Ph.D. Thesis

Investigation of the Osseointegration of Dental Implants and Different Biomaterials Used in Guided Tissue Regeneration

Danica Matusovits M.D.

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PUBLICATIONS RELATED TO THE THESIS

Articles:

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Clin Oral Impl Res, 17; (1) 102-108, 2006 **IF: 2.497**

Suba Zs, Takács D, **Matusovits D,** Fazekas A, Szabó Gy, Barabás J: A maxilla csontregenerációjának mennyiségi és minőségi összehasonlítása β-tricalcium phosphate és autolog csontbeültetés után.
Fogorvosi Szle 99; (1) 21-28, 2006

Matusovits D, Suba Zs, Takács D**,** Turzó K, Donath K, Fazekas A**:** A pilot study of Cerasorb and Bio-Oss enhanced bone formation in animal model.
Acta Biologica Hungarica 59; (3):327-34. 2008 **IF: 0.688**

Matusovits D, Suba Zs, Takács E, Donath K, Turzó K, Fazekas A**:** Bio-Oss és Cerasorb keverék csontképzés serkentő hatásának vizsgálata állatkísérletekben.
Implantológia 5; (2): 60-62, 2008

Published Abstracts:

1. Radnai M, Pelsoczi I, Bereznai M, Toth Z, Turzo K, **Matusovits D,** Bor Z, Fazekas A: Excimer laser treatment of Ti dental implants to promote surface characteristics for bioactivation. Abstr. 16. 6th Interdisciplinary Essen-Symposium of the Working Group on ”Biomaterials and -Tissue Compatibility” 8-10 October 2003, Essen, Germany

2. **Matusovits D**, Perényi J, Turzó K, Radnai M, Donath K, Jennissen HP, Fazekas A: Study of the efficacy of rhBMP-2 on osseogenesis in animal model. Az MFE Fogpótlástani Társasága XVI., a Magyar Fogorvosok Implantológiai Társasága VI., a Magyar Parodontológiai Társaság XIV. kongresszusa. Sopron, 2005. október 13-15. Fogorvosi Szle 99; (2) 74, 2006.

3. Turzo K, Stajer A, Ungvari K, Pelsoczi I, Polyanka H, Oszko A, **Matusovits D,** Mihalik E, Rakonczay Z, Radnai M, Fazekas A: Investigation of the corrosive effects of fluoride on titanium. 0289 (85184) IADR PEF Dublin, September 13-16, 2006 J Dent Res 85: B 289 (85184) PEF, 2006 (IF abstract: 3.192)

4. **Matusovits D,** Laub M, Perenyi J, Turzo K, Radnai M, Donath K, Jennissen HP, Fazekas A: Bone rebuilding enhancement induced by rhBMP-2 solution in animal model. 0417 (85430) IADR PEF Dublin, September 13-16, 2006 J Dent Res 85: B 0417 (85430) PEF, 2006 (IF abstract: 3.192)

5. Laub M, **Matusovits D**, Chatzinikolaidou M, Donath K, Turzo K, Radnai M, Fazekas A, Jennissen HP: Critical Size Femoral Defects in Rabbit as a New Model for Testing rhBMP-2 Osteoinductivity. P-Sat-A-069 8th World Biomaterials Congress Amsterdam, 28 May-1 June 2008

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1. Introduction

1.1. Osseointegration of dental implants

Dental implantology, a special field of dentistry dealing with the rehabilitation of the damaged chewing apparatus due to loss of the natural teeth, is currently the most intensively developing field of dentistry. Missing teeth can be replaced by dental implants (artificial roots), which are inserted into the root-bearing parts of the mandible or maxilla. The success and long-term prognosis of implant prosthetic therapy depend primarily on the anchorage of the implant in the jawbone, *i.e.* on the **osseointegration**. Today, there are ever increasing demands from patients with missing teeth for masticatory function and aesthetic appearance of their replaced teeth to be restored and for shortening of the period of osseointegration of the implants, which takes a relatively long time (3-6 months).

The successful insertion of a biocompatible material into living tissue with little to no evidence of rejection has revolutionized medicine and dentistry. In the 1960s, Brånemark *et al.* stumbled upon this phenomenon when using titanium (Ti) in animal models, with little idea of the impact this discovery would have on the rehabilitation of future medical and dental patients. This phenomenon, described as “osseointegration”, was characterized by a number of clinical and ultrastructural observations. Osseointegration may broadly be defined as the dynamic interaction and direct contact of living bone with a biocompatible implant in the absence of an interposing soft tissue layer [1-3].

Although the clinical term osseointegration describes the anchorage of endosseous implants to withstand functional loading, it provides no insight into the mechanisms of bony healing around such implants. However, in the last decade it became clear that the long-term success of dental implants also depends on the complex **biointegration** of these alloplastic materials, which is determined by the responses of the different surrounding host tissues (the alveolar bone, the conjunctival part of the oral soft tissues and the gingival epithelium). Nevertheless, an understanding of the sequence of bone-healing events around endosseous implants is believed to be critical in developing biologic design criteria for implant surfaces. Bone growth on the implant surface can be phenomenologically subdivided into three distinct phases that can be addressed experimentally [4]. The first, *osteoconduction*, relies on the migration of differentiating osteogenic cells to the implant surface, through a temporary connective tissue scaffold. Anchorage of this scaffold to the implant surface is a function of the implant surface

design. The second, *de novo bone formation*, results in a mineralized interfacial matrix, equivalent to that seen in cement lines in natural bone tissue, being laid down on the implant surface. The implant surface topography determines whether the interfacial bone formed is bonded to the implant. A third tissue response, the *bone remodelling*, creates a bone-implant interface comprising *de novo* bone formation. Treatment outcomes in dental implantology depend critically on the implant surface designs that optimize the biological response during each of these three distinct integration mechanisms.

Today, much effort is devoted to the design, synthesis and fabrication of Ti dental implants in order to obtain long term (lifelong) secure anchoring in the bone. Fundamentally, this means the ability of then implant to carry and sustain the dynamic and static loads that it is subjected to. The bulk structure of the material governs this ability. Evidently, it is important to achieve a proper function in the shortest possible healing time, with a very small failure rate and with minimal discomfort for the patient. These factors are also important for cost reasons. As regards osseointegration, *i.e.* the formation of a direct connection between the living bone and the surface of the load-carrying implants, the important question arises as to how to attain a better integration by modification of the implant surface morphology.

A wide variety of materials have been used to produce endosseous implants [5,6]. Currently, Ti and its alloys are the most commonly utilized dental and orthopaedic implant materials that meet the most important requirements [7,8]. The properties of Ti and its surface, which is covered by a native oxide layer, are appropriate to allow its use as a biocompatible material [9]. At a cellular level, the relationship of an implant with the surrounding tissue is highly dependent on the interaction between the passive titanium oxide (TiO_2) which is formed on the surface of a Ti implant, and biological elements such as collagen, osteoblasts, fibroblasts and blood constituents [2,10]. The TiO_2 layer is very stable, corrosion-resistant and may be manipulated to have variable thickness.

The clinician is often faced with the challenge of identifying the successful osseointegration of a dental implant. Clinical success is determined by a lack of mobility and by the ability of the implant to resist functional loading (chewing force) without mechanical deformation and to transfer the load onto the alveolar bone without deterioration of the bony interface [11]. Radiographically, the bone should appear to be closely apposed to the implant surface. The resolution currently achievable in medical imaging, however, is several orders of magnitude less than what is required to observe a soft tissue cell. Accordingly, radiographic assessment alone is unsuitable to determine with certainty whether a soft tissue is present [12]. A number of studies have analysed this bone to Ti interface histologically and ultrastructurally, often

with inconsistent findings. The difficulty arises primarily with the need to prepare and section the specimens without changing or damaging the interface. Recent studies have utilized CT scanning to obtain a 3-dimensional picture of the implant interface [13,14].

1.2. Biomaterials used in guided bone regeneration (GBR)

The aesthetic and functional demands of the patients have recently increased enormously. In dental implantology, new biomaterials and available surgical techniques furnish excellent possibilities.

However, there are certain fundamental weaknesses in the current technology. Patients must have suitable morphology and a sufficient amount of available jawbone for reconstruction to be a viable option. After extraction or the loss of teeth for any other reason, the edentulous alveolar ridge resorbs. Consequently, its dimensions and morphology, especially as concerns the labial plate, rapidly become inadequate for the appropriate accommodation of artificial roots. To preserve the height and width of the alveolar bone for future implantation therapy, guided tissue regeneration (GTR) procedures are used [15].

GBR has become a routinely applied method in dental implantology. Most of the dentoalveolar regenerative techniques require osteoconductive material in order to establish new bone formation in the necessary anatomical form. GBR is a surgical procedure that makes use of barrier membranes to direct the growth of new bone at sites having insufficient volumes or dimensions for function or prosthesis placement. GBR is similar to any other GTR utilized in dental therapy, but is focused on the development of bony tissues instead of soft tissues of the periodontal attachment. At present, GBR is predominantly applied in the oral cavity to support new hard tissue growth on an alveolar ridge so as to allow the stable placement of dental implants. Used in conjunction with a sound surgical technique, GBR is a reliable and validated procedure [16].

Xenograft bone substitutes originate from a species other than human, *e.g.* bovine. Xenografts are usually distributed only as a calcified matrix. **Bio-Oss** is a safe, effective xenograft: a deproteinized, sterilized bovine bone with 75-80% porosity. It is reported to be highly osteoconductive and biocompatible. It is known that Bio-Oss serves as a scaffold in GBR, but, due to its poor resorbability, it may exert a negative influence on the structure of the newly-formed bone. The large-mesh interconnecting pore system facilitates angiogenesis and the migration of osteoblasts. It has been found clinically that its resorption is very similar to that of human bone [17-19].

Pure beta-tricalcium phosphates (TCP-β) such as **Cerasorb** are widely used osteoconductive materials. The chemical characteristics of Cerasorb allow it to resorb completely and quite rapidly during new bone formation. This may result in too early resorption in some cases without fulfillment of the clinical requirement of the space-maintaining function [20,21]. These bone-substitute materials allow targeted bone regeneration as they facilitate construction of a base on which implants can be positioned and further stabilized. Cerasorb has good osteoconductive and resorption properties [21,23]. Full resorption over a defined period of time, with simultaneous transformation into autologous bone, is of particular significance in this respect. Because of its rounded surface and chemical composition, Cerasorb is remarkably bioinert and is therefore particularly suitable for innovative procedures. The unique open porosity structure increases active cellular in-growth and improves nutrition, while the rough surface further increases osteoconductivity. The result is the rapid in-growth of local bone and a significantly shorter resorption time (6-12 months) compared with other ceramic products [22].

Calcium phosphate cements (CPCs, *e.g.* **Vitalos**) are an emerging class of bone-substitute materials that are capable of rapid setting to a hard mass, providing a scaffold for the bone-remodelling process. The CPCs synthetic bone graft materials invented in the 1980s, consist basically of tricalcium phosphate and anhydrous dicalcium phosphate. Many different combinations of calcium and phosphate have been developed as commercial CPC materials [24].

Hydroxyl-apatite (HA) is the main component of VitalOs and the primary inorganic component of natural bone which makes the hardened cement biocompatible and osteoconductive. Over time, CPC is gradually resorbed and replaced by new bone. CPC has two significant advantages over pre-formed, sintered ceramics. First, CPC paste can be sculpted during surgery to fit the contours of the wound. Second, the nanocrystalline HA structure of the CPC makes it osteoconductive, causing it to be gradually resorbed and replaced by new bone. Recent work with CPCs has focused on improving the mechanical properties, making premixed CPCs, giving the CPCs macroporous properties and seeding cells and growth factors into the cement [25].

CPCs are identified as alloplastic materials appropriate for osseous augmentation because of the unique combination of osseoconductivity, biocompatibility, mouldability and malleability. In contrast with conventional bone graft materials, CPCs can be directly moulded and shaped to fill intrabony defects. Moreover newly-developed CPCs are fully injectable, which ensures easy handling and appropriate application of these materials [26].

1.3. Surface modifications of Ti implants to improve osseointegration

The biological responses of the surrounding tissues to dental implants are controlled largely by their surface characteristics (chemistry and morphology). The biorecognition takes place at the interface of the implant and host tissue [27]. Biological tissues interact mainly with the outermost atomic layers of an implant, which measure about 0.1-1 nm. The molecular and cellular events at the bone-implant interface are not yet fully understood and there are still some uncertainties concerning the molecular structure of the bone-implant interface [28,29].

The rationale for the surface modification of implants is straightforward: to retain the key physical properties of an implant, while modifying only the outermost surface layer to influence the bio-interaction. As a result, much research work is devoted to the elaboration of methods of modifying surfaces of existing implants (biomaterials) in order to achieve the desired biological responses.

These responses can be several: in a healthy patient it may be a regular osseointegration process, but an older or even an ill patient a smaller bone quantity or a not ideal bone quality means a handicap in biointegration. These cases are often avoided by appropriate patient selection. As the length of the average human lifetime is increasing, more and more people are living with missing teeth and in widely differing status of health. There is a demand at present for the optimization of osseo/biointegration processes (reducing the 3-6-month healing period) even for people in different status of health.

For dental implants, as for other biomaterials, the bio- and osseointegration processes can be controlled at molecular and cellular levels by modification of the implant surface. There are various surface-modification possibilities, which are usually subdivided into **physicochemical** and **biochemical methods** [28].

1.3.1. Physicochemical methods

The most common physicochemical treatments are chemical surface reactions, ***e.g.*** oxidation, acid-etching, sand-blasting, ion implantation, laser ablation, surface coating with calcium phosphate, *etc*. These methods alter the energy, charge and composition of the existing surface, but can lead to surfaces with modified roughness and morphology.

The surface energy plays an important role not only with regard to protein adsorption, but also as concerns cell attachment and spreading [30]. The surface charge influences both the molecular or cellular orientation and the cellular metabolic activity [31].

The roughness of the implant surface plays a significant role in anchoring cells and connecting together the surrounding tissues, thereby leading to a shorter healing period. These surfaces display advantages over smooth ones as the area of contact is enlarged by micro-structuring the implant surface. Acid-etching, sand-blasting and Ti plasma-spraying are typical methods for the development of rough surfaces and are well documented with *in vitro* and *in vivo* methods [32-35].

Ion implantation methods are generally used to improve the mechanical quality of an implant. For example iridium has been ion implanted in the Ti-6Al-4V alloy to improve its corrosion resistance [36] and the implantation of nitrogen into Ti reduces wear significantly [37].

To increase the roughness of solid surfaces, a number of laser-based techniques have been applied in the last decade [38]. The advantages of using lasers for the ablation of surfaces are the precise control of the frequency of the light, the wide range of frequencies available, the high energy density, the ability to focus and raster the light, and the ability to pulse the source and control the reaction time. Lasers commonly used for surface modification include ruby, Nd:YAG, argon, CO_2 and excimer [39,40]. Besides the prompt intense heating of the surface, excimer laser illumination may further enhance the sterilizing effect in consequence of the high dose in the UV range [41].

Inorganic materials, such as the bioreactive calcium phosphate (CaP) coatings (or HA), have been extensively applied because of their chemical similarity to bone minerals. Several studies have shown that these coatings achieve a very intimate contact between the implant and bone [42,43]. Clinical investigations have reported a high degree of success with HA-coated implants, with a reduction of the healing period [44]. However, in other studies, HA-coated implants showed signs of the covering material peeling off from the implant surface, which may induce foreign body reactions [45,46]. Furthermore, a long-term clinical study of HA-coated oral implants indicated a significantly lower survival rate (77.8% after 8 years) for HA-coated implants as compared with TPS-coated (Ti-plasma-sprayed) implants (92.7%) [47]. The biodegradation of these coatings may be the reason why HA coatings are no longer the surface modifications of choice.

1.3.2. Biochemical methods

For implants, the goal of biochemical methods is to immobilize peptides, proteins and enzymes on the surface in order to induce specific cell and tissue responses (adhesion, signaling and stimulation) and to control the tissue-implant interface with molecules delivered there directly [28].

Numerous different biologically functional molecules can be immobilized onto Ti surfaces to enhance bone regeneration at the interface of implant devices. One essential aspect is the maintenance of the bioactivity (or the recognizable binding site) of these molecules during their incorporation into a biomimetic coating.

CaP coatings and the purely organic components of bone can serve as carrier systems for osteogenic drugs, thereby rendering them osteoinductive and osteoconductive. The most promising candidates for osteogenic agents are the members of the transforming growth factor-β (TGF-β) superfamily, such as bone morphogenic proteins (BMPs).

Following its successful coprecipitation with the inorganic components and incorporation, **BMP-2** retains its biological activity *in vitro* [48]. The application of BMPs to improve the present implantation techniques appears rather promising [49,50]. BMPs such as rhBMP-2 (recombinant human BMP-2) are growth factors that could be employed to augment the resorbed alveolar ridge prior to implantation.

BMP-2 is a member of the TGF-β superfamily of multifunctional cytokines. Mature BMP-2 is a homodimer of two subunits, each consisting of 114 peptides [49]. The two chains are held together by a single disulphide group. The monomers contain six additional cysteine residues, which are involved in three intrachain disulphide linkages. The cysteine residue is characteristic of all members of the TGF-β superfamily [51] (Fig. 1 a,b).

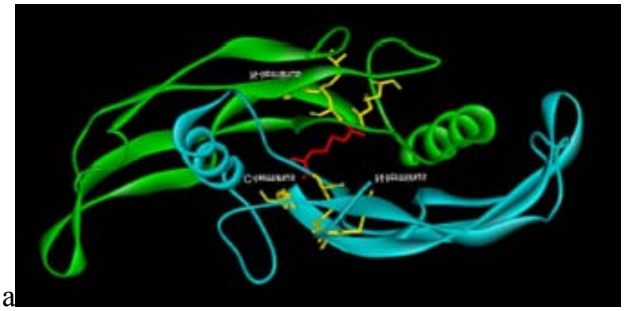
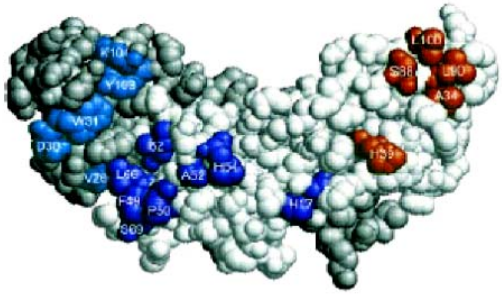
a  b 

Fig. 1a, b *The molecular structure of rhBMP-2*

BMPs induce bone formation and regeneration, and thereby play important roles in repair processes. BMP-2 exhibits high osteoinductive properties as it attracts osteoprogenitor cells and directs their differentiation into osteoblasts. When used in conjunction with implants, BMPs form a monolayer on the surface of the device, which causes cell proliferation, thereby increasing the incorporation of the device. Overall, the main effect of BMPs (including rhBMP-2) is the stimulation of bone growth through an increase in cell differentiation [52,53].

At present, four major strategies exist for organic coating approaches: immobilization of extracellular matrix (ECM) proteins (collagen, *etc*.) or peptide sequences as modulators for bone

cell adhesion, deposition of cell signalling agents (bone growth factors) to trigger new bone formation, immobilization of DNA for structural reinforcement and enzyme-modified Ti surfaces for enhanced bone mineralization [48].

Biomolecules can be immobilized by physical absorption (van der Waals or electrostatic interactions), physical entrapment (use of barrier systems) and covalent attachment. The selection of the immobilization method depends on the working mechanism of the specific biomolecules, which dictates, for instance, a short-term, transient immobilization for growth factors and a long-term immobilization for adhesion molecules and enzymes.

The cell membrane receptor family of integrins is involved in cell adhesion to ECM proteins. These integrins bind to specific amino acid sequences within ECM molecules and in particular to the RGD (arginine-glycine-asparagine) sequence. For this reason, the most commonly used peptide sequence for surface modification is the above-mentioned cell adhesion motive [54,55].

Our group has developed a **polyelectrolyte (PE) multilayer (ML)** surface modification involving the alternating adsorption of poly-cations (poly-L-lysine (PLL)) and poly-anions (poly-L-glutamic acid (PGA)) from aqueous solution onto a charged, solid surface. PE film coatings modify the solid/liquid interface in such a way as to ensure a suitable environment for the adsorption of proteins. The alternating adsorption technique has been successfully applied in different fields of science, as a consequence of its numerous practical applications. It can be automated, it involves the use of aqueous solutions, it is environment-friendly, and various substrates can be covered with films of readily variable thickness [56].

In consequence of its structural properties, DNA is of high potential for application as a biomaterial coating, regardless of its genetic information. Additionally, DNA can be used as a drug delivery system since its functional groups allow the incorporation of growth factors. The studies by van den Beucken *et al.* [57] proved that DNA-based coatings improve the deposition of CaP.

A relatively new approach for surface modification is enzyme-modification of the Ti surface to enhance bone mineralization along the implant surface. In particular the enzyme alkaline phosphatase (AP) is known to increase the local concentration of inorganic phosphate, and to decrease the concentration of extracellular pyrophosphate, a potent inhibitor of mineralization [58].

In the past decade, another viable biomimetic strategy has appeared: organic-inorganic composite coatings. These mimic the bone structure, which is composed of an organic matrix (90% of which comprises collagenous proteins) and an inorganic CaP phase. Collagen-CaP [59],

growth-factor-CaP [60] and PE multilayers-CaP [61] composite coatings have been developed and have furnished promising *in vitro* and *in vivo* experimental results.

Many of the above-mentioned biochemical methods are still in the experimental stage and the *in vivo* applications (animal or clinical studies) are still ahead. It is believed that these surfaces will make an enormous positive contribution to clinical implant science, especially if the older subjects or patients are targeted. Hence, our group has started tests on some of these surface modifications and biomaterials by means of animal (rabbit and pig) experiments.

1.4. Animal models for the investigation of osteogenesis and osseointegration

Researchers often use laboratory animals as models of humans. The use of animal models in oral health science has increased significantly over the past 20 years. In attempts to understand the onset and dissemination of different oral diseases and to identify and develop dental materials and methods suitable for the restoration of the damaged tissues, animal experiments are of fundamental significance. A specific model is chosen because it is believed to be appropriate to the condition being investigated and is thought likely to respond in the same way as humans to the proposed treatment for the character being investigated.

After the model has been chosen, it is essential that any experiments in which it is used are well designed, *i.e*. are capable of demonstrating a response to any treatment applied. If the model happens to be insensitive or the experiments are badly designed (*e.g*. the use of too few animals) so that they are incapable of distinguishing between the treated and control groups, the model is not appropriate for its purpose.

When new animal experiments are introduced five key features of the animal models used in biomedical research must be considered [62-64]:

1. There can be substantial asymmetry between the model and the target in the numbers of similarities and differences. In theory, the model and the target only need to have a single feature in common, but there can be any number of differences. This means that useful models can sometimes be highly abstract, such as a mathematical equation or computer simulation. Moreover, the more fundamental the biological process, the more likely it is that the animal model and humans will respond similarly.

2. Some differences between the model and the target are necessary; otherwise the animal would not be a model. Differences are as important as similarities as they allow us to do things with the model which would not be possible with a human. Mice are widely used because they are

small and prolific, and their genetics can be manipulated in ways not possible with humans. These differences from humans make them more, not less, valuable as models of humans for some applications. Characteristics such as a small size may make them unsuitable for other applications, *e.g.* heart surgery or bone surgery.

3. Models are highly specific to a particular study. Strains of mice and rats which develop cancer, heart disease, diabetes or neurological diseases could be of great interest in the study of these diseases, but these animals would probably be unsuitable for regulatory toxicology, where long-lived strains are usually required. Thus, it is impossible to say whether the rat for example, is a good or bad model of humans without specifying the context of the proposed study.

