to be defined in the histological control sections.

Analysing the images

Osiris image analysis software (Digital Imaging Unit, University Hospital of Geneva, Switzerland) was used for analysing the images. With this software it is possible to calculate the percentage of individual grey values or colour scaling in a grey-scale image, and allows a percentage of each individual image to be given a specific colour. These percentages were classified into bone, bone augmentation materials, and voids.

For control reasons, histological sections were evaluated similarly. However, whereas in the microradiographic image the whole area of the defect could be evaluated, specific regions of interest (ROI) had to be defined in the histological control sections.

Statistics

The microradiographs were each analysed by two workers and the values were added for each specimen. The mean and range of these values were then given for each group. The AXUM program (Math-Software, Cambridge, MA, USA) was used for the graphic images. Box plots were created,
each covering all groups at a certain time point. The results were then verified using the Wilcoxon Rank test (with 95% confidence intervals (CI)). A difference was accepted as significant if the probability was less than 0.05.

Results

All the animals survived the operation and each of the postoperative examinations, and could be evaluated at the end of the experiment. During healing we found no differences between the materials for bone replacement and autogenous bone as regards local tissue compatibility. The histological assessment showed that there was no connective tissue layer between the bone replacement materials and the local bone. The osseointegration of each material could therefore be evaluated.

Materials for the bony augmentation varied in their radiopacity: in the CSB group we found values nearly similar to local bone. However, in the early stages, each material could be clearly distinguished from bone because of their different structures. Newly formed bone had less mineralisation than local bone, the architecture of the spongiosa was different, and there was no alignment of the trabeculae. Shrinkage of the specimens in the dehydration process tended to lower the absolute values of mineralised areas.

One defect at each removal time (control 1) was filled with autogenous bone (Fig. 2), the so-called gold standard of bone substitute materials. The first signs of bone formation were visible in the microradiographic image after 2 weeks. After 4 weeks, new bone had formed in two-thirds of the defect, leaving a residual superior area. At 12 weeks new bone had formed in the cross-sectional area. The structures of the newly formed bone were still partly heterogeneous with sections of compact and spongy bone. At the end of the evaluation period (at 26 weeks) the bony structure was regular and the original margins of the defect were no longer visible. The image largely corresponded with that of physiological bone (Fig. 2).

A second defect (control 2) remained unfilled (Fig. 2) and served as a control. The microradiographic examination of these samples confirmed the validity of the experiment. During the postoperative period of 26 weeks there was only incomplete bony regeneration with a residual defect of about a quarter of the area of the original defect (Fig. 2).

Anorganic bone matrix (ABM 1 and ABM 2)

This showed results similar to those of control 1, with signs of bone formation near the edges after 2 weeks. The formation of bone progressed evenly over the course of the study until its end at 26 weeks. After the insertion of ABM 1, newly formed bone was measured in a mean (S.D.) of 32% (8.6%) of the area of the defect. After the insertion of ABM 2, new bone was found in 44% (4.4%) of the cross-sectional area. There were significant differences in the osteoconductive capabilities between the materials (p = 0.05). However, the limited number of specimens has to be considered when assessing the importance of these data.

At the end of the trial there was virtually complete bone filling of the defect with ABM particles enclosed in a bony covering of the whole cross-section after the use of ABM 2, and the lower two-thirds after the use of ABM 1 (Fig. 3).

Microradiographic evaluation was further used to determine the stability of the bone substitutes. When ABM 1 was applied initially, the percentage of material remaining after 26 weeks was 44% (9.9%). A control evaluation of the histological sections showed a value of 41% (11.4%) (p = 0.2). After the use of ABM 2, the remaining bone substitute material filled 21% (5.4%) after 26 weeks. Histological controls showed 19% (9.8%) (p = 0.7).

After the application of anorganic bone matrix, microradiographic evaluation was easy and precise. There were no significant differences from the control evaluation of the histological sections.

Phycogenic hydroxyapatite (PHA)

After 2 weeks, the defects had been filled with the bone substitute material and there were a few irregular immature bone

Fig. 2. Microradiographic images of the control groups. (1) Control 1 (autogenous bone) after 1 week and (2) after 26 weeks (area of defect indicated by green box), showing complete bony regeneration. (3) Control 2 (empty defect) after 1 week and (4) after 26 weeks showing incomplete bony consolidation of area of defect with a residual defect of roughly a quarter of the cross-section.
Fig. 3. Microradiographic and light microscopy images of the ABM-materials ABM 1 (1–4) and ABM 2 (5–8) after 1 week (1 and 5), 12 weeks (2 and 6), and 26 weeks (3 and 7). Bone substitute materials are visible at all evaluation times; light microscopy of similar samples (4 and 8) after 26 weeks (toluidine blue O staining, original magnification ×10, asterisk marks the remaining particles of bone substitute), showing a picture largely corresponding to the microradiographic imaging and light microscopy at the end of the survey in group ABM 1 and group ABM 2.