4. Models need to be validated. Research using animal models usually aims at the prediction of a response in humans. When a new treatment for a particular disease or condition is developed in animals, clinical trials will normally show whether or not the model was valid. If not, it may either be because the model was biologically invalid, or because the experiments in which the model was utilized were badly designed.

5. Models are subject to improvement through further research. Much of animal research is aimed at achieving an understanding of the animal as a potential model for particular human conditions. Models are not simply found: they need to be developed, and this requires an understanding of the biology of the species and the effects of various interventions.

In investigations of the osseointegration of dental implants and different biomaterials, successful research is seldom limited to an anatomical region, such as the soft and hard tissues of the mouth. Relevant models are often used to answer more general biological questions. To study bone formation and or osseointegration with the application of the maxillofacial region, the long bones and the calvarias are often used, and not only the jawbones.

Bone is a highly differentiated tissue. After an injury, there is a possibility that bone will heal not as bone, but as fibrous connective tissue. Undue heat injury increases this risk of disturbed bone healing. During surgical interventions in bone, frictional energy generates heat, and thereby increases the risk of fibrous bone healing (importance of continuous irrigation!). This basic knowledge is of utmost significance in all animal experiments involving bone cutting and drilling.

The most often used and preferred animal models for investigations of the osseointegration of dental implants and the otseogenesis of different bone substitutes are the rabbit femur and tibia models [65,66].

We have used the rabbit femur model to test the otseogenesis of different biomaterials utilized in bone substitution and also to study the osseointegration of different biocoated dental

implants. Although this model is easy to handle and has made a significant contribution to our studies, it presents disadvantages, too. The main drawback of this model is that, as the femur is a long bone, the new bone is formed according to endochondral ossification (a cartilage model serves as the precursor of the bone), unlike in the skull. The flat bones of the skull and face, the mandible and the clavicle are developed by intramembranous ossification. This is a simpler method, without the intervention of a cartilage precursor. It is emphasized that these concepts (intramembranous and endochondral ossification) refer only to the mechanism by which a bone is initially formed. Because of the rapid bone remodelling that occurs during bone development, the initial bone tissue laid down by intramembranous or endochondral formation is quickly replaced. The replacement bone is established on the pre-existing bone by appositional growth and is identical in both cases.

Another disadvantage of this rabbit femur or tibia model is that, due to the relatively thin cortical bone width, during the operative drilling or after the postoperative healing period there is a risk of unwanted pathological fractures, especially if the drilling is bicortical (*e.g.* an implant inserted through the width of the long bone), not monocortical.

In view of these disadvantages of the rabbit femur model, my goal was to develop a new animal model in rabbit and pig calvarial bones.

Calvarial wound models bear many similarities to the maxillofacial region. Both calvarial and midfacial bones develop from a membrane precursor, and the calvaria and mandible consist of two cortical tables with regions of intervening cancelluos bone. It contains modest amounts of bone marrow, which generally facilitates bone formation, although bone marrow is not indispensable for bone formation. When the aim is to investigate the pattern of bone formation in growth areas, young animals in an intense craniofacial growth period are preferably used. In adult animals, the regenerative capacity of the cranium is reduced; this therefore constitutes a suitable site for research work on agents for the enhacement of bone repair. Small defects (5 mm in diameter) that would correspond to a typical **operative defect** in clinical maxillofacial surgery have been produced and used in rats and rabbits. This size makes spontaneous bone regeneration possible, allows an evaluation of the regenerative influence stemming from the implant material and of the maturation of the newly formed bone, and permits tests on several implant materials. A **critical-size defect** is a defect that will not heal during the lifetime of the animal. When a defect large enough to preclude spontaneous healing is imployed, the osteogenic potential of an implant or a graft may be considered unambiguous. The critical-size model allows an assessment of whether enhancement of bony regeneration occurs [67-72].

We first intented to use a rabbit calvaria model (Fig. 2a,b) for our investigations, but as the calvarial bone of the rabbit is quite thin and not wide enough to test an adequate number of grafts and implants, we rather chose the Vietnamese-pot-bellied pig calvaria, where we could comfortably present 6 critical-size defects (8 mm in diameter and 2 mm in depth) in the parietal bone to test the osteogenic potential of different surface-modified implants and grafts used in the dental implantology (Fig. 3a,b).

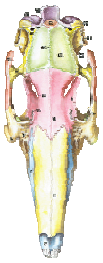
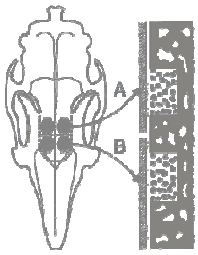
a  b 

Fig. 2a. *New Zealand White rabbit skull;*
b. *in the rabbit calvaria model, a maximum of4 critical-size defects can be developed*

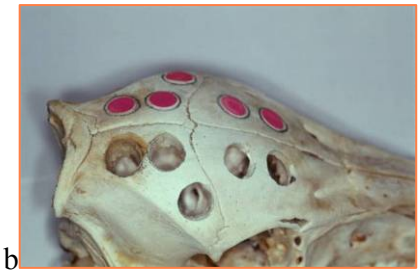
a  b 

Fig. 3a. *Vietnamese pot-bellied pig skull;* ***b.*** *Vietnamese pot-bellied pig skull with the prepared critical-size defects. In the parietal bones 6 wounds, and in the frontal bones 2 additional defects can be drilled*

2. Aims and questions to be answered

The aim of my research was to investigate the osseointegration of different surface-modified dental implants and the osteogenesis of different biomaterials used in GTR in order to improve osseointegration.

This thesis reports on the potential of a mixture of Cerasorb + Bio-Oss, Bio-Oss and Cerasorb alone and of BMPs in implantation technology. A further goal was to develop an *in vivo* animal model suitable for the investigation and comparison of the effects of different materials on osteogenesis and to determine the most advantageous characteristics of these bone substitutes. BMP accelerates, but does not guide new bone formation. Bio-Oss serves as a scaffold, but its resorbability is poor, while Cerasorb is a good bone-developing material, but resorbs too early, not providing a scaffold for the new bone bridges. Both Cerasorb and Bio-Oss are currently in clinical

use, but, as far as we are aware, their effects in a mixture have never been investigated and never been compared with those of rhBMP-2 on new bone formation.

In order to determine the advantageous (osseous) augmentation properties of CPCs, I investigated the osseoconductivity, biocompatibility, mouldability and malleability of VitalOs. In contrast with conventional bone graft materials, CPCs can be directly moulded and shaped to fill intrabony defects. Moreover, newly developed CPCs are fully injectable, which ensures the easy handling and appropriate application of these materials.

As biochemical surface modification is the most important hot topic of Ti dental implant development, different surface modifications (PE-ML and Camlog experimental surface) were tested with the new animal model (Vietnamese pot-bellied pigs). These studies are a natural continuation of the *in vitro* studies performed by our group regarding the PE-ML surface modification.

The challenging problems in my work may be summarized as follows:

- To test different surface-modified implants (discs) and biomaterials which may influence osseointegration and osteogenesis.
- To find the most suitable biomaterial with the most advantageous augmentation properties.
- To develop new, reproducible and efficient animal models suitable for testing different surface-modified dental implants and the different osteoinductive and/or osteoconductive biomaterials.
- To find the most efficient method of evaluation of osseointegration and to compare the results of histomorphometric, push-out and pull-out experiments.

The animal studies presented in this thesis are of great importance concerning the clinical applicability of different biomaterials used in GBR and PE-ML and Camlog experimental surface modified implants.

3. Materials and methods

3.1. Materials

3.1.1. RhBMP-2 solution and rhBMP-2-coated implants

These experiments were carried out in cooperation with the Institute of Physiological Chemistry, Division of Biochemical Endocrinology, University Clinics of Essen, Germany (Head: Professor Herbert P. Jennissen), through a Hungarian–German Intergovernmental S&T Cooperation Grant (2003-2004).

RhBMP-2 was prepared in *E. coli* and purified to homogeneity with the objective of the production of a very high-grade pure protein species. The biological activity of soluble rhBMP-2 was assessed with MC3T3-E1 cells by the induction of *de novo* synthesis of alkaline phosphatase [73].

RhBMP-2-coated implants

RhBMP-coated Ti specimens (dental implants) were first hydrophilized by treatment with chromosulphuric acid (CSA) and then biocoated with rhBMP-2 (200-400 ng/cm^2).

Twenty-eight cylindeórical implants (Camlog, Altatec, Germany) were manufactured from commercially pure Ti. The core diameter of the implants was 3.3 mm and the length was 8 mm. A total of 7 implants were treated with nitric acid, and 7 implants were surface-enhanced by a novel procedure with CSA [74]. The treatment of metals with CSA (CSA–Ti alloy) [75] leads to ultrahydrophilic (contact angles 0–10°, no hysteresis) bioadhesive surfaces [76]. A total of 14 surface-enhanced implants were divided into two subgroups and biocoated with rhBMP-2: 7 implants non-covalently immobilized rhBMP-2 (2.3 μg/cm^2), and 7 covalently immobilized rhBMP-2 (4.9 μg/cm^2) [77]. RhBMP-2 was immobilized by covalent and non-covalent methods on these CSA-treated surfaces [75,77,78]. In brief, the implants were assigned to the following test and control groups:

1. group: 7 control implants treated with nitric acid, without BMP-2

2. group: 7 control implants treated with CSA, without BMP-2

3. group: 7 CSA-treated implants with non-covalently linked BMP at 2 2.3 μg/cm^2

4. group: 7 CSA- treated implants with covalently bound BMP-2 at 4.9 μg/cm^2.

To control the surface produced, the following "sibling method" was employed: In parallel with the preparation of the above dental implants for *in vivo* experiments, miniplates (10×5×1 mm) with identical Promote surfaces were surface-enhanced with CSA and coated with ^{125}I-rhBMP-2 under identical conditions as for the dental implants. In this way, the corresponding contact angles, the amount of immobilized rhBMP-2, and the *in vitro* biological activity [79] could be tested before the implants were placed into the animals. Only those dental implants were released for implantation whose sibling miniplates reached the standard mentioned above and whose surfaces exhibited an intense *in vitro* bioactivity on fluorescence microscopy [79].

All 28 dental implants were prepared under sterile conditions in sterile solutions and packed singly in sterile Eppendorf cups.

3.1.2. Ti discs with different surface modifications

Ti discs 8 mm in diameter and 2 mm in height were cut from commercially pure (grade 4) Ti rods (Camlog Biotechnologies AG, Switzerland) used for the fabrication of dental implants. The surfaces were sand-blasted and acid-etched according to a standardized procedure (Promote). This surface served as the control surface.

We applied two kinds of surface modifications:

(a) A **PE-ML** coating was produced by the alternating adsorption of poly-cations (PLL) and poly-anions (PGA) from aqueous solution onto a Ti surface. **PE solutions** were prepared in an aqueous buffer solution of 25 mM TRIS (tris (hydroxymethyl) aminomethane, Sigma), 25 mM MES (2-(N-morpholino) ethanesulphonic acid, Sigma), and 100 mM NaCl (Fluka), pH 7.4. Before the coating, the samples were exposed to treatment in acetone and ethanol for 15 minutes consecutively in an ultrasonic bath and then sonicated in ultrapure water three times for 10 min, to ensure the cleanliness of the surface of the Ti discs.

The PE-ML films were formed by the alternating adsorption of cationic PLL (M_w = 30 000-70 000, Sigma Aldrich, P-2636) and anionic PGA (M_w = 50 000-100 000, Sigma Aldrich, P-4886) on machined Ti discs. The PE concentration was in all cases 1 mg/ml. Solutions were prepared with ultrapure water (Milli-Q-plus system, Millipore), and all buffer solutions were filtered before use.

(b) A **Camlog experimental surface modification** was produced to increase surface hydrophilicity, which is known to improve osseointegration. This surface modification is a modified Promote surface. After sandblasting and acid etching with inorganic acids, a further etching step is added. Each of the 3 steps creates a special topography to the final surface structure. Sand-blasting creates micro-craters with a diameter of 10 to 50 μm. Acid-etching superimposes micropits with a diameter of 0.5-2 μm. Final etching leads to an overlaying nanostructure with increased surface hydrophilicity.

3.1.3. Biomaterials used in GBR

We used **Cerasorb** M, a synthetic TCP granulate (1-2 mm) produced by Curasan (Kleinostheim, Germany).

The applied **Bio-Oss,** a bovine HA had a granule size of 1-2 mm (Geistlich Pharma AG, Switzerland).

The **Cerasorb + Bio-Oss mixture** consisted of a 50:50 (v/v %) Bio-Oss and Cerasorb, each with a granule size of 1-2 mm of each material.

VitalOs is a VitalOs Cement® synthetic and biocompatible CPC (Produits Dentaires, Switzerland). Its components: tricalcium phosphate 36%, monocalcium phosphate 23%, dicalcium phosphate 11.5%, ultrapure water 27%.

3.2. Animal specimens

The studies involved adult New Zealand White rabbits and Vietnamese pot-bellied pigs. The animal management and the surgical and routine procedures followed "The Guiding Principles for the Care and Use of Animals" approved by the Animal Investigation Review Board of the University of Szeged, in accordance with the principles of the Helsinki Declaration.

3.3. Human specimens

In 17 edentulous healthy patients (10 women, 7 men, with an average age of 52 years), the maxillary sinus floor was so atrophied that dental implantation was impossible. The patients were fully informed about the surgical intervention, the bone substitute and the implants. All gave their written informed constent. The Ethical Committees at Szeged University and Semmelweis University approved the research protocol.

3.4. RhBMP-2 experiments

3.4.1. Experiments with rhBMP-2-coated implants

We investigated the rhBMP-2 coating on implants with a diameter of 3.3 mm and a total length of 8 mm. The goal was to test how the coating accelerates the osseointegration and improves implant-bone bond strength and the bone quality around the implants. 14 rabbits participated in the experiment, divided into 2 groups.

Surgical procedure

In the first group (7 rabbits), 7 non-coated control implants treated with nitric acid were inserted into the left femurs, and 7 test implants treated CSA and non-covalently linked BMP-2 at 2.3 μg/cm^2 were inserted into the right femurs.

In the other group (7 rabbits), the same surgical procedure was performed: 7 control implants treated with CSA, without a BMP-2 coating were inserted into the left femurs, and 7 test implants treated with CSA and covalently bound BMP-2 at 4.9 μg/cm^2 were inserted into the right femurs.

Narcosis was induced with a cocktail of 5.0 mg/body mass (bm) kg (0.25 ml/bmkg) xylazine (Xylazine 2% inj.), 40.0 mg/bmkg (0.4 ml/bmkg) ketamine (Ketavet inj.) and 0.8

mg/bmkg (0.08 ml/bmkg) acepromazine (Vetranquil inj.) intramuscularly. During narcosis the rabbits received a Ringer lactate infusion 0.3 ml/min via a cannula inserted into an ear vein.

After disinfection, isolation and skin incision, the fascia lata was prepared. The femurs were visualized by folding the *m. tensor fasciae latae* and the *m. abductor cruris cranialis* (Fig. 4a).

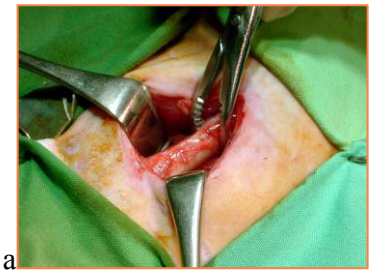
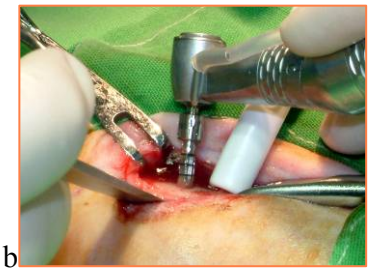
a  b  c 

Fig. 4a, b, c

The sites of insertion of the coated and control implants were marked with a round burr in the proximal third of the femur, approximately 2 cm distally from the *trochanter major* of the femur. A pilot, a pre- and finally a form drill (Fig. 4 b,c) were then used to create the bone bed of the implant in the femur with irrigation (pilot, pre- and form drills, Camlog Biotechnologies AG, Switzerland). The implant was inserted bicortically (Fig. 5a,b).

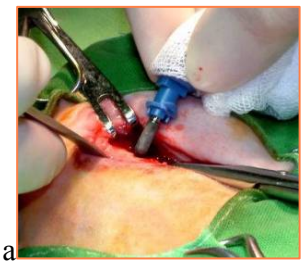
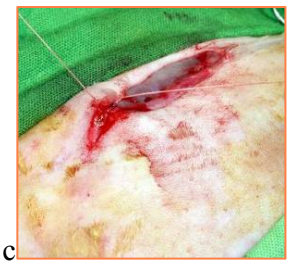
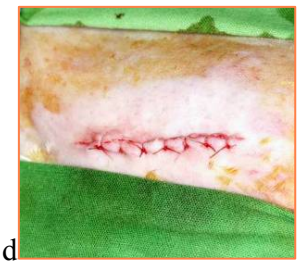
a  b  c  d 

Fig. 5a, b, c, d

Suturing was performed with absorbable Vicril 5.0, in three layers (fascia lata, subcutaneous layers and skin; Fig. 5c,d).

During the postoperative care, the rabbits received an analgetic (4.0 mg/bmkg (0.8 ml/bmkg) carprofen (Rimadyl) inj. sc.) and antibiotic support (15 mg/bmkg (0.15 ml/bmkg) enrofloxacin (Enroxil) inj. sc.) for 5 days following the operation.

Sample harvesting

After 4 weeks of osseointegration, the rabbits were sacrificed under general anaesthesia induced with an intravenous injection of an overdose of ketamine.

Specimen preparation for histological and histomorphometric evaluation

For histological evaluation, perfusional tissue fixation (Fig. 6a,b,c) was carried out with 4% neutral buffered formalin solution, after which the cut specimens were subjected to immersional fixation in 4% neutral buffered formalin solution.

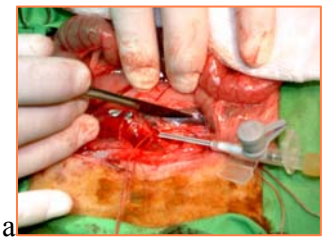
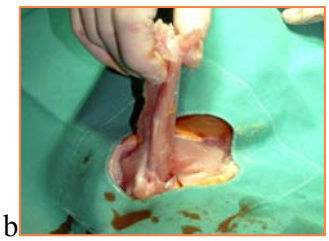
a  b  c 

Fig. 6a. *Cannula insertion in the a. abdominalis for perfusion 4% neutral buffered formalin solution;* ***b.*** *specimen taking; and* ***c.*** *removed femur specimen*

Decalcified paraffin sections, without the osseointegrated implant were prepared using by Goldner, Masson-Goldner, haematoxylin-eosin and toluidine blue staining.

Push-out biomechanical tests

The push-out test (Fig. 7a,b) is a biomechanical test commonly used for the evaluation of osseointegration. We performed these tests to study the quality (*i.e.* the mechanical strength) of the bone-implant connection by determining the peak (maximum) values of the push-out curves, measured with a Lloyd L1000R instrument (Lloyd Instruments, Segensworth West, UK). This value is the force needed to push-out an implant (in our case an osseointegrated control or a BMP-coated implant) from the rabbit femur after a 4-week of osseointegration period. Through measurment of the area of the connection between the bone and the implant (which is different for each femur), the magnitude of the shear strength at the interface can determined.

In order for such push-out tests to be adequate the axes of the push-out force and the implant should coincide and the fixation of the bone segment should be firm enough not to allow any movement during the push-out procedure.

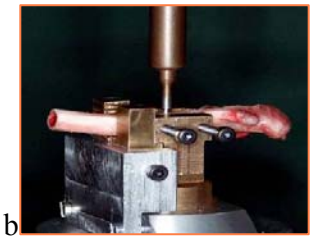
a  b 

Fig. 7a. *Picture of the Lloyd instrument used for push-out evaluation test;* ***b.*** *and the grip, which holds the rabbit femur*

Statistical analysis

The mean, the standard deviation (SD) and the standard error of the mean (SEM) were calculated. The results were analysed by Student's *t*-test (STATISTICA 8 software), with $p = 0.05$ taken as the level of statistical significance.

3.4.2. Experiments with rhBMP-2 solution

The next animal experiment involved an investigation of the effect and activity of rhBMP-2 solution itself. The same surgical protocol was used (section 3.4.1, page 18), but instead of a bicortical bone wound, which was suitable for the insertion of the implants, a monocortical bone wound was drilled to allow testing of how the rhBMP-2 solution influences the bone healing, *i.e.* the osseogenesis.

Surgical procedure

10 rabbits were included in this experiment. They were divided into 2 groups. Again both femurs of the rabbits were used. The first group (5 rabbits) received 5 µl of a solution containing 90 µl rhBMP-2/ml on the test side (right femur), while the other 5 rabbits were injected with 20 µl rhBMP-2 solution in the same concentration into the surgically prepared bone wound. The left femur was regularly the control side, into which the same amount of buffered physiological solution was injected, *i.e.* the solution which served as carrier for the rhBMP-2.

The diameter of the monocortical bone wound was 3.3 mm. Implantation drills with irrigation were used (pilot, pre- and form drills, Camlog Biotechnologies AG, Switzerland) to establish bone wounds of the same size. Spongostan (Johnson & Johnson) was placed into the hole and 20 µl rhBMP-2 solution was injected with a sterile pipette onto the Spongostan in the test (right) femur. The bone wound was covered with Surgicel (Johnson & Johnson), which was fixed around the hole with Histoacryl (Braun) to ensure that the BMP did not leak out. The wound was sutured with absorbable Vicril 5.0, in three layers (fascia lata, subcutaneous layers and skin). On the control side (left femur), the same procedure was performed, except that the same amount of sterile buffered physiological salt solution (the solution which served as carrier for the rhBMP-2) was injected into the bone wound.

Sample harvesting

After 4 weeks of osseogenesis, the rabbits were sacrificed under general anaesthesia induced with an intravenous injection of an overdose of ketamine.

Specimen preparation

For histological evaluation, perfusional tissue fixation (as described previously, page 20) was carried out with 4% neutral buffered formalin solution, after which the cut specimens were subjected to immersional fixation in 4% neutral buffered formalin solution. The specimens were dehydrated and embedded in Technovit 7200VLC resin (Heraeus Kulzer, Germany). Cutting was performed with the Exact cutting and grinding system without decalcification [80]. The thickness of the sections was 5 μm and the slides were stained with toluidine blue.

Histology and histomorphometric analysis

Optical microscopic images (Nikon Eclipse 80i, Japan) were recorded on an Evolution MP 5.1 Mega-pixel FireWire Digital CCD Color Camera Kit (Media Cybernetics, Inc., USA). Measurements were performed with Image-Pro Plus 5.1.1 image-analysing software (Media Cybernetics, Inc., USA).

The histomorphometrical evaluation involved use of the areal bone density, *i.e.* the ratio of the area of newly-formed bone to the total area of the image [81]. This permitted a quantitative comparison of the new bone formation in the control and the different types of test bone wounds.

3.5. Experiments with different surface-modified Ti discs

In further experiments, my goal was to test different surface-modified implants (discs) and biomaterials which may influence osseointegration. I decided to use the parietal bone of the pig, which is a surgical territory relatively easy to reach, where desmogenic ossification occurs, similarly to the conditions in the jaws. Moreover, this site is readily accessible for probing the effects of the different surface-modified implants or biomaterials on osteogenesis and osseointegration. Six 8-mm critical-size bone defects were drilled in the parietal bone of the pig.

Surgical procedure

Narcosis was induced with a cocktail of 5.0 mg/bmkg (0.25 ml/bmkg) xylazine (Xylazine 2% inj.), 40.0 mg/bmkg (0.4 ml/bmkg) nembutal (Nembutal inj.) and 0.8 mg/bmkg (0.08 ml/bmkg) acepromazine (Vetranquil inj.) intramuscularly. During narcosis, the pigs received a Ringer lactate infusion therapy 0.3 ml/min via a cannula inserted into an ear vein.

After disinfection, isolation and a U-shaped skin incision, the periosteum of the parietal bone was prepared. The parietal bone was visualized by preparing the periosteum. Following the protocol of bone surgery, a special burr developed for preparing the critical-size bone defects with irrigation was used (Fig. 8a). Six bone wounds were drilled into the parietal bone of the animal,

which allowed investigations of two different types of test Ti discs (for each type two discs) and the remaining two wounds served for the two control Ti discs (sand-blasted and acid-etched surface (Camlog-Promote)). After preparation of the bone wounds with a special burr with irrigation, the two test Ti discs were inserted, with the surface modification developed by the working group of Camlog (Camlog Biotechnologies AG, Switzerland). The purpose of the Camlog experimental surface modification was to make the Ti surface more hydrophilic; the details of the development have not been published, as the right belongs to the manufacturer.

The other tested surface modification was PE-ML on the CP4 Ti discs, which was assumed to improve the osseointegration (section 3.1.2, page 17). In the remaining two bone wounds, the control discs (sand-blasted and acid-etched) were inserted. To evaluate the osseointegration of the discs according to standardized parameters, Teflon (polytetrafluoroethylene) cylinders were used around the discs and they were also covered with Teflon caps. With this procedure, osseointegration of the walls and the top of the discs was avoided (Fig. 8b).

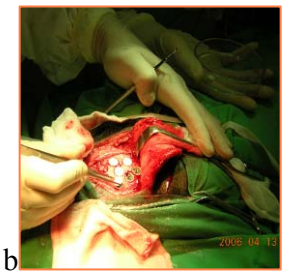
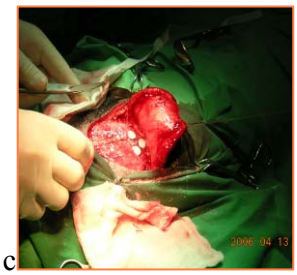
a  b  c  d 

Fig. 8a, b, c, d

The periosteum was enclosed tightly above the discs with Vicril 5.0 absorbable suture (Fig. 8c). The skin was sutured with skin suture (Fig. 8d).

During the postoperative care, the pigs received an analgetic (4.0 mg/bmkg (0.8 ml/bmkg) carprofen (Rimadyl) inj. sc.) and antibiotic support (15 mg/bmkg (0.15 ml/bmkg) cephalosporin (Zinacef) (Enroxil) inj. sc.) for 5 days following the operation. All the pigs remained healthy and the postoperative period was uneventful.

Specimen preparation

For histological evaluation, perfusional tissue fixation was carried out with 4% neutral buffered formalin solution. The *a. carotis* and *v. jugularis* were prepared and the head region was washed with 4% neutral buffered formalin solution (Fig. 9a). The cut specimens (containing the Ti discs; Fig. 9b) were then subjected to immersional fixation in 4% neutral buffered formalin solution. X-ray images were taken to identify the exact locations of the area of interest (Fig. 9c).

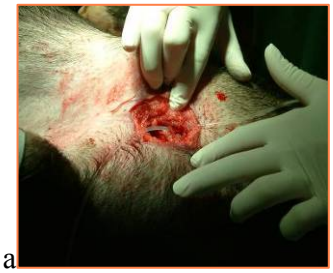
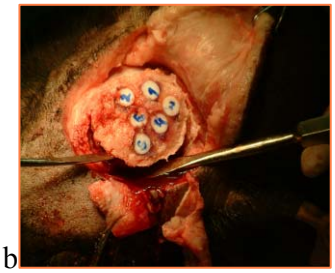
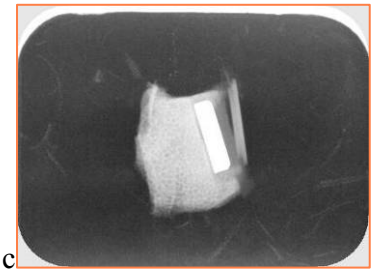
a  b  c 

Fig. 9a, b, c

The specimens were dehydrated and embedded in Technovit 7200VLC resin (Heraeus Kulzer, Germany). Cutting was performed with the Exact cutting and grinding system without decalcification and together with the discs [80]. The thickness of the sections was 5 µm. The slides were stained with toluidine blue.

Histology and histomorphometric analysis

This was as described previously (see page 22).

3.6. Bio-Oss experiments

To test other materials used in augmentation techniques, experiments were continued in Vietnamese pot-bellied pigs. The parietal bone of the animal permits at least 6 critical-size bone defects (8 mm in diameter and 2 mm in depth). The developed model proved suitable and reproducible for other kinds of biomaterials.

Surgical procedures

Narcosis was induced with the same procedure as described in section 3.5 (page 22).

After disinfection, isolation and a U-shaped skin incision, the periosteum of the parietal bone was prepared. The parietal bone was visualized by preparing the periosteum (Fig. 10a).

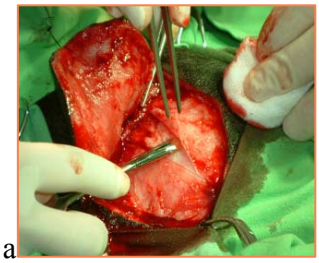
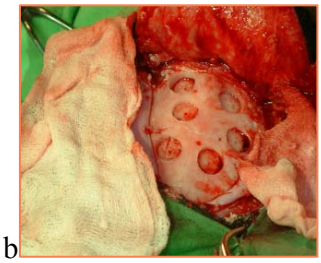
a  b 

Fig. 10a, b

Following the protocol of bone surgery, a special burr developed for preparing the critical-size bone defects with irrigation was used. Six bone wounds (Fig. 10b) were drilled into the parietal bone of the animal, which allowed investigations of two test materials (*e.g.* Cerasorb in two wounds, Bio-Oss in two wounds) and the remaining two wounds served as the control bone

defects. After preparations of the bone defects, Bio-Oss mixed with the animal blood was inserted in 2 bone wounds (Fig. 11a), the other four bone wounds serving as control or for tets of another material. The wounds were covered with Lyoplant membrane (Fig. 11b), a pure collagen derived from the bovine pericardium (Braun, Germany).

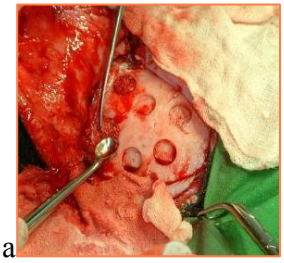
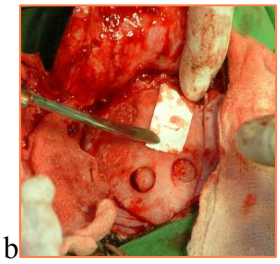
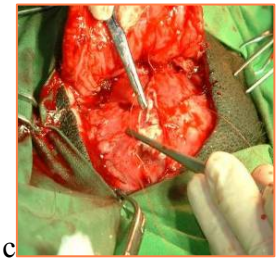
a  b  c  d 

Fig. 11a, b, c, d

The periosteum above the wounds was enclosed tightly with Vicril 5.0 absorbable sutures (Fig. 11c). The skin was sutured with skin sutures (Fig. 11d).

During the postoperative care, the pigs received an analgetic (4.0 mg/bmkg (0.8 ml/bmkg) carprofen (Rimadyl) inj. sc.) and antibiotic support (15 mg/bmkg (0.15 ml/bmkg) cephalosporin (Zinacef) inj. sc.) for 5 days following the operation. All the pigs remained healthy and the postoperative period was uneventful.

Sample harvesting

To test the difference in the early and late ossification, after 2 and 4 weeks of osteogenesis, the pigs were sacrificed under general anaesthesia, induced with an intravenous injection of an overdose of ketamine.

Specimen preparation

For histological evaluation, perfusional tissue fixation was carried out with 4% neutral buffered formalin solution. The cut specimens (Fig. 12a) were then subjected to immersional fixation in 4% neutral buffered formalin solution.

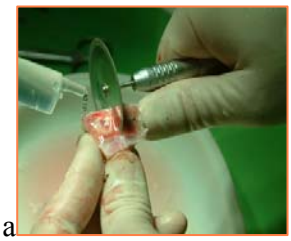
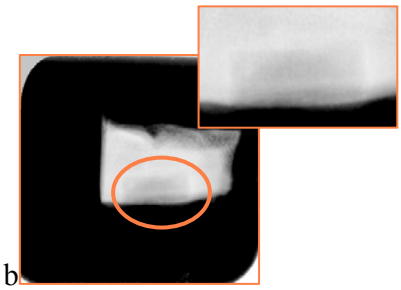
a  b 

Fig. 12a, b

X-ray images were taken to identify the exact locations of the bone wounds (Fig. 12b - orange circle). The specimens were dehydrated and embedded in Technovit 7200VLC resin

(Heraeus Kulzer, Germany). Cutting was performed with the Exact cutting and grinding system without decalcification [80]. The thickness of the sections was 5 μm and the slides were stained with toluidine blue.

Histology and histomorphometric analysis

This was as described previously (see page 22).

3.7. Cerasorb experiments

Surgical procedures

3.7.1. Pig experiments

The same pig model and the same surgical procedure were used as for Bio-Oss to test other otseoconductive materials, already described above (Fig. 13a,b).

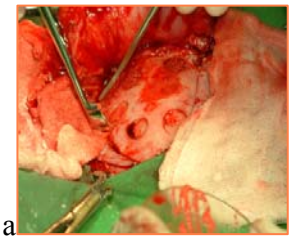
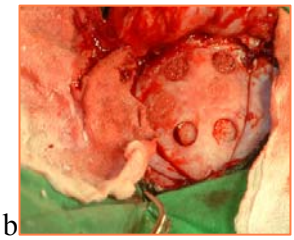
a  b 

Fig. 13a. *Insertion of Cerasorb;* ***b.*** *inserted Cerasorb*

Two different healing periods were chosen: 2 and 4 weeks, to test the difference in the early and late ossification.

Sample harvesting, specimen preparation, histology and histomorphometric analysis were performed as previously described in connection with Bio-Oss experiments (see pages 22 and 25).

3.7.2. Human experiments

Graft insertion can be one of the alternatives for the augmentation of injured bone. The effects of an alloplastic bone-replacing material, TCP-β (Cerasorb), were tested in comparion with autologous bone as concerns osteogenesis.

In 17 edentulous healthy patients (10 women, 7 men, with an average age of 52 years) the maxillary sinus floor was so atrophied that dental implantation was impossible.

Preoperative examinations were performed with panoramic images and CT scans. All patients had a bone height in the subantral maxillary sinus floor that was insufficient for dental implantation (average 1.9 mm). In all cases, surgery was performed under general anaesthesia with an autotransplant from the iliac crest to the control side (3-4 cm^3). The Schneiderian membrane was elevated by the insertion of Cerasorb (1.5-2 g Cerasorb granules 500-1000 μm in

diameter; Curasan AG, Kleiostheim, Germany) on the experimental side, while on the control side autogenous bone graft was inserted. After surgery, the healing period was followed both clinically and radiologically. After a healing period of 6 months, cylinders were excised from the grafted areas immediately before dental implantation; samples were taken from each side the with a trephine burr with 2 mm in inner diameter and 3 mm in outer diameter. During the implantation procedure, this bone would otherwise have been lost. Ankylos implants (Degusa, Friadent, Germany) were then inserted into their places.

Histology

For histological and histomorphometric analysis, undecalcified bone samples were fixed in 4% buffered formalin for 24 hours and then rinsed thoroughly in running water. The samples were dehydrated in ascending alcohol series and then embedded in methyl methacrylate resin at 4 °C. Histological sections 5 μm in thickness were cut, using a diamond knife. Sections were stained with toluidine blue, haematoxylin and eosin and Goldner trichrome for light microscopy. Photos were taken by the means of Olympus BH_2 microscope equipped with an Olympus DP50 digital camera (Olympus Optical Company Ltd., Melville, NY, USA) and a Nikon Eclipse 80i microscope and Evolution MP 5.1 Mega-pixel FireWire Digital CCD Color Camera Kit (Media Cybernetics, Inc., Rochester, NY, USA).

Histomorphometry

Histomorphometric measurments were performed according to the principles of Parfitt *et al.* [81]. (Image-Pro Plus 5.1. image-analysing software, Media Cybernetics, Inc., Rochester, NY, USA). The density of the newly formed bone was measured via the trabecular bone volume (TBV), which was defined as the area of the bone trabeculae to the total area analysed. The percentage of the grafted area was also determined. The trabecular bone pattern factor (TBPf) was also quantified. This factor marks the microarchitecture of the newly-formed bone. The trabecular bone area and perimeter were measured, before and after arithmetic dilatation of the binary image, to determine the relation of the convex and concave trabecular structures of the sections. The higher the degree of trabecular connectivity, the lower the value of TBPf.

Statistical analysis

The mean and the standard deviation (SD) values were calculated. The data obtained were analysed by Student's *t*-test, with the significance level set at $p < 0.05$.

3.8. Experiments with Bio-Oss + Cerasorb mixture

Surgical procedures

The main aim of this study was to establish a gold standard for the artificial bone growth-accelerating effect of a Cerasorb + Bio-Oss mixture on osteogenesis in order to determine and utilize the most advantageous characteristics of these bone substitutes. Bio-Oss serves as a scaffold, but its resorbability is poor, while Cerasorb is a good bone-developing material, but resorbs too early, not providing a scaffold for the new bone bridges. Both New Zealand white rabbits and Vietnamese pot-bellied pigs were involved in these experiments.

3.8.1. New Zealand white rabbit experiment

The surgical protocol was the same as described previously in connection with the rhBMP-2 solution experiments (section 3.4.2, page 21). The diameter of the monocortical bone wound was 3.3 mm. A mixture of the bone graft materials Cerasorb + Bio-Oss was inserted into the bone wound. The mixture consisted of a 50:50 (v/v %) mixture Bio-Oss and Cerasorb. This combination was applied mixed with rabbit blood to fill the 3.3-mm-diameter monocortical bone wound in the test (right) femur. On the control side (left femur), the bone wound was simply covered with Surgicel (Johnson & Johnson), which was fixed around the hole with Histoacryl (Braun). The wound was sutured with absorbable Vicril 5.0, in three layers (fascia lata, subcutaneous layers and skin).

During the postoperative care, the rabbits received an analgetic (4.0 mg/bmkg (0.8 ml/bmkg) carprofen (Rimadyl) inj. sc.) and antibiotic support (15 mg/bmkg (0.15 ml/bmkg) enrofloxacin (Enroxil) inj. sc.) for 5 days following the operation. All the rabbits remained healthy and the postoperative period was uneventful.

Sample harvesting

After 4 weeks of osteogenesis, the rabbits were sacrificed under general anaesthesia induced with an intravenous injection of an overdose of ketamine.

Specimen preparation and histology and histomorphometric analysis were carried out with the same protocol as described previously (see pages 21 and 22).

3.8.2. Vietnamese-pot bellied pig experiments

For tests of the osteogenic effect of this mixture under desmogenic circumstances (as in the oral cavity), the parietal bone of the animal permits at least 6 critical-size bone defects (8 mm in diameter and 2 mm in depth).

The surgical protocol was the same as described previously in connection with the Bio-Oss (see section 3.6, page 24). Six bone wounds were drilled into the parietal bone of the animal, which allowed the investigation of two test materials, and the remaining two wounds serving as control bone defects. After preparation of the bone defects, a mixture of the bone graft materials Cerasorb and Bio-Oss was inserted into the bone wound. The mixture consisted of a 50:50 (v/v %) mixture Bio-Oss and Cerasorb. This combination was applied mixed with the blood of the animal to fill two of the critical-size bone wounds (Fig. 14a,b).

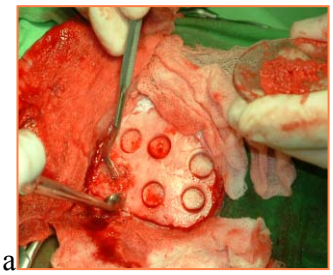
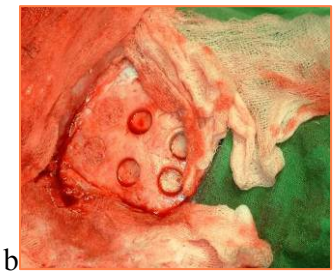
a  b 

Fig. 14a, b. *Insertion of Cerasorb and Bio-Oss mixture*

The other four bone wounds served as control (two bone wounds) and for tests of another material (VitalOs cement). The wounds were covered with Lyoplant membrane (pure collagen derived from the bovine pericardium, Braun, Germany). The periosteum was enclosed tightly above the wounds with Vicril 5.0 absorbable sutures. The skin was sutured with skin sutures. During the postoperative care, the pigs received an analgetic (4.0 mg/bmkg (0.8 ml/bmkg) carprofen (Rimadyl) inj. sc.) and antibiotic support (15 mg/bmkg (0.15 ml/bmkg) cephalosporin (Zinacef) inj. sc.) for 5 days following the operation. All the pigs remained healthy and the postoperative period was uneventful. The healing period was 4 weeks, similarly as in the rabbit experiments.

Sample harvesting, specimen preparation and histology and histomorphometric analysis were carried out as described previously (see pages 22 and 25).

3.9. VitalOs cement experiments

Surgical procedures

To test the effect of **VitalOs** CPC on osteogenesis, the same pig model was used as described previously. The surgical protocol was the same as detailed in connection with Bio-Oss (see section 3.6, page 24). Six bone wounds were drilled in the parietal bone of the animal, which allowed the investigation of two test materials, and the remaining two wounds serving as control bone defects. After preparation of the bone defects VitalOs cement was inserted into the bone wound (Fig. 15a,b).

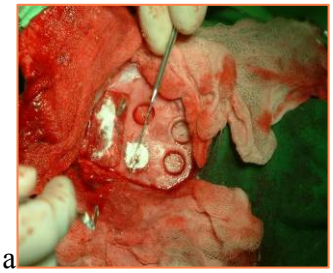
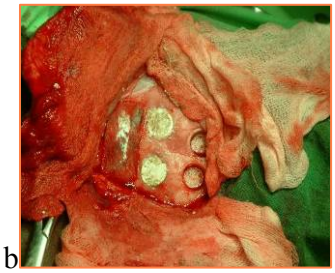
a  b 

Fig. 15a, b. *Insertion of VitalOs.*

The other four bone wounds served as control (two bone wounds) and for investigation of another test material (Cerasorb + BioOss mixture). The wounds were covered with Lyoplant membrane (pure collagen derived from the bovine pericardium; Braun, Germany). The periosteum was enclosed tightly above the wounds with Vicril 5.0 absorbable sutures. The skin was sutured with skin sutures. The postoperative care was the same as for Cerasorb + BioOss mixtur (see section 3.8.2, page 29). The healing period was 4 weeks, similarly as in the rabbit experiments.

Sample harvesting, specimen preparation and histology and histomorphometric analysis were carried out as described previously (see page 22 and 25).

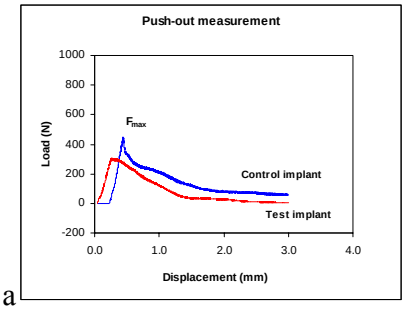
4. Results and discussion

4.1. Results of rhBMP-2 experiments

4.1.1. Evaluation of osseointegration of rhBMP-2-coated implants with push-out test

After 4 weeks of osseointegration, the animals were sacrificed under general anaesthesia and their femurs were removed by surgical intervention. From each group, two kinds of evaluation were performed: push-out and histomorphometry.

Figure 16a presents typical push-out curves, on both femurs of the rabbit, one curve (blue) depicting the data relating to an osseointegrated control implant, and the other (red) the data relating an implant covered by rhBMP-2 with covalent binding.

a 

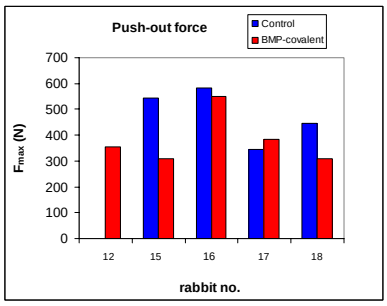
b 

Fig. 16a. *Typical push-out curves;* ***b.*** *push-out force values for the second group of rabbits. Blue bars indicate the control implants (treated with CSA) and the red ones the test implants covered with covalently bound BMP*

Figure 16b presents the bar-graph of the push-out force values (F_{max} (N)) of the second group of rabbits. For rabbit no. 12, the force value for the control implant is missing, because the femur broke during the 4 weeks bone healing period. In the bar graph, the blue bars denote the push-out forces for the control implants (without BMP covering) and the red bars the values for the test implants with covalently bound BMP. The average push-out force for the control implants was 479.8 ± 53 N (mean ± SEM), while that for the test implants was 381.4 ± 45 N.

The statistical analysis (Student's *t*-test) of the two rabbit groups showed that neither the covalently (Fig. 17), nor the non-covalently bound BMP (data not shown) exerted an enhancing effect on osseointegration (p = 0.224 and p = 0.886, respectively) under the given experimental circumstances.

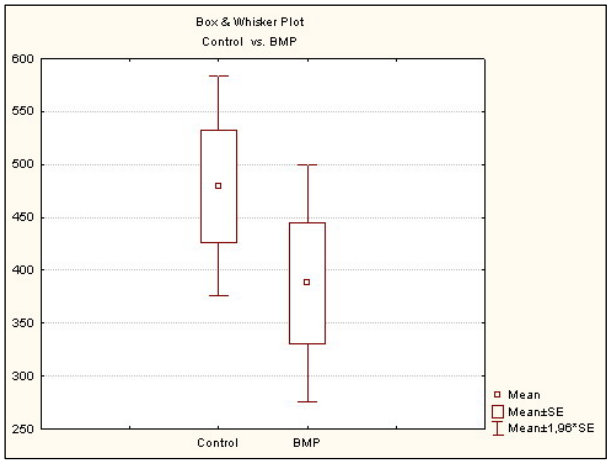


Fig. 17. *Box & Whisker plot of control implants (treated with CSA) and test implants treated with CSA and covalently bound BMP-2 at 4.9 µg/cm^2.*

We also compared the two control groups (K-Alta-CSB and K-Alta+CSB) and the two test groups (covalently and non-covalently bound BMP), using the Student's *t*-test, and we did not find a significant difference between them (p = 0.258 and p = 0.784, respectively). On the basis of this result, the data for the two test implant groups were combined for the analysis of the interface shear strength.

The measured push-out forces were converted to interface shear strengths. The bar graph in Fig. 18 presents the summarized interface shear-strength data, T_{max} (MPa), for the control (without BMP coating) and test implants (with BMP coating). In order to evaluate and compare the level of osseointegration, primary stability tests were also performed with 10 implants.