Trabeculae at the borders. The bone substitute could be differentiated by the fact that the particles were round. However, the microradiographic image produced similar grey values in the bone substitute and the surrounding bone so computer recognition of the material sometimes failed to differentiate between them, and a visual adjustment of the computer recognition was made in all samples. A total of 52% (6.1%) of the defect was filled with new bone after 26 weeks. On microradiography 14% (4.2%) of material was remaining at that time. The histological sections showed a much better contrast between new bone and the material remaining (Fig. 4). These sections showed 20% (5.2%) material remaining ($p = 0.38$).

**Chemically sterilised bone (CSB)**

The area of the defect and the material used for filling it were clearly visible after 1 week (Fig. 4). Resorption became

Fig. 4. Microradiographic and light microscopy images in PHA group (1–4) and CSB group (5–8) after 1 week (1 and 5), 12 weeks (2 and 6), and 26 weeks (3 and 7). In the PHA group converging grey values disturb the calculation by the software, so visual marking is sometimes necessary. (4) Light microscopy of a similar sample after 26 weeks with PHA-remnants visible, (toluidine blue O staining, original magnification ×10, asterisk indicates PHA). Microradiographic imaging and light microscopy correspond at the end of the trial even though computer detection of the bone substitute in the radiograph may fail. In the CSB group similar grey values of bone substitute and local bone are shown from the beginning of the study, and though discrimination by different architecture of the spongiosa and lack of trabeculae is possible early on, differentiation in the micrographic images is impossible at 12 and 26 weeks. (8) Light microscopy at high magnification of a similar sample after 26 weeks shows the remaining particles of CSB, (toluidine blue O staining, original magnification ×20, asterisk indicates CSB). Only light microscopy allows definite identification of bone substitute.
obvious after 2 weeks. From 4 weeks onwards, microradiographic imaging failed to differentiate precisely between remaining bone substitute and newly formed bone (Fig. 4). After 26 weeks the microradiographic image largely coincided with that of physiological bone. Only the total amount of mineralised tissue in the area of the former defect could be measured, and showed 52% (7%). Histological examination at a high magnification (×20) showed small particles of the material enclosed in new bone at the end of the trial (Fig. 4).

Discussion

After the insertion of an anorganic bone matrix, microradiographic assessment was easy and precise, and there were no significant differences between these and the control histological sections. Microradiography is therefore a valuable technique for such materials. After the insertion of phycogenic hydroxyapatite, computer measurement of the remaining PHA in the microradiographic images was difficult and tended to produce incorrect values. However, we found no significant difference between the results of the microradiographic and histological examinations. In contrast, it was not possible to make a reasonable quantitative evaluation of chemically sterilised bone by computer after 4 weeks. Neither microradiographic imaging nor histological examination differentiated between remaining bony substitute and newly formed bone.

Substantial advances have been made in developing substitutes for bone grafts during the past 20 years. These materials share numerous advantages over autografts including unlimited supply, and easy sterilisation and storage. Before being applied in the treatment of patients, new bone substitute materials must be tested using standard protocols. Microradiography has been used in both experimental and clinical studies. It provides high-resolution microradiographs in the plane of the defect, so that patterns of bony growth may be seen. They allow the evaluation of formation of new bone without the undesirable effects of the action of a decalcifying solution on bony tissue. Our method of preparation of specimens not only allows microradiographic and light microscopic evaluation of different portions of the same sample of embedded bone, but an immunohistological assessment of the sample can also be done if necessary.

However, microradiography does have limitations. It is impossible to follow bony growth at the cellular level (in contrast with histology), and only calcified tissue can be detected. Although microradiography is capable of establishing whether or not bone is present (qualitative), a lateral microradiograph does not allow the calculation of the volume of bone (quantitative). In a recent study, Schortinghuis et al. showed that defects in the mandibles of rats were about 5% larger when measured by histology than when measured by microradiography. We conclude that microradiography is a helpful and reliable method in the evaluation of many clinically applied substitutes for bone.

References