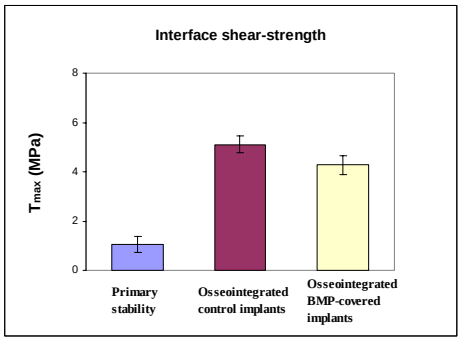


Fig. 18. *Interface shear strength* (T_{max} (MPa)) *values for control and test implants. Primary stability values are also shown*

According to these results, there was a statistically significant difference ($p < 0.05$) between the shear strengths of the primary stability (1.04 ± 0.2 MPa) and osseointegrated control implant groups (5.10 ± 0.3 MPa). Similarly, there was a significant difference ($p < 0.05$) between the interface shear strengths of the primary stability and osseointegrated BMP-covered implant groups (4.27 ± 0.4 MPa). Conversely, there was no significant difference between the two osseointegrated implant groups: $p = 0.139$.

4.1.2. Histological and histomorphometric results with rhBMP-2-coated implants and rhBMP-solution

RhBMP-2-coated implants

The histological evaluation methods (decalcified paraffin samples) and the results of the push-out tests in the animal experiments with rhBMP-2-coated implants allowed some preliminary conclusions. The histomorphometric results from the first experiment supported the outcome of the push-out test: under the given circumstances, the BMP coating on the test implants did not exert a significant effect on the osseointegration during the 4-week healing period.

RhBMP-2 solution

After a 4-week period of healing and osseointegration, the rabbits were sacrificed and their femurs were removed. Undecalcified sections were made and revealed the following findings: in the control samples, the bone wound was only partially filled with newly-formed bone, while the reconstruction of the surgically prepared bone wound was almost completed (Fig. 19a, orange arrows). The bone at the border of the bone wound showed signs of resorption and new bone formation. This was young, immature lamellar bone with centrifugal orientation of the

newly-formed bone in the bone wound. The outer layer of the newly-formed bone was younger woven bone and cross-oriented.

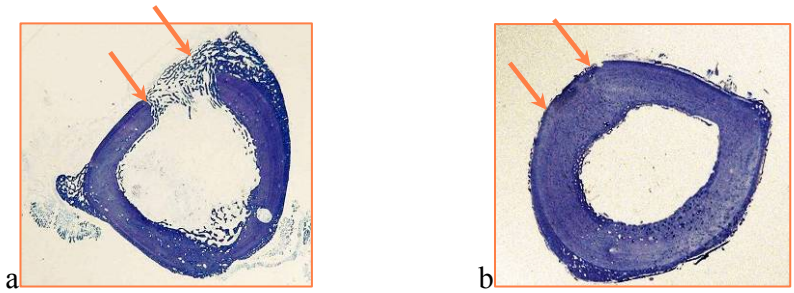


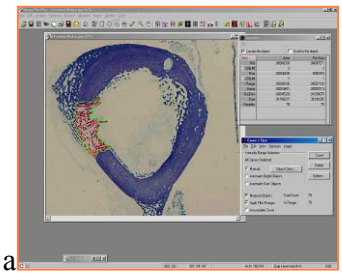
Fig. 19a. *Control sample;* ***b.*** *test, slides stained with toluidine blue (bright field, 1x magnification)*

In the test samples (Fig. 19b), the original bone wounds could not be detected. The surgically prepared bone wound was completely replaced by newly-formed bone. The site of the original bone wound could be only discovered from the different histological structures and the density of the bone by using polarized light and higher magnification (Fig. 20a,b).

Fig. 20a. *Centrifugally grown collagen fibres (10 x magnifications, polarized light);* ***b.*** *secondary and primary osteons (20 x magnification, polarized light) toluidine blue staining*

In this repaired area, the centrifugally grown collagen fibres predominated (Fig. 20a). It was also very characteristic of this bone specimen that, on the entire inner surface of the cortical, endosteal bone formation was seen as a line in tight contact with the old cortical bone. Remodelling occurred in all parts of the bone.

New bone formation was evaluated via the areal bone density (Fig. 21a,b), *i.e.* ratio of the area of newly-formed bone to the total area of the image. This makes it possible to compare the bone formation in the control bone wound (where natural bone healing occurs) and in the rhBMP-2-treated wound in a quantitative way.

a 

b

Sample	Areal bone density ± SD (%)
Control	**34.0 ± 0.1**
Test (rhBMP-2-treated)	**96.3 ± 4.5**

Fig. 21a. *Image of the evaluating procedure;* ***b.*** *mean SD values of areal bone density in the control and test groups*

With the Image-Pro Plus 5.1.1 image-analysing software, the total area of the bone wound was measured; after this, the area of new bone formation was measured in the setout area (Fig. 25a). These measured areas were transferred into Excel files. For the control samples, the mean areal bone density was 34.0 ± 0.1%**,** while for the test samples it was 2.8 times more: 96.3 ± 4.5%.

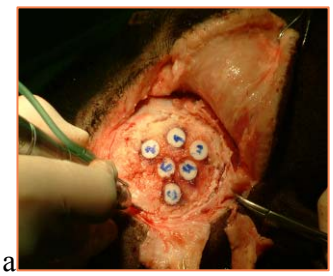
Evaluation of the prepared slides by histological and histomorphometric methods led to the conclusion that, in the monocortically drilled bone wound where the rhBMP-2 solution was inserted, the closure of the bone wound was almost totally complete.

4.2. Results of experiments with surface-modified Ti discs

After a 2-week period of osseointegration, the animals were sacrificed under general anaesthesia and the parietal bone segments were removed by surgical intervention. Two types of evaluation methods were performed: pull-out tests and histological and histomorphometric observations.

4.2.1. Evaluation of osseointegration with pull-out tests

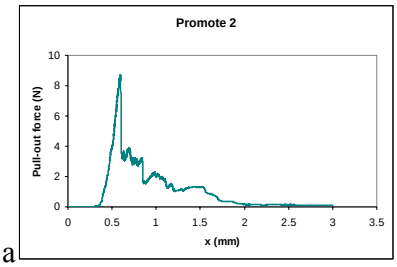
The bone segments containing all 6 discs inside were removed (Fig. 22a) and pull-out tests were performed (Fig. 22b).

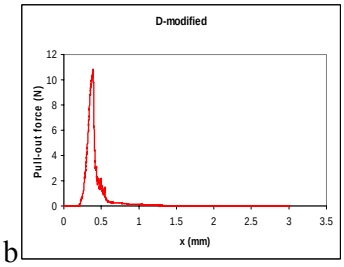
a 

b 

Fig. 22a, b

For the control discs (Promote surface: sand-blasted and acid-etched), the maximum pull-out force was 8.7 N (Fig. 23a), while for the Camlog experimental surface discs the corresponding value was 10.8 N (notation: D-modified in Fig. 23b), and for the PE-ML discs it was only 2.2 N (Fig. 23c).

a 

b 

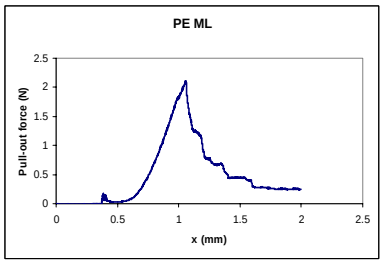
c 

Fig. 23a, b, c

Although we could not perform more measurements in this case, the results of this preliminary study permitted the conclusion that the Camlog experimental surface discs presented a tendency for enhanced osseointegration, during the 2-week healing period. More experiments are needed to allow further conclusions.

4.2.2. Histological and histomorphometric results with different surface-modified discs

After 2-week period of osseointegration the animals underwent perfusional fixation and were sacrificed under general anaesthesia, and the parietal bone segments were removed by surgical intervention. With the help of Image-Pro Plus 5.1.1 image-analysing software, the area of new bone formation was measured on the histological slides. The histomorphometric evaluation involved use of the areal bone density [81]. Only the osseointegrated surface was evaluated (Fig. 24a,b, orange line, box), which was in contact with the bone bed. The teflon cylinder and cap prevented osseointegration on the other surfaces of the Ti disc. The measured areas were transferred into Excel files.

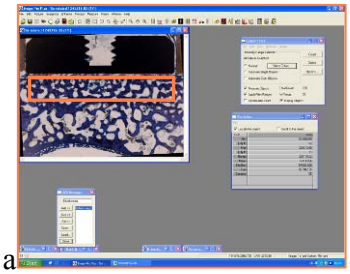
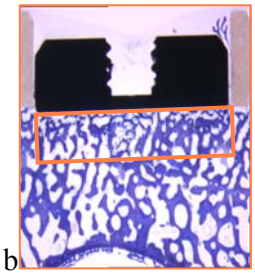
a 
b 

Fig. 24a, b. *Marked (orange) area indicating the evaluated area*

Quantitative comparisons were made of the new bone formation in the control and the two types of test surface-modified discs were permitted: for the control discs, the areal bone density of new bone formation was 42.5%. As may be seen in Fig. 25a, young and thick woven bone trabecules were attached to the surface of the Ti.

The evaluation of the PE-ML-coated discs revealed less new bone formation: the areal bone density was 38.8%. The young bone trabeculae were thin and fewer (Fig. 25b), and the bone-free areas exhibited numerous macrophages.

The Camlog experimental surface modification yielded rather promising results: the areal bone density was 53.9%, which supports the findings of the pull-out tests. The roughness of the disc surface was marked (Fig. 25c) and young woven bone trabeculae could be seen in dense compact bands towards the surface.

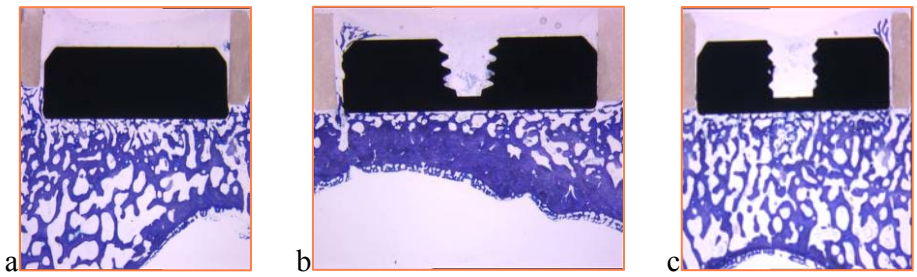


Fig. 25a. *Control;* ***b.*** *PE-ML-coated;* ***c.*** *Camlog experimental surface modification discs (bright field, 1x magnification, toluidine blue staining)*

4.3. Results of Bio-Oss experiments

After 2-or 4-week periods of osseointegration, the animals were sacrificed under general anaesthesia and the parietal bone segments were removed by surgical intervention. Histological and histomorphometric evaluation methods were performed.

As the bone wounds had the same size, with the Image-Pro Plus 5.1.1 image-analysing software, the area of new bone formation could be measured properly. This allowed quantitative comparison of the new bone formation in the control and the different types of test bone wounds. The measured areas were transferred into Excel files.

In the experiments involving the 2-week healing period, the mean value of the new bone formation in the bone wounds of the control group, was 19.1 ± 0.2% (mean ± SD, Fig. 26a). As may be seen in Fig. 26b, the bone trabeculae were oriented radicularly towards the surface of the bone wound.

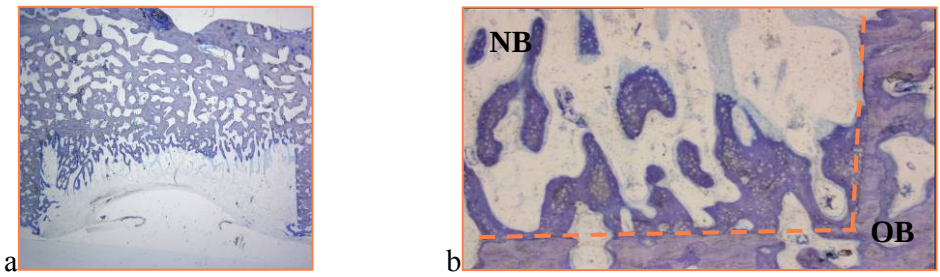


Fig. 26a. *Control (bright field, 1x magnification, toluidine blue staining);* ***b.*** *old (OB) and new bone (NB) contact (bright field, 20 x magnification, toluidine blue staining)*

In the test samples (Fig. 27a), the new bone formation in the bone wound was not significantly higher than in the control: 31.2 ± 2.5% (p = 0.085). The new bone formation, together with the Bio-Oss particles, was significantly higher: 46.2 ± 2.8% (p = 0.043). At the bottom of the bone bed, around the Bio-Oss particles, new bone was formed with bone bridges between the particles (Fig. 27b).

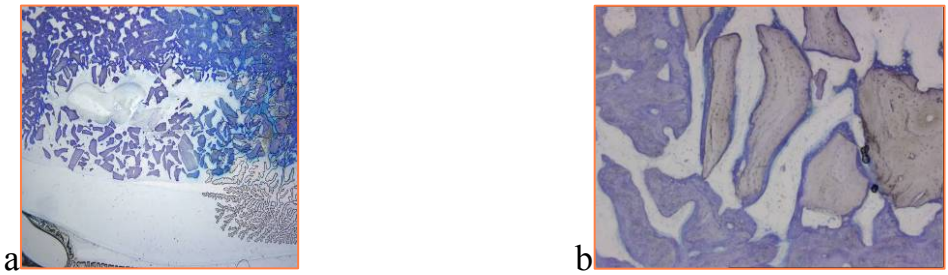


Fig. 27a. *2-week treatment Bio-Oss (bright field, 1x magnification, toluidine blue staining);* ***b.*** *bone bridges between the Bio-Oss particles (bright field, 20 x magnification, toluidine blue staining)*

The mean areal bone density for the control samples after the 4-week osseointegration period was 24.3 ± 5.6% (Fig. 28a). Young immature bone could be detected at the bottom of the bone wound (Fig. 28b).

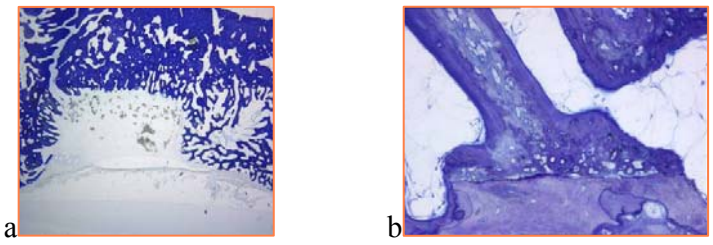


Fig. 28a. *4-week control (bright field, 1x magnification, toluidine blue staining);* ***b.*** *contact of the lamellar new bone with the original bone particles (bright field, 40 x magnification, toluidine blue staining)*

In the case of the 4-week osseointegration period with Bio-Oss, the bone wound was filled with woven bone and Bio-Oss particles (Fig. 29a). It was hard to distinguish between the particles and the newly-formed bone (Fig. 29b). The areal bone density, which included the new bone formation and Bio-Oss particles, was not significantly higher: 56.5 ± 1.5% (p = 0.056).

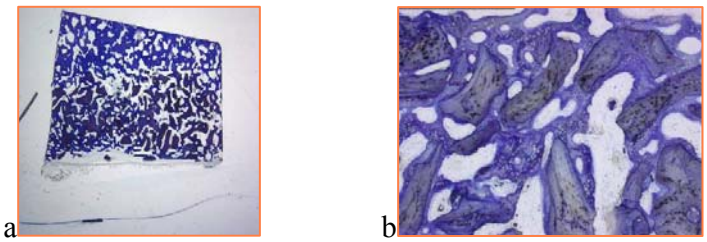


Fig. 29a. *4-week Bio-Oss treatment (bright field, 1x magnification, toluidine blue staining);* ***b.*** *new bone formation between the Bio-Oss particles (bright field, 20 x magnification, toluidine blue staining)*

4.4. Results of Cerasorb experiments

4.4.1. Pig experiments

Similar osseointegration periods (2 and 4 weeks) and the same evaluation methods were applied as described in connection with the Bio-Oss experiments. The control samples were the same as for the 2-week healing period for the Bio-Oss samples (mean areal bone density: 19.1 ± 0.2%). For the 2-week healing period, the areal bone density was significantly higher for new bone formation (27.7 ± 1%; $p = 0.041$) and for the samples including the Cerasorb particles (Fig. 30a): 50.9 ± 1.2% ($p = 0.014$).

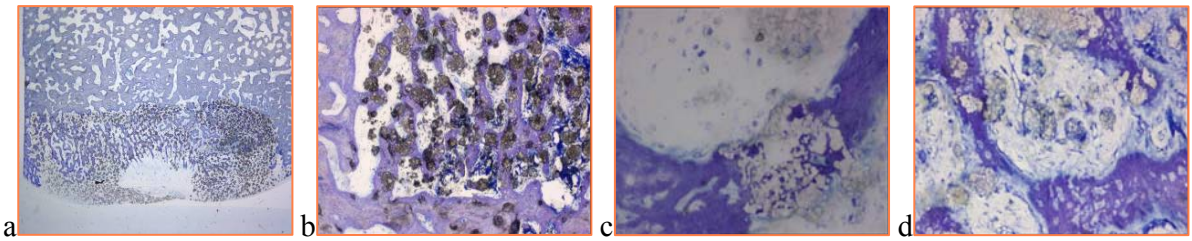


Fig. 30a. *2-week Cerasorb treatment (bright field, 1x magnification, toluidine blue staining);* ***b.*** *Cerasorb particles with newly-formed bone (bright field, 10 x magnification, toluidine blue staining);* ***c.*** *transition from woven to lamellar bone at the osteoblast line (bright field, 20x magnification, toluidine blue staining);* ***d.*** *osteoid and woven bone (bright field, 20 x magnification, toluidine blue staining)*

Two-thirds of the bone wound was filled with Cerasorb granules and newly-formed bone trabeculae (Fig. 30b). The new bone was a transition between woven and lamellar bone (Fig. 30c). Within the densely compacted Cerasorb granules, osteoid and woven bone could be found (Fig. 30d).

For the 4-week osseointegration (Fig. 31a), the mean areal bone density did not differ significantly from the control value: for new bone formation it was 30.8 ± 0.3% ($p = 0.328$), and for the Cerasorb particles it was 56.0 ± 0.3% ($p = 0.074$). The control value was the same as in case of the 4-week healing period for the Bio-Oss samples (24.3 ± 5.6%). In the test samples, new bone formation was more pronounced in the centre of the bone wound. New bone bridges were evident between the original and the newly-formed bone (Fig. 31b).

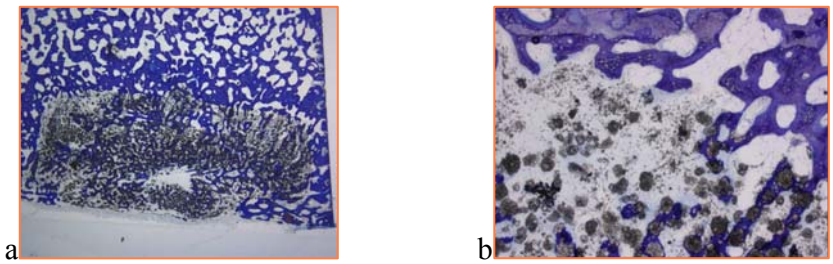


Fig. 31a. *4-week Cerasorb treatment (bright field, 1x magnification, toluidine blue staining);* ***b.*** *new bone bridges between the Cerasorb particles (bright field, 20 x magnification, toluidine blue staining)*

4.4.2. Human experiments

On the test side (augmentation site), in sections stained with haematoxylin-eosin and Goldner's trichrome method, many graft particles proved to have dissolved. However, their previous location could easily be recognized via their characteristic round form (Fig. 32a).

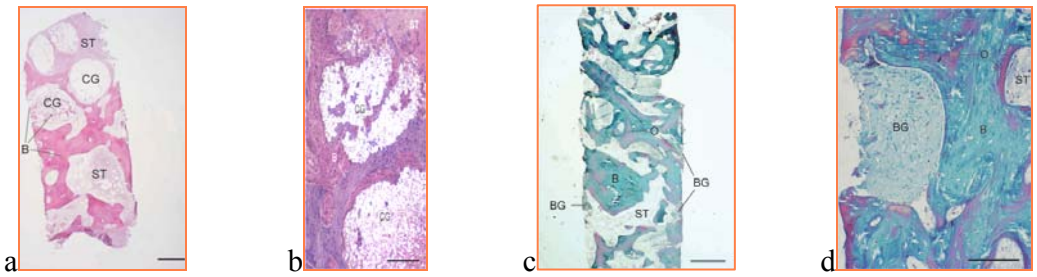


Fig. 32a. *Biopsy taken from the experimental side 6 months after grafting. CG: Cerasorb granule; B: bone; ST: soft tissue; H&E staining (scale bar=500 mm);* ***b.*** *biopsy taken from the experimental side 6.5 months after grafting; peri- and intragranular bone strands can be seen; toluidine blue staining, polarized light (scale bar=400 mm);* ***c.*** *biopsy taken from the control side 6 months after grafting; B: bone; O: osteoid; BG: bone graft; ST: soft tissue, Goldner staining (scale bar=500 mm);* ***d.*** *biopsy taken from the control side 6 months after grafting; bone (B) and osteoid (O) production and a focus of the resorbing bone graft (BG) can be seen; ST: soft tissue, Goldner staining (scale bar=200 mm)*

After 6 months, the newly-formed, predominantly lamellar bone was tightly intermingled with the graft particles at the tissue/graft interface. Bony sheathing of the Cerasorb granules was extensive, and where the graft particles had become completely sheathed, the osteoblastic activity had disappeared. The graft remnants exhibited achromatic birefringence under polarized light (Fig. 32b).

On the control side, the cancellous bone grafts had undergone considerable resorption by the sixth month (Fig. 32c). Graft foci were entrapped in the newly-formed, predominantly mature lamellar bone (Fig. 32d). They were homogeneous acellular particles that stained almost like living bone. There was a continuous transition at the interface of the cancellous graft and the new bone.

Bone density, graft density, TBPf and bone area were measured in 68 bone biopsy samples. The mean bone density for the 17 cases was 32.4 ± 10.8% on the experimental side and

34.7 ± 11.9% on the control side; the difference was not significant ($p > 0.05$). In an overwhelming majority of the patients (14 of 17 cases), the intensity of new bone formation was similar on the two sides.

The graft density was markedly higher on the experimental side than on the control side. The mean density of the graft area was 13.1 ± 4.5% and 8.2 ± 1.7%, respectively, this difference being highly significant ($p < 0.001$).

The TBPf values generally displayed an inverse correlation with the bone density: the higher the bone density, the lower the TBPf. In 11 of the 17 cases, the TBPf value on the control side was lower than that on the experimental side. The mean values were - 0.53 ± 1.74 and - 0.11 ± 1.43 mm^{-1}, respectively, but the difference was not significant ($p > 0.05$).

The mean areas of the biopsy samples taken from the two sides were quite similar: 8.85 ± 1.7 mm^2 on the experimental side and 9.12 ± 2.28 mm^2 on the control side.

4.5. Results of Bio-Oss + Cerasorb mixture experiments

4.5.1. New Zealand white rabbit experiment

After a 4 week period of healing and osseointegration the rabbits were sacrificed and their femurs were removed. The control samples were the same as in case of the 4-week healing period for rhBMP-2 solution (areal bone density: 34.0 ± 0.1%). In the test samples, the closure of the monocortical bone wound (Fig. 33a) was not complete and the areal bone density was significantly higher than for the control: 48.7 ± 0.1% ($p = 0.005$).

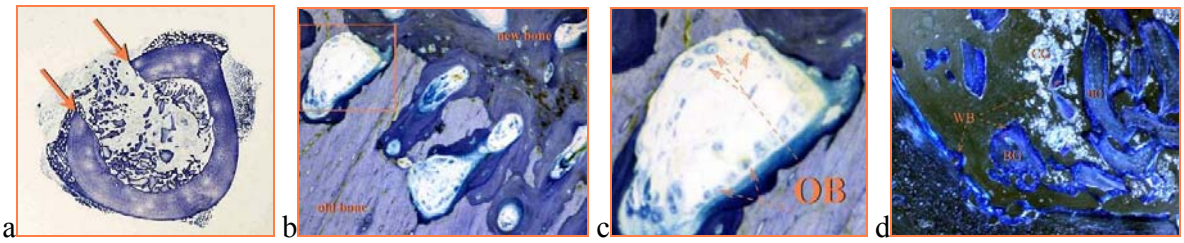


Fig. 33a. *Macroscopic histological slide of the osteogenesis induced by the Cerasorb + Bio-Oss mixture, toluidine blue staining;* ***b.*** *picture of old bone and new bone (bright field, 20 x magnification, toluidine blue staining);* ***c.*** *osteobast line (OB); (bright field, 40 x magnification, toluidine blue staining);* ***d.*** *polarized light microscopic image (20 x magnification, toluidine blue staining); CG: Cerasorb granules, BG: Bio-Oss particles, WB: woven bone.*

Around the bone substitutes, new bone formation had started, containing bone bridges between and around the granules. Mostly young immature woven bone was situated around the granules with osteoblastic activity. In Fig. 33b, the border of the old and the new bone can be clearly differentiated.

In the woven bone (Fig. 33d) an osteoblast line indicated the formation of bone (OB) and primary osteons in the lamellar bone. Both Bio-Oss (BG) and Cerasorb (CG) granules were visible (Fig. 33c), as a sign that the resorption of the material had not yet finished. At the same time, an osteoid bone (woven bone: WB) network could be discerned around the granules.

4.5.2. Pig experiments

The osseointegration period was 4 weeks and the same evaluation methods were applied as described previously in connection with Bio-Oss and Cerasorb experiments. The control samples were the same as in case of the 4-week healing period for the Bio-Oss and Cerasorb samples, with an areal bone density of 25.1 ± 1.7%. The corresponding value for the newly-formed bone (Fig. 34a) was significantly higher (48.3 ± 0.9%; $p = 0.014$), as was that for new bone formation in the presence of Bio-Oss and Cerasorb particles: 57.5 ± 1.1% ($p = 0.008$)

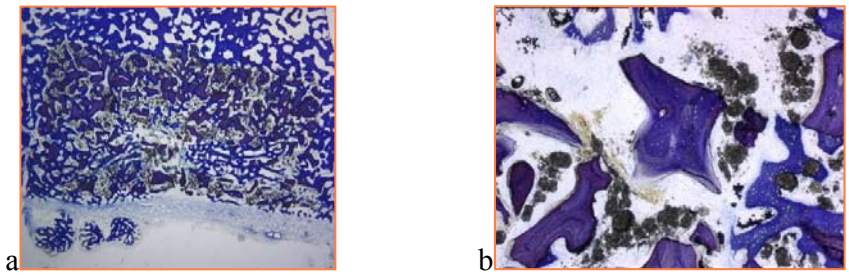


Fig. 34a. *4-week treatment Cerasorb + Bio-Oss mixture (bright field,1x magnification, toluidine blue staining);* ***b.*** *new bone bridges between the Cerasorb and Bio-Oss particles (bright field, 20 x magnification, toluidine blue staining)*

The network of newly-formed bone could be detected in the two types of bone substituents (Fig. 34b). The scalloped surface of the Cerasorb granules could be discerned. The peri- and intragranular bone strands were also evident and the woven bone formation was prominent.

Around the Cerasorb and Bio-Oss particles, new bone formation had started. The dissolution of the Cerasorb particles was proved by the macrophages around the Cerasorb particles. The new woven bone formation was expressed.

4.6. Results of VitalOs experiments

Pig experiments

The osseointegration period was 4 weeks and the same evaluation methods were applied as described previously for the Bio-Oss, Cerasorb and Cerasorb + Bio-Oss mixture experiments. The control samples were the same as in case of the 4-week healing period with Bio-Oss, Cerasorb and Cerasorb-Bio + Oss mixture samples, with an areal bone density of 25.1 ± 1.7%.

For VitalOs cement, the areal density of the newly-formed bone, 24.4 ± 1.3%, was not significantly different ($p = 0.207$) from the control value (Fig. 35a).

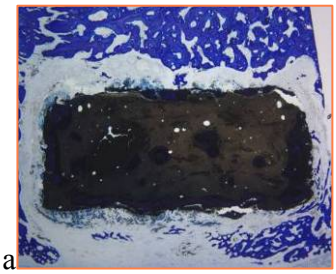
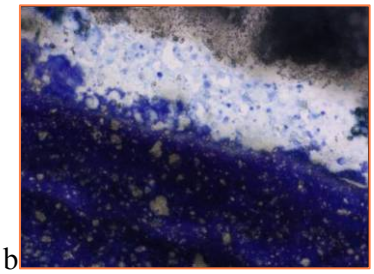
a  b 

Fig. 35a. *4-week VitalOs treatment (bright field, 1x magnification, toluidine blue staining);* ***b.*** *fibrotic zone around VitalOs (bright field, 20 x magnification, toluidine blue staining)*

The VitalOs cement was surrounded by woven bone in a ringlike form. This new woven bone was rather low in quantity. Between the bone substitute material and the bone, a fibrotic zone was formed, which contained granulocytes, macrophages and hystiocytes due to foreign body reactions (Fig. 35b).

4.7. Summary of results with different bone substitutes

Tables of mean areal bone densities are presented for different biomaterials. Differences are significant at $p < 0.05$.

New Zealand white rabbit experiment

Sample	Areal bone density ± SD (%)	*p*
Controls	34.0 ± 0.1	
Rh-BMP-2 solution	96.3 ± 4.5	*0.033*
Bio-Oss + Cerasorb mixture	48.7 ± 0.1	*0.005*

Vietnamease pot-bellied pig experiments

Sample	2 weeks+ Areal bone density ± SD(%) *p*	2 weeks- Areal bone density ± SD(%) *p*	4 weeks+ Areal bone density ± SD(%) *p*	4 weeks- Areal bone density ± SD(%) *p*
Control 1	-	19.1 ± 0.2	-	24.3 ± 5.6
Bio-Oss	46.2 ± 2.8 *0.043*	31.2 ± 2.5 *0.085*	56.5 ± 1.5 *0.056*	37.5 ± 0.8 *0.158*
Cerasorb	50.9 ± 1.2 *0.014*	27.7 ± 1 *0.041*	56.0 ± 0.3 *0.074*	30.8 ± 0.3 *0.328*
Control 2	-	-	-	25.1 ± 1.7
Bio-Oss+ Cerasorb mixture	-	-	57.5 ± 1.1 *0.008*	48.3 ± 0.9 *0.014*
VitalOs	-	-	-	24.4 ± 1.3 *0.207*

2, 4 weeks +: new bone formation together with bone substitute granules
2, 4 weeks -: new bone formation alone without bone substitute granules

5. Summary and Conclusions

In modern implantology, the need for bone augmentation techniques demands adequate osteoinductive and osteoconductive effects from the bone substitutes. It is very important that the original form of the bone should be reconstructed and also that an appropriate bone structure should be achieved as soon as possible.

Bio-Oss is a highly osteoconductive xenograft material certified for the regeneration of bone defects. In our experiments, we found that it displays very low resorbability and acts as an inert scaffold onto which bone-forming cells and blood vessels creep, forming the new bone. The areal density value of the new bone formed together with the Bio-Oss particles after 2 weeks was slightly higher than the control value, but significantly so.

Both in human and in animal experiments, Cerasorb seemed to have good bioresorptive and osteoconductive properties. In the human experiments, the histologic and histomorphometric examination of 68 bone biopsies taken from the 17 cases indicated nearly equal activities of bone regeneration on the two sides. The bone density data in the augmented sinus floor were similar, irrespective of whether autogenous bone or Cerasorb particles had been applied.

The ideal bone substitute maintains biological support during healing and is gradually replaced by the newly-formed bone. We have found that Cerasorb has a higher ’bone induction’ capacity than that of Bio-Oss in the early phase (2 weeks) of new bone formation. The areal bone density was also significantly higher in the case of new bone formation and for the samples including Cerasorb particles. In the late phase (4 weeks) of new bone formation, our results did not indicate a better capacity.

With a combination of the two materials in a Bio-Oss + Cerasorb mixture, we achieved rather promising results in both animal models (New Zealand white rabbits and Vietnamese pot-bellied pigs). In the rabbit femur model, the extent of new bone formation was 1.4 times higher when the two bone substitutes were mixed together. In the pig model (4 weeks), the areal bone density measurements revealed that the induced osteogenesis was increased 2.3- and 1.9-fold for new bone formation together with the granules and without the granules, respectively. The reason for this may be that Bio-Oss serves as a scaffold, but its resorbability is poor, while Cerasorb is a good bone-developing material, but resorbs too early, not providing a scaffold for the new bone bridges. Combining these characteristics of the two materials could give a promising result.

For VitalOs, the results of our pig experiments did not prove an increased bone formation.

The application of rhBMP-2 in dental implantology is an alternative to bone grafting in patients not originally regarded as candidates. The positive effect of rhBMP-2 solution on osteogenesis is proven by the results of our animal (rabbit) experiments. As regards the rate of osteogenesis, significant differences were found between the control and the two different test groups. In the rhBMP-2-treated rabbit femur, the new bone formation was more enhanced than that due to the effect of the Cerasorb + Bio-Oss mixture. It is evident from the measurements that for the test samples, (rhBMP-2 solution induced new bone formation) approximately 2.8 times as much new bone was formed as compared with the control specimens. The demonstrated efficacy of rhBMP-2 alone on bone regeneration is of great importance as concerns its biomedical applications. The combination of an osteoinductive agent (rhBMP-2) with a mostly osteoconductive bone substitute (Cerasorb or Bio-Oss) would give a promising result for reconstructive bone surgery.

The histological evaluation methods (decalcified paraffin samples) and the results of the push-out tests permitted some preliminary conclusions from the animal experiments with rhBMP-2-coated implants. The histomorphometric results of this experiment supported the outcome of the push-out tests: under the given circumstances, and at the given concentrations, BMP did not exhibit a significant effect on osseointegration during the 4-week healing period. The disadvantage of using decalcified samples, is that the interface is ruined almost completely on the removal of the

implant from the specimen. During decalcification, much valuable information could be lost. In order to save the interface, which is the most important and informative part of the samples, the only method that should be used is to cut the sample together with the implant, without any decalcification.

The evaluation of the osseointegration of RhBMP-2-coated implants with push-out tests demonstrated a significant difference between the shear strengths in the primary stability and osseointegrated control implant groups. Similarly, there was a significant difference between the interface shear strengths of the primary stability and osseointegrated BMP-covered implant groups. Conversely, there was no statistically significant difference between the two osseointegrated implant groups.

As mentioned in the Introduction of this thesis, most of the surface modification methods (physicochemical and biochemical) are in the experimental stage and the *in vivo* (animal or clinical) studies are still ahead. Our group has started to test some of these surface modifications by means of animal (rabbit and pig) experiments. Although, we could not perform sufficient measurements to draw exact conclusions, we can say that both animal models presented pull-out and push-out preliminary results which were in accordance with the findings of the histological and histomorphometric results. This strongly suggests that our models are appropriate for these types of studies. Furthermore, these experiments showed that there is potential in the Camlog experimental surface modification discs, as they presented a tendency for enhanced osseointegration during the 2-week healing period. The PE-ML modified samples did not afford signs of improving osseointegration. We plan to continue these experiments, in order to draw further conclusions.

As a final conclusion, our results proved that, although the animal models applied (pig and rabbit) are appropriate for comparisons of the effects of both osteogenic factors and biocompatible graft materials on osteogenesis; the pig calvaria model is more suitable for an analysis of these factors, as it is more effective and economical.

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I.

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Maxillary sinus floor grafting with β-tricalcium phosphate in humans: density and microarchitecture of the newly formed bone

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Key words: bone regeneration, histology, histomorphometry, sinus grafting, β-tricalcium phosphate

Abstract

Objectives: Graft insertion can effectively enhance the regeneration of debilitated bone. The effects of an alloplastic bone-replacing material, β-tricalcium phosphate (Cerasorb), and of autogenous bone graft were compared.

Materials and methods: In 17 edentulous patients, the maxillary sinus floor was extremely atrophied to such an extent that implant placement was impossible. The Schneiderian membrane was surgically elevated bilaterally by insertion of Cerasorb (experimental side) and autogenous bone graft (control side). After surgery, the recovery was followed clinically and radiologically. After 6 months, 68 bone cylinders were excised from the grafted areas and implants were inserted into their places. The bone samples were embedded into resin, and the osteointegration of the grafts was studied histologically. Trabecular bone volume (TBV) and trabecular bone pattern factor (TBPf) were quantified by histomorphometry.

Results: Cerasorb proved to be an effective bone-replacing material with osteoconductivity; it was capable of gradual disintegration, thereby providing space for the regenerating bone. The new bone density was not significantly different on the experimental and control sides (32.4 ± 10.9% and 34.7 ± 11.9%, respectively). However, the graft biodegradation was significantly slower on the experimental side than the control side. The TBPf value was lower on the control side than on the experimental side (-0.53 ± 1.7 and $-0.11 \pm 1.4\,mm^{-1}$, respectively), but this difference was not significant.

Conclusions: Six months after insertion of the grafts, the bone of the augmented sinus floor was strong and suitable for anchorage of dental implants, irrespective of whether autogenous bone or Cerasorb particles had been applied.

Bone regeneration can be facilitated both by systemic factors and by local insertion of bone-substitute materials. In maxillofacial surgery, various types of bone defects (atrophied alveolar ridge, periodontal bone destruction, cystic and tumorous jaw lesions and traumatic bone deformities) give rise to a need for local bone replacement (Moy et al. 1993; Aygit et al. 1999; Groeneveld et al. 1999). Maxillary and mandibular bone alterations require excellent cosmetic reconstruction and also a good load-bearing capacity because of the masticatory forces. The strength of the newly formed bone is especially important when a bony bed is to be prepared for anchorage of dental implants.

Reduction of the chewing forces as a consequence of advanced age and the loss of teeth results in a gradual thinning of the

bony floor of the maxillary sinus (Ariji et al. 1994). The alveolar recesses gradually extend even into the edentulous alveolar process (pneumatization). At the same time, vertical and horizontal bone loss of the alveolar ridge caused by outer resorption makes denture fixation impossible (Cawood & Howell 1988).

Augmentation of the bony sinus floor by insertion of graft materials is an excellent method to construct a suitable bony bed for implant placement (Tatum 1986; Misch 1987). Clinical and pathological evidence from many studies indicates that the use of an autogenous bone graft is favored as a gold standard (Boyne & James 1980; Wood & Moore 1988). However, there are many problems associated with harvesting of an adequate quantity of autogenous bone. It requires a second surgery, causing increases in the time demands and costs of the therapy, and giving rise to considerable complications at the donor site (Kalk et al. 1996).

The availability of suitable biomaterials to be used as a bone replacement that facilitates the bone regeneration would eliminate the need for an extra surgical site. Various grafts have been used for this purpose, including allogenic bone, alloplastic bone substitutes and their combinations (Moy et al. 1993; Aygit et al. 1999; Hanish et al. 1999; Tadjoedin et al. 2000; Yildirim et al. 2000). The effects of these bone-replacing materials have been extensively studied in animal models (McAllister et al. 1999; Liu et al. 2000; Haas et al. 2002; Suba et al. 2004) and *in vitro* experiments (Laurencin et al. 1996; Anselme et al. 1999), but a direct comparison of the results with the clinical data on the patients is impossible.

Clinical observations in humans require non-invasive techniques, such as radiology and macromorphometry. However, the most effective way of evaluation of the density and stability of newly formed bone is histology and histomorphometry. An advantageous approach is the application of a two-stage technique: when the first step is the graft insertion and the second, after several months, is the implant placement in the grafted site (Lundgren et al. 1997). This second step provides an excellent possibility for taking biopsy specimens from the regenerating bone.

In a preliminary study, the two-stage technique was applied in four edentulous patients (Szabó et al. 2001). Bilateral sinus elevations revealed a similar bone-forming capacity of β-tricalcium phosphate (Cerasorb) and of an autogenous bone graft. The present study continues the previous one, with a prospective-controlled histologic and histomorphometric analysis of 17 cases, subjected to statistical analysis.

Materials and methods

Patient selection

The patient population comprised 17 completely edentulous individuals (10 women and 7 men) with an average age of 52 years (range, 39–66 years). The patients had no disease that might influence the treatment outcome. They were fully informed about the procedures, including the surgery, the bone substitute material and the implants. All gave their written informed consent. The Ethics Committee of the Semmelweis University approved the research protocol.

Preoperative examinations with panoramic images and in some cases, computed tomographic scans were performed. All patients had an insufficient bone height in the subantral maxillary floor (average 1.9 mm), being unsuitable for immediate implant placement. Improving the vertical bone height through surgical sinus floor augmentation created suitable implant sites. In the 17 cases, bilateral sinus floor elevations were completed.

Surgical procedures

In all 17 cases, surgery was performed under general anesthesia. The autotransplant was harvested before the time of sinus grafting; 4–5 cm^3 spongiosa were taken from the left iliac crest. The bilateral sinus grafting was completed according to the classic method of Tatum (1986). The cavity thus created under the Schneiderian membrane was filled with 1.5–2 g β-tricalcium phosphate granules 500–1000 μm in diameter (Cerasorb, Curasan AG, Kleiostheim, Germany) on the experimental side, and with 3–4 cm^3 of autogenous spongiosa on the control side. After an average of 6.5 months of healing (range, 6–7.5 months), 68 cylindrical bone biopsies were taken from the grafted posterior maxilla (two each from the experimental and control sides of all patients) using a trephine bur of 2 mm inner diameter and 3 mm outer diameter. Ankylos implants (Degussa, Friadent, Germany) were placed in osteotomy sites thus prepared.

Histology

For histologic and histomorphometric analyses, undecalcified bone biopsies were fixed in 4% buffered formalin for 24 h and then rinsed thoroughly in running water. Samples were dehydrated in ascending alcohol series and then embedded in methylmethacrylate resin at 4°C. Histological sections of 5 μm thickness were cut parallel to the longitudinal axis of the biopsy specimen, using a diamond knife and a Jung-K microtome. Sections were stained with toluidine blue, hematoxylin and eosin and Goldner's trichrome stain for light microscopy. Graft particles often broke off the sections during handling, but their characteristic shapes were easily recognizable. Under polarized light, Cerasorb graft remnants and new collagenous bone trabeculae could be clearly distinguished by virtue of their different birefringence.

Tissue reactions, bone regeneration around the graft particles, microscopic structure of the bone/graft interface, graft bioresorption and new bone quantity and quality were histologically assessed.

Photomicrographs were taken by an Olympus BH2 microscope equipped with an Olympus DP50 digital camera (Olympus Optical Company Ltd., Melville, NY, USA).

Histomorphometric analysis

Measurements on the histological sections were performed by a computerized technique; the operating system applied was the Windows XP service pack 1 (©:Microsoft Corporation, Redmond, OR, USA). Image processing was performed with AnalySIS® of Soft Imaging System (Münster, Germany). Measurement fields were selected by visual monitoring of the microscopic image on screen. RGB images were converted to gray scale, and a manually refined luminescence threshold was then created to define the structures to be measured. Automatic calculation of the perimeter and area was achieved via pixel counting. Arithmetic dilatation of the chosen areas was performed by adding one pixel to each

surface. After calibration, the area was given in mm^2 and the length in mm.

Histomorphometric measurements were performed according to the principles of Parfitt et al. (1987). The density of the newly formed bone was characterized by the trabecular bone volume (TBV), which was defined as the area of the bone trabeculae as a percentage of the total area analyzed. The percentage of the graft area was also determined. The trabecular bone pattern factor (TBPf), which is an indicator of the microarchitecture of the newly formed bone, was also quantified (Hahn et al. 1992). Measurements of the trabecular bone area and perimeter, before and after arithmetic dilatation of the binary image, were performed to determine the relation of the convex and concave trabecular structures in the two-dimensional section. The higher the degree of trabecular connectivity, the lower the value of TBPf.

Statistical analysis

The mean and SD values were calculated. The data obtained were analyzed by a Student's *t*-test, with a significance level set at $P<0.05$.

Results

Clinical observations

The healing period following maxillary sinus augmentation was completed for nearly all patients without complications. Minor nosebleeds occurred in two cases. No clinical symptoms indicating maxillary sinusitis occurred in any of the 17 patients. Postoperative complications at the donor site were not observed. On average, the radiographic vertical height of the grafted sinus floor was 15 mm (range: 12–16 mm) on the experimental side and 14.5 mm (range: 12–15 mm) on the control side.

Histology

Experimental side

In sections stained with hematoxylin & eosin and Goldner's trichrome method, many graft particles had been dissolved. However, their previous location could easily be recognized by their characteristic round or scalloped form and size (Fig. 1). The partly resorbed graft particles were not only invaginated by apposition of the newly formed bone, but the individual granules had an osteiod or woven bone network in their pore system (Fig. 2). Intragranular invasion of the newly formed bone was conspicuous without any sign of osteoclastic activity. However, cytoplasmic accumulation of small achromatic graft remnants in the macrophages was a notable finding (Fig. 3). After 6 months, the newly formed, predominantly lamellar bone was tightly intermingled with the graft particles at the tissue/graft interface. Bony sheathing of the Cerasorb granules was extensive, and where the graft particles had become completely sheathed, the osteoblastic activity disappeared. The graft remnants exhibited achromatic birefringence under polarized light (Fig. 4).

In one sample, there was a lack of bone formation in a demarcated area, in association with an intense inflammatory reaction.

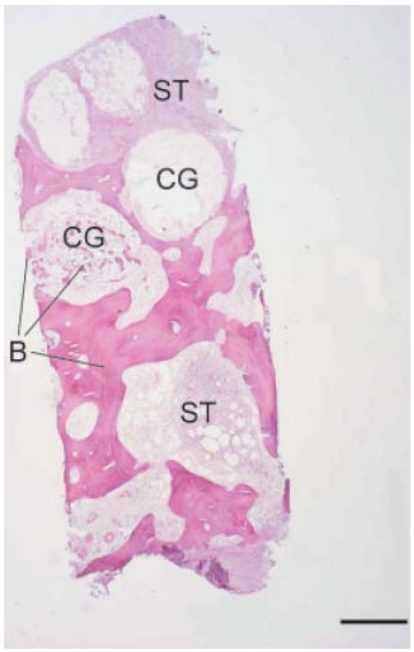

Fig. 1. Biopsy taken from the experimental side after 6 months of grafting. CG, cerasorb granule; B, bone; ST, soft tissue. H&E staining (scale bar 500 μm).

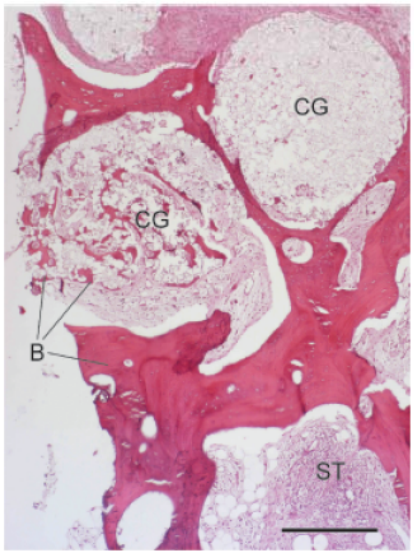

Fig. 2. Biopsy taken from the experimental side after 6 months of grafting. Porosity of the cerasorb granule (CG) is filled by branching bone strands (B). ST, soft tissue. H&E staining (scale bar 600 μm).

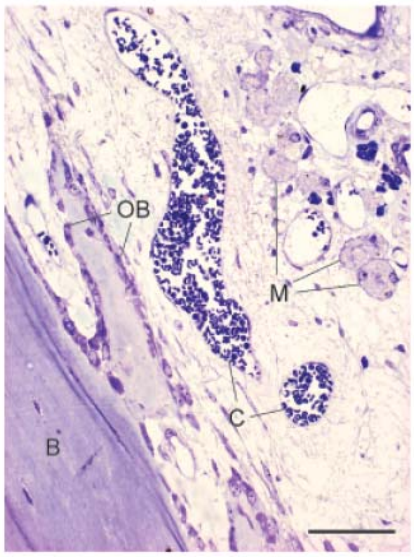

Fig. 3. Biopsy taken from the experimental side after 7 months. New bone formation (B) osteoblast activity (O) and achromatic particle-laden macrophages (M) can be seen. C, capillaries. Toluidine blue staining (scale bar 80 μm).

Control side

The cancellous bone grafts had undergone considerable resorption by the sixth month (Fig. 5). Graft foci were entrapped in the newly formed, predominantly mature lamellar bone (Fig. 6). They were homogenous acellular particles that stained almost like living bone. There was a continuous transition at the interface of the cancellous graft and the new bone. Some trabeculae demonstrated active bone remodelling with a chain of plump osteoblasts on one side, and resorption lacunae with multinucleate osteoclasts on the opposite side. Under polarized light, new bone formation was clearly recognized by the bright red birefringence of its collagenous fibers. Several

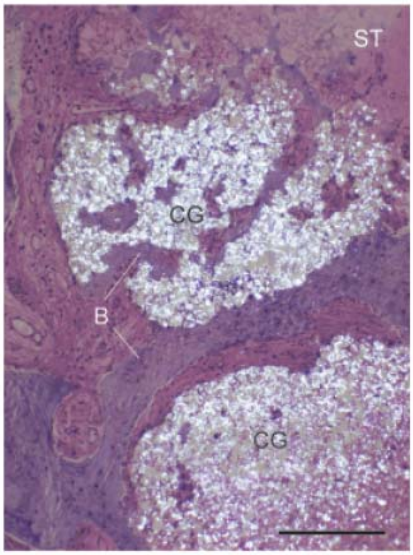

Fig. 4. Biopsy taken from the experimental side after 6.5 months of grafting. Scalloped surface of the cerasorb granule (CG), and peri- and intragranular bone strands (B) can be seen. ST, soft tissue. Toluidine blue staining. Polarized light (scale bar 400 μm).

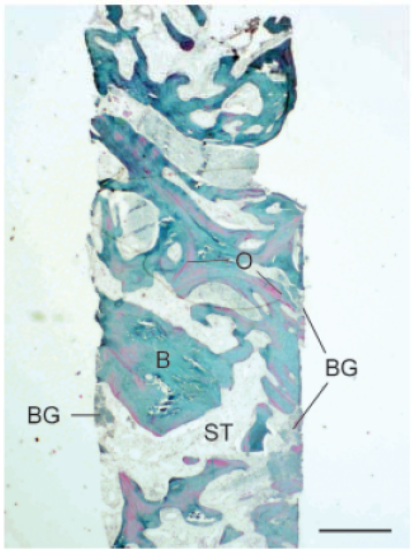

Fig. 5. Biopsy taken from the control side after 6 months of grafting. B, bone; O, osteoid; BG, bone graft; ST, soft tissue. Goldner staining (scale bar 500 μm).

samples showed minor bone formation, a predominantly fibrous marrow and a diffuse, thin network of newly formed trabeculae.

Histomorphometry

Bone density, graft density, TBPf and bone area were measured in 68 bone biopsy samples (Table 1).

Bone density

The mean bone density for the 17 cases was 32.4 ± 10.8% on the experimental side and 34.7 ± 11.9% on the control side; the difference was not significant ($P > 0.05$).

In an overwhelming majority of the patients (14 of 17 cases), the intensity of new bone formation was similar on the two sides.

The new bone was markedly less dense on the experimental side in two of the 17 cases (nos. 4 and 9). In one of these patients, the minimal bone formation was associated with a local inflammatory reaction. In the other case, the Cerasorb graft density was quite high (21.2%).

The bone-forming capacity on the control side was smaller than on the experimental side in one case (no. 8).

In two cases (nos. 10 and 17), the ossification process was similarly weak on the two sides; the respective densities of the newly formed bone were 19.2% and 18.5% on the experimental side, and 18.4% and 17.6% on the control side. In these two cases, the new bone trabeculae were uniformly thin, with no focal inflammatory lesion.

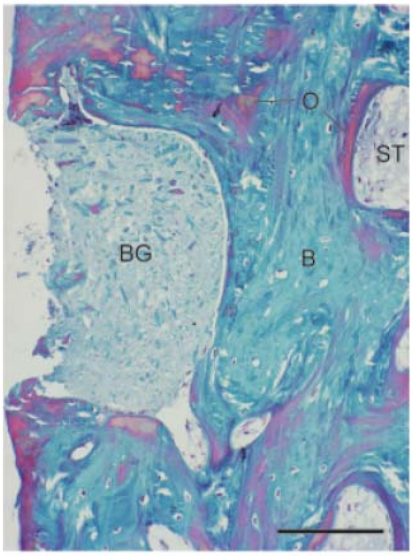

Fig. 6. Biopsy taken from the control side after 6 months of grafting. Bone (B) and osteoid (O) production and a focus of the resorbing bone graft (BG) can be seen. ST, soft tissue. Goldner staining (scale bar 200 μm).

Graft density

The graft density was markedly higher on the experimental side than on the control side. The mean density of the graft area was 13.1 ± 4.5% and 8.2 ± 1.7%, respectively, this difference being highly significant ($P < 0.001$).

Connective tissue and bone marrow density

The mean densities of the connective tissue areas were similar on the experimental

Table 1. **Histomorphometric values* on the experimental and control sides**

No.	Experimental side			Control side		
	Bone density (%)	Graft density (%)	TBPf (1/mm)	Bone density (%)	Graft density (%)	TBPf (1/mm)
1	41.8	13.1	− 0.16	51.5	7.6	− 0.12
2	32.6	11.3	1.56	36.3	8.9	− 0.88
3	24.8	17.5	0.31	27.6	6.7	− 1.01
4	16.7	21.2	0.22	33.6	10.5	− 1.87
5	23.8	10.4	0.55	24.25	8.6	1.22
6	36.5	8.7	− 0.11	34.6	11.3	− 0.57
7	29.9	11	− 0.17	31.4	7.9	− 0.29
8	27.6	9.8	− 1.86	16.4	6.8	0.94
9	25	19.4	− 1.96	44.2	7.8	− 1.86
10	19.2	12.2	1.63	18.4	10.6	2.14
11	47.2	10.8	0.27	50.8	5.4	− 3.23
12	50.7	8.1	− 2.19	46.6	6.9	− 2.08
13	40.4	10.3	− 1.25	42.8	7.1	− 1.99
14	26.7	12.2	1.85	24.5	8.7	1.11
15	45.7	11.8	− 1.86	49.2	5.8	− 2.25
16	43.4	11.2	− 1.03	40.2	8.1	− 1.43
17	18.5	23.5	2.41	17.6	9.9	3.13
Mean	32.38	13.09	− 0.11	34.7	8.15	− 0.53
SD	10.85	4.49	1.43	11.86	1.69	1.74
	Bone density	Graft density	TBPf			
P	0.10693659	0.00012541	0.1156			

*Each value is an average of two measured data on parallel biopsy samples.
TBPf, trabecular bone pattern factor.

and control sides (49.4 ± 16% and 50.4 ± 19%, respectively). The mean bone marrow density was higher on the control side (6.8 ± 2.9%) than on the experimental side (5.1 ± 2.2%) ($P>0.05$).

TBPf

These values generally displayed an inverse correlation with the bone density (Table 1). The higher the bone density, the lower the TBPf. In 11 of the 17 cases, the TBPf values on the control side were lower than that on the experimental side. The mean values were −0.53 ± 1.74 and −0.11 ± 1.43 mm $^{-1}$, respectively, but the difference was not significant ($P>0.05$).

Area and length of biopsy samples

The mean areas of the biopsy samples taken from the two sides were quite similar: 8.85 ± 1.7 mm^2 on the experimental side and 9.12 ± 2.28 mm^2 on the control side. The mean length of the biopsy samples was 7.83 ± 1.9 mm on the experimental side and 8.08 ± 2.2 mm on the control side.

Discussion

The quantity and quality of the cancellous host bone are crucial for the stability of endosseous implants placed in the alveolar ridge. Bone graft insertion is commonly used in an effort to increase the bony support for oral implants (Boyne & James 1980; Lundgren et al. 1997; Groeneveld et al. 1999). The incorporation of the graft and the integration of the implants are both complex healing situations and must result in a direct contact between the implant and the remodelled, grafted bone. The maxillary sinus floor seems to be ideally suited for the use of various bone substitutes, because it has a high osteoregenerative potential.

In totally edentulous patients and in cases of serious local atrophy, a two-stage technique is the correct choice (Lundgren et al. 1997). The first step is the sinus floor grafting, which requires a longer time to be revascularized and incorporated. The second step is the implant placement, several months later; this promotes an immediate healing response, similar to that in natural viable bone. At the same time, histological analysis of bone biopsies taken from the grafted site allows an evaluation of the integration and resorption of the bone substitute used.

Bilateral sinus grafting in the same patient, under nearly identical circumstances, is an excellent method for comparison of the bone-regenerating effects of different graft materials (Moy et al. 1993; Groeneveld et al. 1999; Tadjoedin et al. 2000). As autogenous bone grafting is regarded as the gold standard, such an insertion is favored as a control method (Wood & Moore 1988; Klinge et al. 1992; Becker et al. 1994).

Clinical and radiological evaluations are widely used to assess the results of bone regeneration. However, the quantity and structural quality of the healing cancellous bone can be exactly assessed only by means of histologic and histomorphometric methods.

Histologic and histomorphometric examination of 68 bone biopsies taken from the 17 cases in the present study indicated nearly equal activities of bone regeneration on the two sides. The bone density data in the augmented sinus floor were similar, irrespective of whether autogenous bone or Cerasorb particles had been applied.

New bone formation was concentrated primarily on the surface and in the pore system of the Cerasorb granules on the experimental side. Penetrating inward bone growth reflected a gradual biological degradation of the bone-substitute material. Zerbo et al. (2001) histologically observed active osteoclastic resorption of Cerasorb granules. Accordingly, the rate of remodelling may be the main factor causing loss of the graft particles. Other mechanisms, primarily physical dissolution, can also operate in the removal of this graft material. The histologic findings in the present study did not support osteoclastic graft resorption; however, small cytoplasmic Cerasorb particles in the mononuclear macrophages suggested active cellular elimination.

In the present study, there were three cases with unilateral lower rates of bone regeneration, on the control side in one patient and on the experimental side in two. One case on the experimental side could be explained by a local inflammatory reaction; in the other case, the biopsy sample was crowded by graft remnants. On the control side, there was no plausible explanation for the minimal bone healing. These cases supported the role of local factors such as microvascular deficiency of the atrophied bone (Solar et al. 1999).

In two cases of the present study, there was only minor bone formation on both sides. One of them involved the oldest patient, while the other case was a postmenopausal woman.

Statistically evaluable morphometric results are not available with respect to Cerasorb-grafted sinus floor elevations in humans. The effect of bioactive glass mixed with autogenous bone particles was earlier compared with that of bone particles alone in bilateral sinus floor augmentation cases (Tadjoedin et al. 2000). After 6 months, the trabecular bone densities were higher than the present data, on both the experimental and control sides (44% and 38%, respectively).

Maxillary sinus floor augmentation with a mixture of Bio-Oss xenograft and autogenous bone was also studied (Yildirim et al. 2001). The histomorphometric results revealed a markedly lower bone density (18.9%) and a higher graft density (29%) as compared with the present data. However, the average density of the bone–graft complex was close to 50%, which was similar to the present findings.

The combination of Bio-Oss and venous blood in human maxillary sinus elevation cases yielded modest results (Yildirim et al. 2000). The average density of the newly formed bone was only 14.7%, while the proportion of the xenogenic graft residue remained at 29.7%.

In addition to the bone quantity, another factor requiring consideration is the bone microarchitecture. TBPf measurements on grafted bone samples give rise to special problems, depending on the nature of the graft. In porous, alloplastic materials, the intragranular weblike bony network exhibits many thin branching-free endings, and this will negatively influence the trabecular connectivity measurements. In the present study, the better trabecular connectivity resulted in lower TBPf values on the control side. However, these differences did not prove significant.

Graft biodegradation is also an important factor in bone substitution (Zerbo et al. 2001; Suba et al. 2004). In our cases, graft resorption was followed by new bone de-

position on both sides. However, after 6 months, the graft density was significantly higher on the experimental side than on the control side. A further question is whether persistence of the graft inclusions can affect the stability of the newly formed bone. The present results revealed that there was no significant difference in bone density between the two sides. This suggests that alloplastic granules persisted predominantly on account of the bone marrow space.

The size of the biopsy samples can also influence the quantitative comparison of the effects of bone substitutes, as the grafted bone samples are not homogeneous. The greater the bone area, the more representative the measurement. In our study, the areas of the bone biopsies from the two sides did not differ significantly.

In clinical practice, long-term studies under prosthetic loading will be necessary to clarify the success rate of implantation in the augmented regions. However, histologic and histomorphometric results can predict the load-bearing capacity of the bony bed, and reveal the weakest points requiring more careful handling.

Résumé

L'insertion d'un greffon peut augmenter de manière efficace la régénération de l'os affaibli. Les effets d'un matériel de remplacement osseux alloplastique, le phosphate β-tricalcique (Cerasorb) et un greffon osseux autogène ont été comparés. Chez dix-septs édentés, le plancher sinusal maxillaire était extrêmement atrophié à telle enseigne que tout placement implantaire s'avérait impossible. La membrane de Schneiderian a été chirurgicalement élevée bilatéralement par l'insertion de Cerasorb (site expérimental) ou d'os autogène (site contrôle). Après la chirurgie, la guérison a été suivie cliniquement et radiographiquement. Après six mois, 68 cylindres osseux ont été prélevés des zones greffées et des implants ont été insérés à ces endroits. Les échantillons osseux ont été enfouis dans la résine et l'ostéoïntégration des greffons a été évaluée histologiquement. Le volume osseux trabéculaire (TBV) et le facteur du modèle osseux trabéculaire (TBPf) ont été quantifiés par histomorphométrie. Le Cerasorb était un matériau de remplacement osseux efficace montrant une ostéoconductivité; qui était capable d'une désintégration graduelle, apportant ainsi un espace pour l'os régénéré. La densité de l'os néoformé n'était pas significativement différente entre les sites expérimentaux et contrôles (respectivement 32 ± 11% et 35 ± 12%). Cependant, la biodégradation du greffon était significativement plus lente au niveau du site expérimental qu'au niveau du contrôle. La valeur TBPf était inférieure au niveau du site contrôle vis-à-vis de l'expérimental (−0.53 ± 1.7 mm ^{1} et −0.11 ± 1.4 mm ^{1}) mais cette différence n'était pas significative. Six mois après l'insertion des greffons, l'os du plancher sinusal épaissi était fort et acceptable pour le placement d'implants dentaires que cela soit de l'os autogène ou des particules de Cerasorb qui avaient été utilisés.

Zusammenfassung

Ziel: Das Einsatz eines Transplantates kann die Regeneration von verlorengegangenem Knochen effektiv beschleunigen. Man verglich die Einflsse eines alloplastischen Knochenersatzmaterials, dem -Tricalciumphosphat (Cerasorb), und einem autologen Knochentransplantat.

Material und Methode: Bei 17 zahnlosen Patienten war der Boden des Sinus maxillaris so extrem tief abgesunken, dass die verbleibende Knochendicke am Sinusboden fr die Implantation nicht ausreichte. Daher hob man in einem chirurgischen Eingriff beidseits die Schneider'sche Membran ab und fllte den Hohlraum mit Cerasorb (Testseite) oder autologem Knochen (Kontrollseite) auf. Nach dem operativen Eingriff verfolgte man klinisch und rntgenologisch die weitere Entwicklung. 6 Monate spter entnahm man 68 bei der Implantation als Nebenprodukt entstehende Knochenzylinder aus den aufgebauten Regionen und setzte in die entstandenen Bohrstollen je ein Implantat. Die Knochenbiopsien bettete man in Kunststoff ein und berprfte histologisch die Osteointegration der Transplantate. Histomorphometrisch bestimmte man anschliessend das Knochenvolumen des trabekulren Knochens (TBV) und einen Faktor, der den Knochenersatz des trabekulren Knochens wiederspiegelt (TBPf).

Resultate: Cerasorb zeigte sich als effizientes Knochenersatzmaterial mit Osteokonduktivitt; es kann schrittweise aufgelst werden und hinterlsst hierbei Raum fr den sich regenerierenden Knochen. Die Knochendichte des sich neu bildenden Knochens unterschied sich nicht siginifikant von der Kontrollseite (32.4 ± 10.9%, beziehungsweise 34.7 ± 11.9%). Der biologische Abbau des Transplantates erfolgte aber auf der Testseite signifikant langsamer als auf der Kontrollseite. Der TBPf-Wert war auf der Kontrollseite kleiner als auf der Testseite (−0.53 ± 1.7 mm ^{1}, beziehungsweise −0.11 ± 1.4 mm ^{1}); dieser Unterschied war aber nicht signifikant.

Zusammenfassung: Sechs Monate nach dem Einbringen der Transplantate war der Knochen im aufgebauten Sinusbodenbereich stabil und zur Verankerung von Zahnimplantaten geeignet, unabhngig davon ob autologer Knochen oder Cerasorb-Partikel verwendet worden waren.

Resumen

Objetivos: La inserción de injertos puede efectivamente realzar la regeneración de hueso debilitado. Se compararon los efectos de un material de sustitución ósea aloplástico, â-fosfato tricálcico (Cerasorb), y de injerto de hueso autógeno.

Material y métodos: En 17 pacientes edéntulos, el suelo del seno maxilar estaba extremadamente atrófico hasta tal extremo que la colocación de implantes fue imposible. Se elevó quirúrgicamente la membrana de Schneider bilateralmente insertándose Cerasorb (lado experimental) e injerto de hueso autógeno (lado de control). Tras la cirugía, la recuperación fue seguida clínica y radiograficamente. Tras seis meses, se extirparon 68 cilindros de hueso de las áreas injertadas y se insertaron implantes en su lugar. Las muestras de hueso se embebieron en resina, y se estudió histológicamente la osteointegración de los injertos. Se cuantificaron por histomorfometría el volumen de hueso trabecular (TBV) y el factor de patrón de hueso trabecular (TBPf).

Resultados: El Cerasorb demostró ser un material sustituto óseo efectivo con osteoconductividad; fue capaz de una desintegración gradual, por ello suministrando un espacio para el hueso de regeneración. La nueva densidad ósea no fue significativamente diferente en los lados experimentales y de control (32.4 ± 10.9% y 34.7 ± 11.9% respectivamente). De todos modos, la biodegradación fue significativamente mas lenta en el lado experimental que en el lado de control. El valor del TBPf fue menor en el lado de control que en el lado experimental (−0.53 ± 1.7 mm ^{1} y −0.11 ± 1.4 mm ^{1}, respectivamente); pero esta diferencia no fue significativa.

Conclusiones: Seis meses tras la inserción de los injertos, el hueso del suelo del seno aumentado era fuerte y adecuado para el anclaje de implantes dentales, sin tener en cuenta si se había aplicado hueso autógeno o partículas de Cerasorb.

要旨

目的：移植材料の埋入は、弱体化した骨の再生を効果的に促進できる。人工骨代替材料の一つであるβ燐酸３カルシウム（**Cerasorb**）と自家骨移植を比較した。

材料と方法：無歯顎患者１７名において上顎洞が極度に萎縮しており、インプラントの埋入が不可能であった。**Cerasorb**（実験部位）と自家骨移植片（対照部位）を両側に埋入し、シュナイデル膜を外科的に挙上した。術後の回復を、臨床的検査とレントゲンによって経過観察した。６ヵ月後に６８箇所の移植部位で骨床を形成し、インプラントを埋入した。切除した骨標本を樹脂に包埋し、移植片の骨性結合を組織学的に検討した。海綿骨量（TBV）と海綿骨パターン・ファクター（TBPf）を組織形態計測法によって定量化した。

結論：Cerasorb は骨伝導性を有する有効な骨代替材料であることが証明されているが、徐々に分解していき、再生骨のための空間を提供した。新生骨の密度について、実験部位と対照部位の間に統計学的な有意差はなかったが（各々３２．４±１０．９%と３４．７%±１１．９%）、移植片の生体分解の速度は対照部位より実験部位が有意に遅かった。ＴＢＰｆ値は、実験部位より対照部位の方が低かったが（各々－０．５３±１．７mm^{-1}と－０．１１±１．４mm^{-1}）、この差は有意差ではなかった。

結果：移植片埋入後６ヵ月後に造成された上顎洞底の骨は、自家骨あるいは **Cerasorb** のいずれにおいても、インプラントの固定に適した強度であった。

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II.

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A maxilla csontregenerációjának mennyiségi és minőségi összehasonlítása β-tricalcium phosphate és autolog csontbeültetés után

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A graftbeültetés hatékonyan fokozza a károsodott csont regenerációját. A szerzők egy alloplasztikus csontpótló anyag, a β-tricalcium phosphate (Cerasorb) hatását hasonlították össze az autológ csontgraft hatásával. 17 fogatlan beteg vett részt a vizsgálatban, akiknek maxillaris sinus aljazata rendkívül athrophiás volt, ezért implantátum behelyezésére nem volt alkalmas. A Schneider-membránt sebészi úton megemelték mindkét oldalon, és a kísérletes oldalon Cerasorbot, a kontroll oldalon autológ csontgraftot ültettek be. A sebészi beavatkozást követően a gyógyulást klinikailag és radiológiailag ellenőrizték. Hat hónap után 68 csonthengert távolítottak el a regenerálódó csont területéről, és helyükre implantátumokat ültettek be. A csontmintákat ezután műgyantába ágyazták, és szövettanilag tanulmányozták a beültetett graftok osteointegrációját. A csontsűrűséget és a csontgerendák mintázatát kvantitatíve értékelték hisztomorphometrias mérésekkel. A Cerasorb hatékony csontpótló anyagnak bizonyult, melynek jelentős oszteokonduktív hatása van. Fokozatos lebomlásra képes, ezáltal teret biztosít a regenerálódó csontnak. Az új csont denzitása nem különbözött szignifikánsan a kísérletes és a kontroll oldalon. A graft biodegradációja viszont jelentősen lassúbb volt a kísérletes oldalon, a kontroll oldalhoz hasonlítva. A gerendás csontmintázat (TBPf) érték kedvezőbb volt a kontroll oldalon, mint a kísérletes oldalon, de ez a különbség nem volt szignifikáns. A szerzők megállapították, hogy 6 hónappal a műtét után a kialakuló csont megfelelő elhorgonyzást biztosított a műgyökér implantátumoknak, függetlenül attól, hogy autológ csont vagy Cerasorb volt a beültetett graft.

Kulcsszavak: sinus-graft, csontregeneráció, hisztológia, hisztomorfometria, β-tricalcium phosphate

Bevezetés

A csontregenerációt szisztémás tényezők és lokálisan beültetett csontpótló anyagok egyaránt élénkíthetik. A maxillofaciális sebészetben különféle csontdefektusokkal találkozunk (athrophiás alveoláris taraj, parodontális csontdestrukció, cysták, daganatok, fejlődési rendellenességek és traumás csontdeformitások), amelyek lokális csontpótlást igényelnek [3, 7, 18]. A maxilla és mandibula csontdefektusai kitűnő kozmetikai rekonstrukciót igényelnek, ugyanakkor nagyon jó teherbíró képességre van szükség a rágóerők hatása miatt. Az újdonképzett csontnak különösen erősnek kell lenni akkor, ha műgyökér implantátum elhorgonyzását tervezzük.

A rágóerők időskori csökkenésével és a fogak elvesztésével a sinus maxillaris csontos aljzatának folyamatos vékonyodása következik be [2]. A sinus recessusok fokozatosan beterjednek a fogatlan processus alveolaris állományába (pneumatizáció). Ugyanakkor az alveolaris taraj vertikális és horizontális sorvadása is bekövetkezik, ezáltal a műfogsor rögzítése lehetetlenné válik [6].

A sinus maxillaris csontos aljzatának vastagítása graftbeültetéssel kitűnő módszer arra, hogy megfelelő csontos támasztékot alakítsunk ki a beültetendő műgyökereknek [17, 24]. Klinikai és pathológiai vizsgálatok támasztják alá, hogy az autológ csontgraftbeültetés a legelőnyösebb, ezt arany standardként alkalmazzák [5, 25]. Kétségtelen azonban, hogy megfelelő mennyiségű autológ csont kinyerése számos problémát vet fel. Szükséges egy második sebészi beavatkozás, amely megnöveli a terápia költségeit, és a kezelés időbelileg is elhúzódik. Ezenkívül jelentős szövődmények is felléphetnek a donor csont területén [11].

A megfelelő bioanyagok használata csontpótlás céljára lehetővé teszi, hogy elkerüljük a további sebészi beavatkozást. Különféle csontpótló graftok használhatók erre a célra, idetartoznak az allogén és alloplasztikus csontpótló anyagok és ezek kombinációi [3, 10, 18, 23, 26]. Ezen csontpótló anyagok hatását nagyon jól lehet tanulmányozni állatkísérletes modelleken [16, 8, 14, 22] és in vitro kísérletek során [13, 1], de a kísérletes eredményeket nem lehet közvetlenül összehasonlítani az emberi leletekkel.

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Elfogadva: 2005. október 26.

A klinikai kutatások a human gyakorlatban megfelelő, nem invazív technikát igényelnek, pl. a radiológia és a makromorfometria jó lehetőségeket biztosít. Mindenesetre a leghatékonyabb módszer az újdonképzett csont denzitásának és stabilitásának vizsgálatára a szövettani és a hisztomorfometriai értékelés. Előnyös a kétlépcsős technika alkalmazása, amikor az első lépcső a graftbeültetés, és néhány hónap múlva a második lépcső az implantátum behelyezése a regenerálódó csontba [18]. Ez a második lépcsőfok kitűnő lehetőséget nyújt a biopsziás szövetmintavételre, így mennyiségileg és minőségileg értékelhetjük a csontregenerációt.

Előzetes vizsgálatok során kétlépcsős technikát alkalmaztunk 4 fogatlan betegnél [22]. Kétoldali sinus elevatio során, hasonló csontképzési aktivitást találtunk a Cerasorbbal és az autológ csonttal kezelt oldalon. Jelen vizsgálatunk a korábbiak folytatásának tekinthető. Prospektív, kontrollált, hisztológiai és hisztomorfometriai elemzést végeztünk 17 esetben, és az eredményeket statisztikailag értékeltük.

Anyag és módszer

A betegek. A vizsgálatot teljesen fogatlan egyéneken végeztük el (10 nő és 7 férfi), akiknek átlagos életkora 52 év volt (szélsőértékek: 39–67 év). A betegeknek nem volt olyan betegsége, amely a csontgyógyulást jelentősen befolyásolta volna. A betegeket részletesen tájékoztattuk a tervezett eljárással kapcsolatban, beleértve a sebészi beavatkozást, a csontpótló anyagot és az implantátumokat. Valamennyien írásos beleegyezésüket adták a vizsgálathoz. A Semmelweis Egyetem Etikai Bizottsága engedélyezte a vizsgálat elvégzését.

A preoperative vizsgálatok során panoráma röntgenfelvételeket készítettünk, és indokolt esetben komputertomográfiás vizsgálatot is alkalmaztunk. Valamennyi betegnél rendkívül elvékonyodott a sinus maxillaris aljzat (átlagosan 1,9 mm). Az ilyen mértékű sorvadás az implantátum-behelyezést lehetetlenné tette. A vertikális csontdimenzió növelésére kétoldali sinusaljzat-emelést végeztünk, hogy a beültetendő műgyökereknek megfelelő támasztékuk legyen.

Elő- és utókezelés. A műtétet megelőzően gondos depurálással és 1 napos Corsodyl-öblögetéssel biztosítottuk a megfelelő szájhigiénét. A műtét előtt 1 órával profilaktikus antibiotikum kezelést kezdtünk 2 g Amoxicillin tablettával, majd 5 napon át napi 2 x 1 g-ot alkalmaztunk. Műtét után Nasivin orrcseppet rendeltünk a nyálkahártya-duzzanat enyhítésére.

Műtéti eljárások. Valamennyi esetben általános érzéstelenítésben történt a műtéti beavatkozás. Az autotransplantátumot a sinusaljzat-emelést megelőzően nyertük a bal oldali csípőcsontból. 4-5 cm^3 spongiosát használtunk fel esetenként. A kétoldali sinus-elevatiót *Tatum* klasszikus módszere szerint végeztük [24]. A kísérletes oldalon a Schneider-membrán megemelésével keletkezett üregbe 1,5-2 g β-tricalcium phosphate granulatumot helyeztünk, a szemcsék átmérője 500-1000 μm volt (Cerasorb, Curasan AG Kleinostheim Germany). Hasonló technikával 3-4 cm^3 autológ spongiosát ültettünk be a kontroll oldalon. Átlagosan 6 és ½ hónap múlva (szélsőértékek: 6,0–7,5) 68 csontcylindert nyertünk a regenerálódó hátsó maxillaris területről (2-t a kísérletes oldalról, 2-t a kontroll oldalról minden beteg esetében). Olyan trepánt használtunk, amelynek 2 mm volt a belső és 3 mm a külső átmérője. Ankylos implantátumokat (Degussa Friadent, Germany) helyeztünk be a kimetszett csontbiopsziák helyére.

Szövettani vizsgálatok. Szövettani és hisztomorfometriai vizsgálatokat végeztünk a csontmintákon dekalcinálás nélkül. A szövetmintákat 4%-os pufferelt formalinban 24 óráig rögzítettük, utána alaposan kiöblítettük folyóvízben. A dehidrálást felszálló alkoholsoron végeztük, utána methylmethacrylat gyantába ágyaztuk az anyagokat 4 °C-on. A szövettani metszeteket a csontminták hosszanti tengelyével párhuzamosan készítettük gyémántkéssel Jung-K Mikrotom segítségével. A metszetek átlagosan 5 μm vastagok voltak. A festést toluidin-kékkel, haematoxilin-eosinnal, valamint Goldner-féle trichróm technikával végeztük, fénymikroszkópos vizsgálatok céljára. A graftrészecskék gyakran kitörtek a metszetek készítése során, de jellegzetes alakjukat könnyen fel lehetett ismerni. Polarizált fényben a Cerasorb-graft-maradványok és az újdonképzett collagenrost-dús csonttrabekulák kettős törésük segítségével könnyen felismerhetők voltak.

Vizsgáltuk a szöveti reakciókat, a csontregenerációt a graftrészecskék körül, a csont/graft határzóna mikroszkópos képét, a graft bioresorpciót, valamint az új csont mennyiségét és minőségét. A mikrofotókat Olympus BH2 mikroszkópon készítettük Olympus DP50 digitális kamera csatlakoztatásával.

Hisztomorfometriai vizsgálatok. A szövettani metszeteken a méréseket számítógépes technikával végeztük, az alkalmazott operatív rendszer Windows XP Service Pack 1 volt (Microsoft Corporation). A képanalízist az AnalySIS® of Soft Imaging System (Münster, Germany) programmal végeztük. A mérendő területeket vizuális megítélés alapján választottuk ki. A színes képeket fekete-fehér skálára konvertáltuk, és kézileg finomított megvilágítási küszöböt alakítottunk ki, hogy meghatározzuk azokat a struktúrákat, amelyeket mérni akarunk. A kerület- és területméréseket automatikus pixel-számlálással végeztük el. A választott terület aritmetikus tágítását úgy hajtottuk végre, hogy a felszínt egyenletesen 1 pixellel növeltük. Kalibrálást követően a területet mm^2-ben, a kerületet mm-ben adtuk meg.

A hisztomorfometriai méréseket *Parfitt és mtsai* alapelvei alapján végeztük el [19]. Az újdonképzett csont denzitását a trabekuláris csont-térfogat (TBV) segítségével határoztuk meg, amely kifejezi a csont-trabekulák területének %-os arányát a teljes analizált területhez viszonyítva. A graftanyag %-os arányát szintén meghatároztuk. A csont gerendás szerkezetét a trabekuláris csontmintázat-faktorral (TBPf) határoztuk meg, amely kiváló

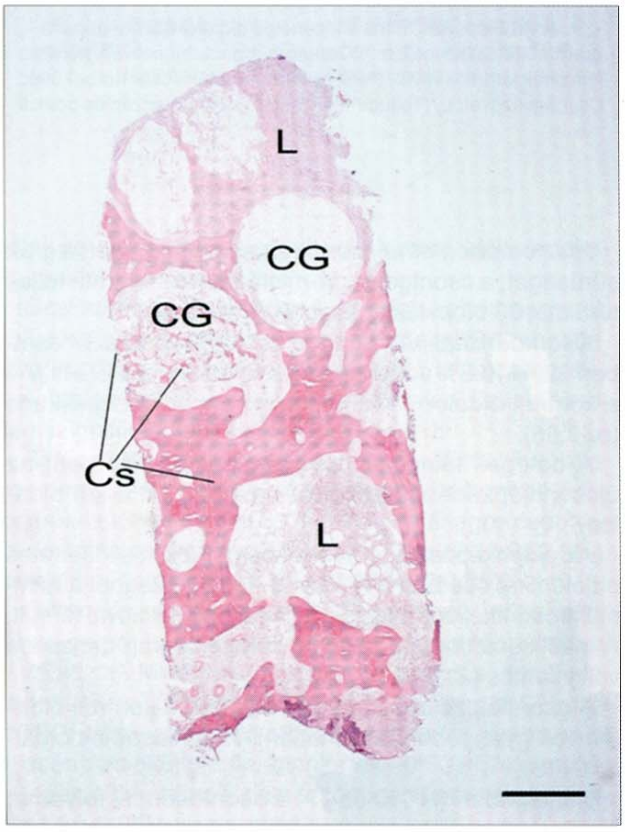
mutatója az újdonképzett csont mikrostruktúrájának [9]. A trabekuláris csont terület- és kerületmérését elvégeztük az aritmetikai dilatációt megelőzően és azt követően, hogy meghatározzuk a konvex és konkáv felszínek arányát a kétdimenziós metszetben. Minél magasabb a csont-trabekulák konnektivitása, annál alacsonyabb a TBPF érték.

Statisztikai elemzés. A mérési adatokból kiszámítottuk az átlagot és a standard deviációt. Az adatokat student t teszt segítségével elemeztük, és $p<0.05$ értéknél tekintettük szignifikánsnak.

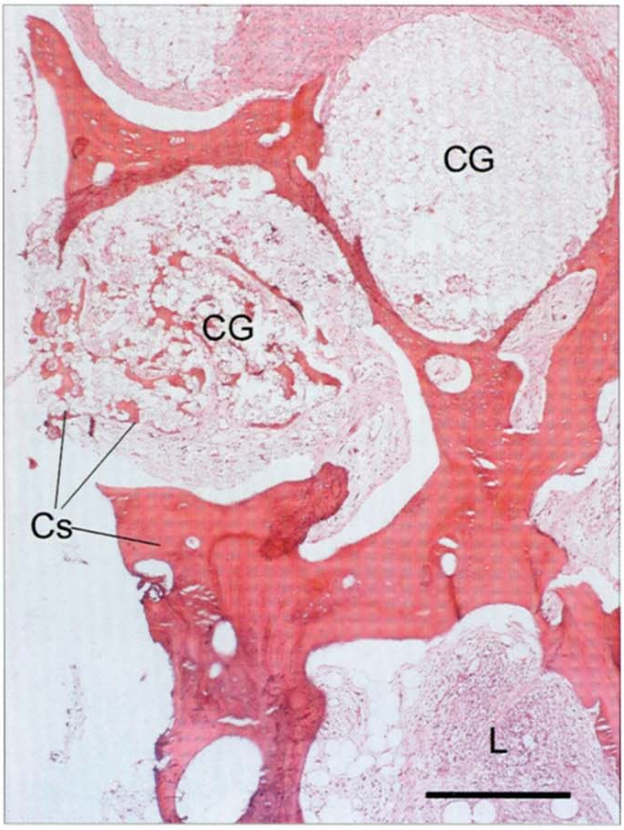
Eredmények

Klinikai megfigyelések

A sinus-aljzat emelése a gyógyulási periódus során csaknem valamennyi betegnél szövődménymentes volt. Kisebb orrvérzés fordult elő 2 betegnél. Gyulladásos szövődmény egyetlen betegnél sem jelentkezett. A donor terület postoperatív szövődményeket egy esetben sem mutatott. A radiológiailag mért csontmagasság átlagosan 15 mm volt a grafttal magasított sinus aljzatban (szélsőértékek: 12–16 mm) a kísérletes oldalon és 14,5 mm (szélsőértékek: 12–15 mm) a kontroll oldalon.



1. ábra. Biopsziás minta 6 hónappal a csontpótlást követően a kísérletes oldalról. Cerasorb granulum: CG, csont: Cs, lágyszövet: L. (HE festés; x4)



2. ábra. Biopsziás minta a kísérletes oldalról 6 hónappal a csontpótlást követően. A Cerasorb granulum (CG) pórusai elágazódó csonthálózattal vannak kitöltve. Lágyszövet: L. (HE festés; x20) Új csontképződés (Cs), osteoblast aktívitás (OB) machrophagok (M) citoplazmatikus graftmaradványokkal, és kapillarisok (C). (Toluidin-kék festés; x40)

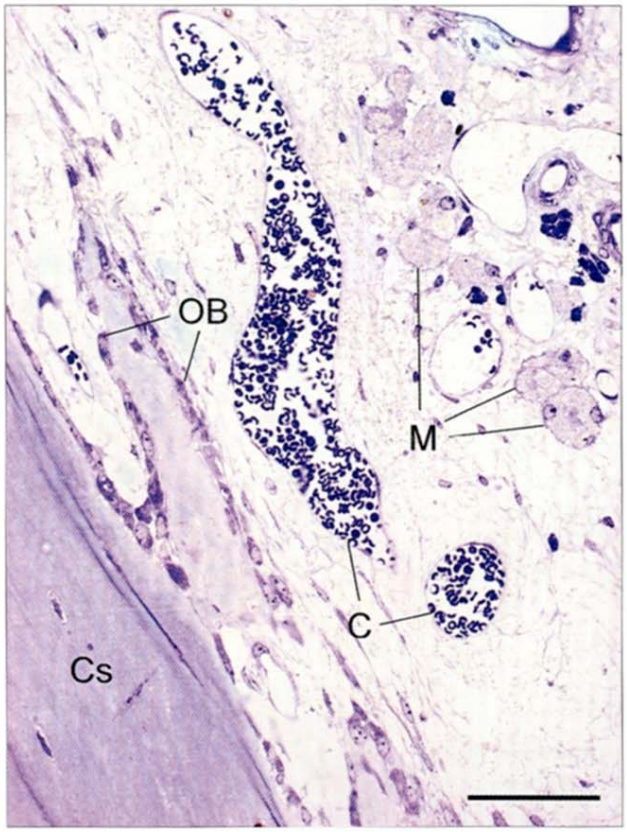
Szövettani vizsgálatok

Kísérletes oldal. A haematoxin-eosinnal festett metszetekben és a Goldner-féle trichróm módszerrel festett anyagokban számos graftrészecske kioldódott. Korábbi elhelyezkedésüket azonban könnyen fel lehetett ismerni jellegzetes kerek formájuk vagy kivájt konkáv felszínük alapján.

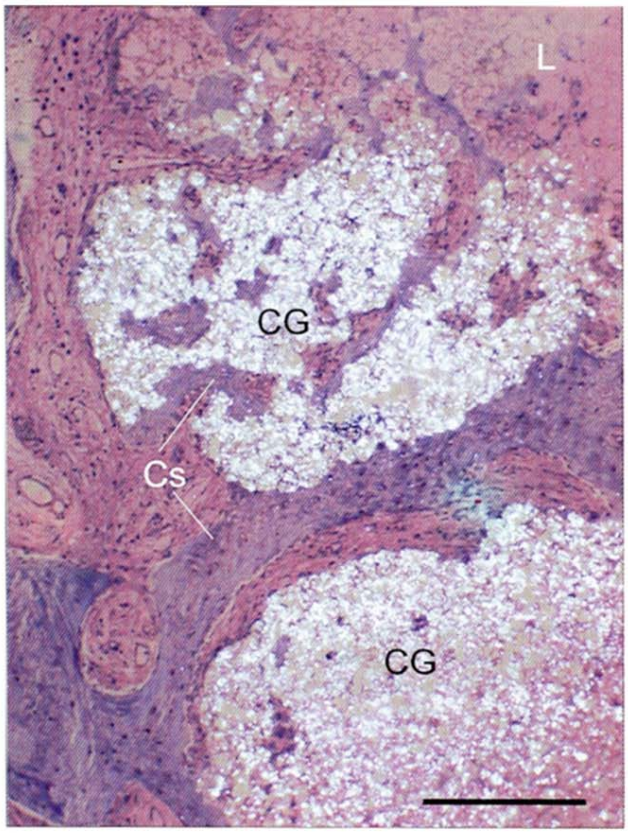
A részlegesen felszívódott graftrészecskék felszíne konkávvá vált az újdonképzett csont lerakódásától, ezenkívül egyes granulumokban osteoid, illetve fonatos csonthálózat alakult ki a pórusrendszerben.

Az újdonképzett csont inváziója a granulumokon belül nem társult osteoclast aktívitással. Előfordult azonban, hogy a környező makrofagokban kis achromatikus graftrészecskéket találtunk a cytoplasmában *(3. ábra).*

Hat hónap után az újdonképzett, elsősorban lemezes szerkezetű csont szoros kapcsolatot mutatott a graftrészecskékkel a csont/graft határzónában. A Cerasorb granulumokat a regeneráló csont beborította, és ahol ez a csontboríték teljesen elfedte a granulum felszínét, teljesen eltűnt az osteoclast aktivitás. A graftmaradvá-



3. ábra. Biopsziás minta a kísérletes oldalról 7 hónappal a graftbeültetés után.



4. ábra. Biopsziás minta a kísérletes oldalról 6,5 hónappal a graftbeültetést követően. A Cerasorb granulumon kivájt, homorú felszínek láthatók (nyíl). Peri- és intragraniuláris csonttrabekulák: Cs. Lágyszövet: L. (Toliudin-kék festés; x20) Polarizációs optikai felvétel

nyok achromatikus kettős törést mutattak poláros fényben *(4. ábra)*.

Egy esetben fordult elő, hogy a körülírt területen elmaradt a csontképzés intenzív gyulladásos reakció következtében.

Kontroll oldal. A szivacsos csontgraft jelentős felszívódáson ment keresztül 6 hónap után *(5. ábra)*. A graftszigetek zárványként fordultak elő az újdonképzett, dominálóan érett lamelláris csontszövetben *(6. ábra)*. Ezek a graftmaradványok homogén, sejtmentes anyagnak bizonyultak, melyek festődése hasonló volt az élő csontéhoz. Több területen folyamatos átmenetet találtunk a szivacsos csontgraft és az újdonképzett csont határzónájában. Egyes csontgerendák aktív remodelling jelenségét mutatták, felszínükön gömbölyű osteoblast láncokkal, és a túlsó oldalon resorpciós lacunák látszottak többmagvú, osteoclast típusú óriássejtekkel. Poláros fényben az újdonképzett csont élénkvörös kettős törést mutatott gazdag kollagénrost-tartalma miatt *(7. ábra)*. Néhány mintában szerényebb csontképzés látszott, elsősorban fibrosus csontvelő képződött, és diffúz, vékony újdonképzett csontgerenda-hálózatot lehetett megfigyelni.

Hisztomorfometria. Mértük a csont sűrűségét, a graft sűrűségét, a csontgerenda-mintázatot és a minta teljes területét 68 biopsiás anyagban *(I. táblázat)*.

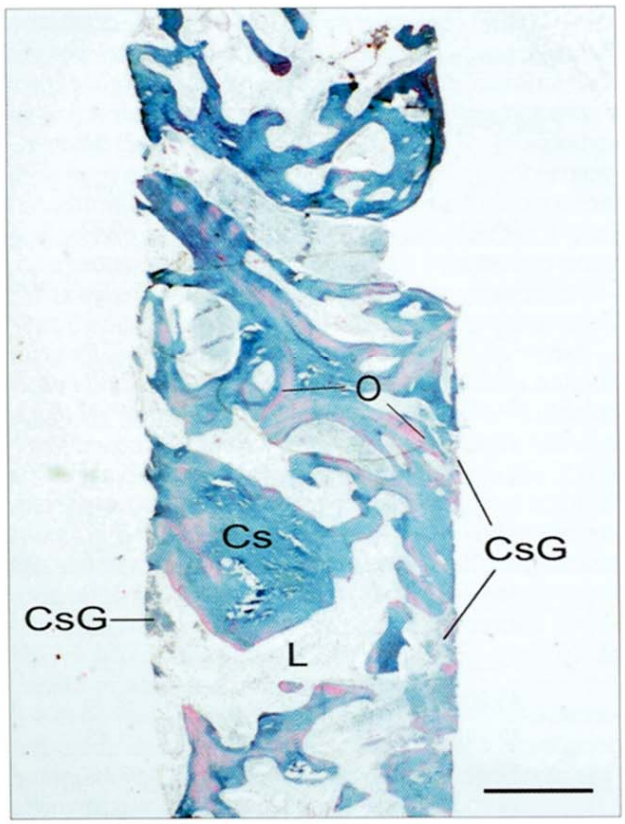
Csontdenzitás. Az átlagos csontdenzitás a 17 esetben 32,4±10,8% volt a kísérletes oldalon és 34,7±11,9% a kontroll oldalon. A különbség nem volt szignifikáns ($p>0,05$).

A betegek túlnyomó többségénél (14 esetben) az újcsontképződés intenzítása hasonló volt a két oldalon.

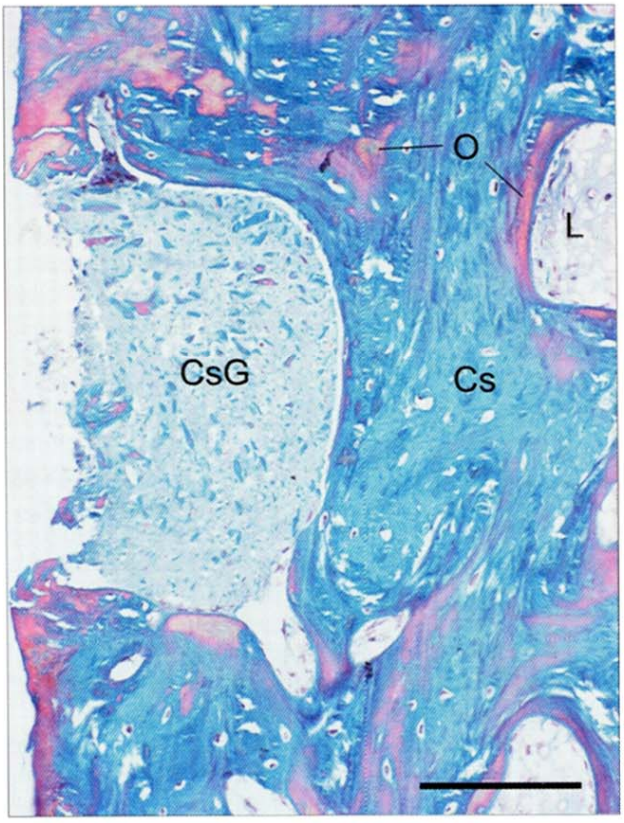
Az újdonképzett csont kevésbé denz volt a kísérletes oldalon két esetben (№ 4 és 9). Egyik betegnél a minimális csontképzés lokális gyulladásos reakcióval társult. A másik esetben a beültetett Cerasorb-graft denzitása volt jelentős (21,2%).

A csontképző aktivitás egy esetben a kontroll oldalon volt gyengébb, összehasonlítva a kísérletes oldallal (№ 8).

Két esetben (№ 10 és 17) a csontosodási folyamat hasonlóan gyengének bizonyult mindkét oldalon, az újdonképzett csont denzitása 19,2% és 18,5% volt a kísérletes oldalon, és 18,4% valamint 17,6% a kontroll oldalon. Ezen két esetben az újdonképzett csonttrabe-



5. ábra. Biopsziás minta a kontroll oldalról 6 hónappal a graftbeültetés után. Csont: Cs, oszteoid: O, csontgraft: CsG, lágyrész: L. (Goldner trikróm festés; x4)



6. ábra. Biopsziás minta a kontroll oldalról hat hónappal a graftbeültetést követően. Csont (Cs) és oszteoid (O) képződés a felszívódó csontgraft (CsG) körül. Lágyrész; L. (Goldner tichromfestés; x20)

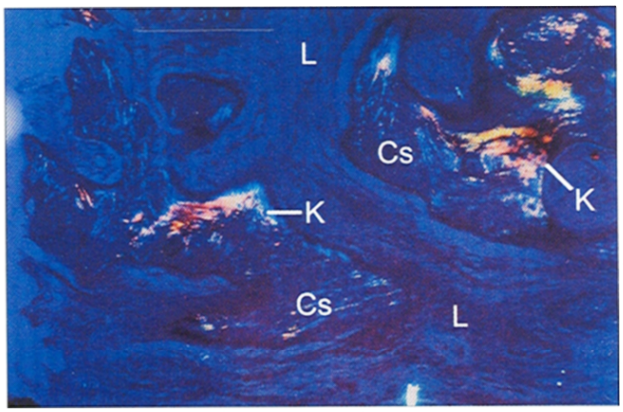
kulák egyenletesen vékonyak voltak, nem találtunk fokális, gyulladásos reakciót.

Graftsűrűség. A graftdenzitás kifejezetten nagyobb volt a kísérletes oldalon, a kontroll oldallal összehasonlítva. Az átlagos graftdenzitás 13,1±4,5% volt a kísérletes oldalon és 8,2±1,7% a kontroll oldalon. Ez a különbség szignifikánsnak bizonyult ($p<0,001$).

Kötőszövet- és csontvelő-denzitás. A kötőszövet átlagos denzitása hasonló volt a kísérletes és kontroll oldalon (49,4±16% valamint 50,4±19%). Az átlagos csontvelő-denzitás nagyobb volt a kontroll oldalon (6,8±2,9%), mint a kísérletes oldalon (5,1±2,2%) ($p>0,05$).

Gerendás csontmintázat (TBPf). Ezek az értékek általában inverz kapcsolatot mutattak a csontdenzitással *(I. táblázat)*. Minél nagyobb a csontdenzitás, annál alacsonyabb a TBPf érték. Tizenegy esetben a 17 közül a TBPf érték a kontroll oldalon volt alacsonyabb, mint a kísérletes oldalon. Az átlag érték $-0,53\pm1,74$ mm^{-1} és $-0,11\pm1,43$ mm^{-1} volt, de a különbség nem volt szignifikáns ($p>0,05$).

A biopsziás minták területe és hosszúsága. Az átlagos terület a két oldalról származó biopsziás mintákra vonatkozóan hasonló volt: 8,85±1,7 mm^2 a kísérletes oldalon és 9,12±2,28 mm^2 a kontroll oldalon. A biopsziás minták átlagos hosszúsága 7,83±1,9 mm volt a kísérletes oldalon és 8,08±2,2 mm a kontroll oldalon.



7. ábra. Újdonképzett csont (Cs) a kontroll oldalról. Kettős törés a kollagéndús területeken (K). (Goldner trikróm festés; x10). Polarizációs optikai felvétel

I. táblázat

Mérési eredmények a kísérletes és a kontroll oldalon*

	Kísérletes oldal			Kontroll oldal		
	Csontdenzitás (%)	Graftdenzitás (%)	TBPf (1/mm)	Csontdenzitás (%)	Graftdenzitás (%)	TBPf (1/mm)
	41,80	13,10	-0,16	51,50	7,60	-0,12
	32,60	11,30	1,56	36,30	8,90	-0,88
	24,80	17,50	0,31	27,60	6,70	-1,01
	16,70	21,20	0,22	33,60	10,50	-1,87
	23,80	10,40	0,55	24,25	8,60	1,22
	36,50	8,70	-0,11	34,60	11,30	-0,57
	29,90	11,00	-0,17	31,40	7,90	-0,29
	27,60	9,80	-1,86	16,40	6,80	0,94
	25,00	19,40	-1,96	44,20	7,80	-1,86
	19,20	12,20	1,63	18,40	10,60	2,14
	47,20	10,80	0,27	50,80	5,40	-3,23
	50,70	8,10	-2,19	46,60	6,90	-2,08
	40,40	10,30	-1,25	42,80	7,10	-1,99
	26,70	12,20	1,85	24,50	8,70	1,11
	45,70	11,80	-1,86	49,20	5,80	-2,25
	43,40	11,20	-1,03	40,20	8,10	-1,43
	18,50	23,50	2,41	17,60	9,90	3,13
Átlag:	32,38	13,09	-0,11	34,70	8,15	-0,53
SD:	10,85	4,49	1,43	11,86	1,69	1,74
	Csontdenzitás	Graftdenzitás	TBPf			
p:	0,10693659	0,00012541	0,1156			

*Az adatok két biopsiás mintán párhuzamosan mért értékek átlagai

Megbeszélés

A regenerálódó szivacsos csont mennyisége és minősége igen nagy jelentőségű a műgyökér implantátumok beültetése során. A csontgraft beültetés általánosan elterjedt módszer, ezáltal növeljük a műgyökér implantátumok csontos támasztékát [5, 7, 15]. A graftanyag beépülése és az implantátum integrálódása komplex gyógyulási folyamat, és eredményeként direkt kapcsolatnak kell létrejönni a beültetett implantátum és a grafttal serkentett újdonképzett csont között. A sinus maxillaris aljzata ideális a különböző csontpótló anyagok beültetésére, mivel nagyon komoly regenerációs képessége van.

Teljesen fogatlan betegeknél és súlyos lokális atrophia esetében a kétlépcsős technika alkalmazható [15]. Az első lépcső a graft beültetése a sinus aljzatába. Ennek integrálódása hosszabb időt igényel, melynek során a graft ereződik, és végbemegy a csontos regeneráció. A második lépcső az implantátum behelyezése, amely hónapok múlva történik. Az implantátum egy azonnali gyógyulási folyamatot indukál a grafttal stimulált csontban, amely hasonló ahhoz a folyamathoz, amely a természetes csontban is végbemegy. Ugyanakkor ezen technika során lehetővé válik a kiemelt csontbiopsziák szövettani vizsgálata, amely segítséget nyújt abban, hogy értékeljük a csontpótló anyag integrálódását és felszívódását.

A kétoldali sinusaljzat-emelés ugyanabban a betegben, azonos körülmények között, kitűnő módszer arra, hogy összehasonlítsuk különböző csontpótló anyagok csontregenerációs hatását [7, 18, 23]. Mivel az autológ csontot tekintjük arany standardnak, ezen graft beültetését érdemes alkalmazni kontroll módszerként [4, 12, 25].

Klinikai és radiológiai értékelést egyaránt alkalmazhatunk a csontregeneráció eredményeinek felmérésére. A szivacsos csontképződés mennyiségileg és minőségileg csak szövettani és hisztomorfometriai módszerekkel értékelhető pontosan.

A szövettani és hisztomorfometriai vizsgálat a 17 esetből származó 68 csontbiopszián azt bizonyította, hogy a csontregeneráció mértéke csaknem azonos volt mindkét oldalon. A csontdenzitás a grafttal vastagított sinus aljzatban hasonló volt, függetlenül attól, hogy autológ csontot vagy Cerasorb-szemcséket ültettünk be.

Az új csont képződése elsősorban a Cerasorb-granulumok felszínére és a pórusrendszerre koncentrálódott

a kísérletes oldalon. A szemcse belsejébe terjedő csontos növedék arra utal, hogy a csontpótló anyag folyamatos biodegradáció jeleit mutatja. *Zerbo és mtsai* szövettanilag aktív osteoclastos resorptió jeleit figyelték meg a Cerasorb-granulumokon [8]. Ebből arra következtethetünk, hogy az aktív remodelling tekinthető a graftrészecskék eltüneséhez vezető útnak. Más mechanizmusok, elsősorban fizikai oldódás is szerepet játszhat a graft resorptióban. Szövettani vizsgálataink nem támasztották alá az osteoclastos graftresorptiót, bár apró cytoplasmaticus Cerasorb-részecskéket találtunk a mononukleáris makrofágokban. Ez aktív celluláris eliminációra utal.

A jelen vizsgálatok során 3 esetben tapasztaltunk egyoldali renyhe csontregenerációt. A kontroll oldalon 1 esetben, a kísérletes oldalon 2 esetben. Egy esetben a kísérletes oldalon lokális gyulladásos reakció alakult ki, a másik esetben a szövetminta graftszemcsékkel volt telezsúfolva, ami magyarázhatja a csökkent mértékű csontképzést. A kontroll oldalon nem volt ésszerű magyarázat arra, hogy miért volt renyhébb mértékű a csontgyógyulás. Ezek az esetek alátámasztják, hogy lokális tényezők, pl. a kapilláris rendszer elégtelensége az atrophiás csontban jelentős szerepet játszhat [20].

Két esetben mindkét oldalon csekély mértékű csontregenerációt tapasztaltunk. Az egyik eset a legidősebb betegünk volt, a másik esetben postmenopausalis aszszonyról volt szó.

Statisztikailag értékelhető morfometriás eredmények nem találhatók az irodalomban a Cerasorbbal történő sinusaljzat emelésre vonatkozólag. Bioaktív üveget kevertek autológ csontrészecskékkel, és összehasonlították az egyedül alkalmazott csontrészecskék hatásával bilaterális sinusaljzat-emelés során [23]. Hat hónappal később a trabekuláris csontdenzitás jóval magasabb értéket mutatott, mint jelen vizsgálataink során, a kísérletes és a kontroll oldalon egyaránt (44% és 38%).

A maxillaris sinusaljazat-emelést Bio-Oss xenograft és autológ csont keverékével ugyancsak vizsgálták [26, 27]. A hisztomorfometriás adatok kifejezetten alacsonyabb csontdenzitást (18,9%) és magasabb graftdenzitást (29%) mutattak, összehasonlítva saját adatainkkal. Megjegyezzük azonban, hogy a csontgraft komplex denzitása közel volt az 50%-hoz, amely a mi eseteinkben is hasonlóan alakult. A Bio-Oss és a vénás vér kombinációját alkalmazták humán sinus elevációs esetekben, melynek során szerényebb eredmények születtek [26, 27]. Az újdonképzett csont átlagos denzitása csak 14,7% volt, míg a xenogén graftmaradvány aránya magas maradt 29,7%.

Az újdonképzett csont mennyiségi vizsgálata mellett nagyon fontos tényező a csont mikrostruktúrája is. A TBPf-mérés a grafttal stimulált csontmintákban speciális problémákat vet fel attól függően, hogy milyen természetű a graftanyag. Porózus, alloplasztikus anyagok esetén a granulum pórusrendszerében kialakuló csontos hálózat számos vékony, elágazódó szabad végződést tartalmaz, és ez negatívan befolyásolja a trabekuláris konnektivitás méréseket. Jelen vizsgálatunk során a kedvezőbb trabekuláris konnektivitás alacsonyabb TBPf-értékeket eredményezett a kontroll oldalon. Ez azonban nem mutatott szignifikáns különbséget a kísérletes oldalhoz viszonyítva.

A graft lebomlása szintén nagyon fontos tényező a csontpótlás során [22, 28]. Eseteinkben a graft felszívódását új csont képződése követte mindkét oldalon. Megállapítottuk, hogy hat hónap múlva a graft denzitása szignifikánsan magasabb értéket mutatott a kísérletes oldalon, a kontroll oldalhoz viszonyítva. További kérdésként merült fel, hogy a graftzárványok fennmaradása az újdonképzett csontban mennyire befolyásolja a stabilitást. Jelen vizsgálataink arra utaltak, hogy nem volt szignifikáns különbség a két oldalon. Ez arra utal, hogy az alloplasztikus szemcsék elsősorban a csontvelő területének rovására perzisztáltak.

A biopsziás minták mérete szintén befolyásolhatja a csontpótló anyagok hatásának kvantitatív értékelését, mivel a graftbeültetés után a minták szerkezete nem homogén. Minél nagyobb csontterületet tudunk mérni, annál reprezentatívabb a mérési eredmény. Vizsgálataink során a csontbiopsziák mérete mindkét oldalon hasonlóan alakult, nem különböztek szignifikánsan.

A klinikai gyakorlatban hosszú távú vizsgálatok szükségesek annak megítéléséhez, hogy a fogpótlással megterhelt műgyökerek mennyire stabilizálódtak a grafttal kezelt régióban. A szövettani és hisztomorfometriai eredmények jelentős információt nyújtanak arról, hogy milyen teherbíró kapacitása lesz az implantátum csontos ágyának, és felderítheti azokat a gyenge pontokat, amelyek gondosabb kezelést igényelnek.

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Dr. Suba Zs, Dr. Takács D, Dr. Matusovicz D, Dr. Fazekas A, Dr. Szabó Gy, Dr. Barabás J:

Quantitave and qualitative comparison of the maxillary bone regeneration after β-tricalcium phosphate and autogenons bone implantation

Graft insertion can effectively enhance the regeneration of debilitated bone. The effects of an alloplastic bone-replacing material, β-tricalcium phosphate (Cerasorb), and of autogenous bone graft were compared.
In 17 edentulous patients, the maxillary sinus floor was extremely atrophied to such an extent that implant placement was impossible. The Schneiderian membrane was surgically elevated bilaterally by insertion of Cerasorb (experimental side) and autogenous bone graft (control side). After surgery, the recovery was followed clinically and radiologically. After 6 months, 68 bone cylinders were excised from the grafted areas and implants were inserted into their places. The bone samples were embedded into resin, and the osteointegration of the grafts was studied histologically. Trabecular bone volume (TBV) and trabecular bone pattern factor (TBPf) were quantified by histomorphometry.
Cerasorb proved to be an effective bone-replacing material with osteoconductivity; it was capable of gradual disintegration, thereby providing space for the regenerating bone. The new bone density was not significantly different on the experimental and control sides (32.4±10.9% and 34.7±11.9%, respectively). However, the graft biodegradation was significantly slower on the experimental side than on the control side. The TBPf value was lower on the control side than on the experimental side (−0.53±1.7 mm^{-1} and −0.11±1.4 mm^{-1}, respectively); but this difference was not significant.
Six months after insertion of the grafts, the bone of the augmented sinus floor was strong and suitable for anchorage of dental implants, irrespective of whether autogenous bone or Cerasorb particles had been applied.

Key words: sinus grafting, bone regeneration, histology, histomorphometry, β-tricalcium phosphate

III.

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A PILOT STUDY OF CERASORB AND BIO-OSS ENHANCED NEW BONE FORMATION IN ANIMAL MODEL

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The aim of this pilot investigation was to develop a new animal model for studying the effects on osteogenesis of agents used in the guided bone regeneration technique. As test material, a mixture of two osseoconductive materials with different physico-chemical characteristics was used. One component of the mixture was Bio-Oss, a bovine hydroxyapatite; the other was Cerasorb, a synthetic tricalcium phosphate. The mixture consisited of 50 volume percent of Bio-Oss and 50 volume percent of Cerasorb. In *in vivo* pilot experiment, bone wounds were prepared in the proximal third of both femurs of rabbits. A Cerasorb+Bio-Oss mixture was inserted on the test side and the same amount of sterile buffered physiological solution on the control side. After healing for 4 weeks, the bone segments were embedded and cut without decalcification, using the Exact cutting and grinding system. The density of the newly-formed bone was evaluated histomorphometrically. On the Cerasorb+Bio-Oss test side the bone density was almost 1.5 times higher than that on the control side. These results demonstrated that the applied animal model is appropriate for investigation of the effects on osteogenesis of biocompatible graft materials such as Bio-Oss and Cerasorb.

Keywords: Bio-Oss – Cerasorb – osteogenesis – rabbit model – histomorphometry

INTRODUCTION

Guided bone regeneration has become a routinely applied method in dental implantology. Most of the dentoalveolar regenerative techniques require osseoconductive material in order to establish new bone formation in the necessary anatomical form.

Bio-Oss is a safe, effective xenograft: a deproteinized, sterilized bovine bone with 75–80% porosity. It is reported to be highly osteoconductive and biocompatible

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[3, 5]. It is known that Bio-Oss serves as a scaffold in guided bone regeneration, but, due to its poor resorbability it may exert a negative influence on the structure of the newly-formed bone. It has been found clinically that its resorption is very slow, requiring many years [13].

The chemical characteristics of Cerasorb, another widely used osteoconductive material (pure beta-tricalcium phosphate), allow it to resorb completely and quite rapidly during new bone formation. This may result in too early resorption in some cases, not fulfilling the clinical requirements, the space-maintaining function [1, 15].

The main aim of the present study was to establish a gold standard for the artificial bone growth-accelerating effect of a Cerasorb and Bio-Oss mixture on osteogenesis in order to determine and utilize the most advantageous characteristics of these bone substitutes. Bio-Oss serves as a scaffold, but its resorbability is poor, while Cerasorb is a good bone-developing material, but resorbs too early, not providing a scaffold for the new bone bridges [12, 13].

Bone-substitute materials such as Cerasorb allow targeted bone regeneration as they facilitate construction of a base on which implants can be positioned and further stabilized [12]. Full resorption over a defined period of time, with simultaneous transformation into autologous bone, is of particular significance in this respect. Because of their rounded surface and chemical composition, the Cerasorb particles are remarkably bioinert and is therefore particularly suitable for innovative procedures [11, 15].

The present pilot study reports on the potential of a mixture of Cerasorb and Bio-Oss in implantation technology. A further goal was to develop an *in vivo* animal model suitable for the investigation and comparison of the effects of different materials. Both Cerasorb and Bio-Oss are currently in clinical use, but as far as we are aware their effects in a mixture have never been investigated.

MATERIAL AND METHODS

The applied Bio-Oss had a granule size of 1–2 mm (Geistlich Pharma AG, Switzerland); the Cerasorb was Cerasorb M (1–2 mm) produced by Curasan (Kleinostheim, Germany).

Animals

The pilot study involved adult New Zealand White rabbits. The animal management and the surgical and routine procedures followed "The Guiding Principles for the Care and Use of Animals" approved by the Animal Investigation Review Board of the University of Szeged, in accordance with the principles of the Helsinki Declaration.

Surgical procedures

Narcosis was induced with a cocktail of 5.0 mg/body mass (bm) kg (0.25 ml/bmkg) xylazine (Xylazine 2% inj.), 40.0 mg/bmkg (0.4 ml/bmkg) ketamine (Ketavet inj.) and 0.8 mg/bmkg (0.08 ml/bmkg) acepromazine (Vetranquil inj.) intramuscularly. The rabbits received Ringer lactate infusion therapy at a rate of 0.3 ml/min during narcosis via, a cannula inserted into an ear vein.

After disinfection, isolation and skin incision, the fascia lata was prepared. The femurs were visualized by folding the *m. tensor fasciae latae* and the *m. abductor cruris cranialis*. The site of the bone wound was marked by a round burr in the proximal third of the femur, approximately 2 cm distally from the *trochanter major* of the femur. The diameter of the monocortical bone wound was 3.3 mm. To establish a bone wound of the same size, implantation drills with irrigation were used (pilot-, pre- and form drills, CAMLOG Biotechnologies AG, Germany). In the test (right) femur a 1 : 1 mixture of the bone graft materials Cerasorb and Bio-Oss was inserted. This combination mixed with rabbit blood, was applied to fill the 3.3-mm-diameter monocortical bone wound. On the control side (left femur), sterile buffered physiological salt solution was injected. The bone wound was covered by Surgical (Johnson & Johnson), which was fixed around the hole with Histoacryl (Braun). Suturing was performed with absorbable Vicril 5.0, in three layers (fascia lata, subcutaneous layers and skin).

During the postoperative care, the rabbits received an analgetic [4.0 mg/bmkg (0.8 ml/bmkg) carprofen (Rimadyl) inj. sc.] and antibiotic support [15 mg/bmkg (0.15 ml/bmkg) enrofloxacin (Enroxil) inj. sc.] for 5 days following the operation. All the rabbits remained healthy and the postoperative period was uneventful.

Sample harvesting

After 4 weeks of osteogenesis, the rabbits were sacrificed under general anaesthesia induced by an overdose of an intravenous injection of ketamine.

Specimen preparation

For histological evaluation, perfusional tissue fixation was carried out with 4% neutral buffered formalin solution, after which the cut specimens were subjected to immersional fixation in 4% of neutral buffered formalin solution. X-rays were taken to identify the exact locations of the bone wounds. The specimens were dehydrated and embedded in Technovit 7200VLC resin (Heraeus Kulzer, Germany). Cutting was performed with the Exact cutting and grinding system without decalcification [2]. The thickness of the sections was 20 μm. The slides were stained with toluidine blue.

Histology and histomorphometric analysis

Optical microscopic images (Nikon Eclipse 80i, Japan) were recorded on an Evolution MP 5.1 Mega-pixel FireWire Digital CCD Color Camera Kit (Media Cybernetics, Inc., USA). Measurements were performed with Image-Pro Plus 5.1.1 image-analysing software (Media Cybernetics, Inc., USA).

The histomorphometrical evaluation involved use of the areal bone density, i.e. the ratio of the area of newly-formed bone to the total area of the image [8, 9]. This permitted a quantitative comparison of the new bone formation in the control and the test bone wounds.

RESULTS

Histological observations

In the control samples (Fig. 1a), the reconstruction of the surgically prepared bone wound was not complete. The bone at the border of the wound exhibited signs of resorption and new bone formation. This was young immature lamellar bone with centrifugal orientation of the newly-formed bone in the wound. The outer layer of newly-formed bone was a younger woven bone with cross-oriented collagen fibres.

The closure of the monocortical bone wound bone formation induced by the Cerasorb + Bio-Oss mixture was not complete (Fig. 1b). Around the bone substitutes, new bone formation had started, containing bone bridges between and around the granules. Mostly young immature woven bone was situated around the granules with osteoblastic activity.

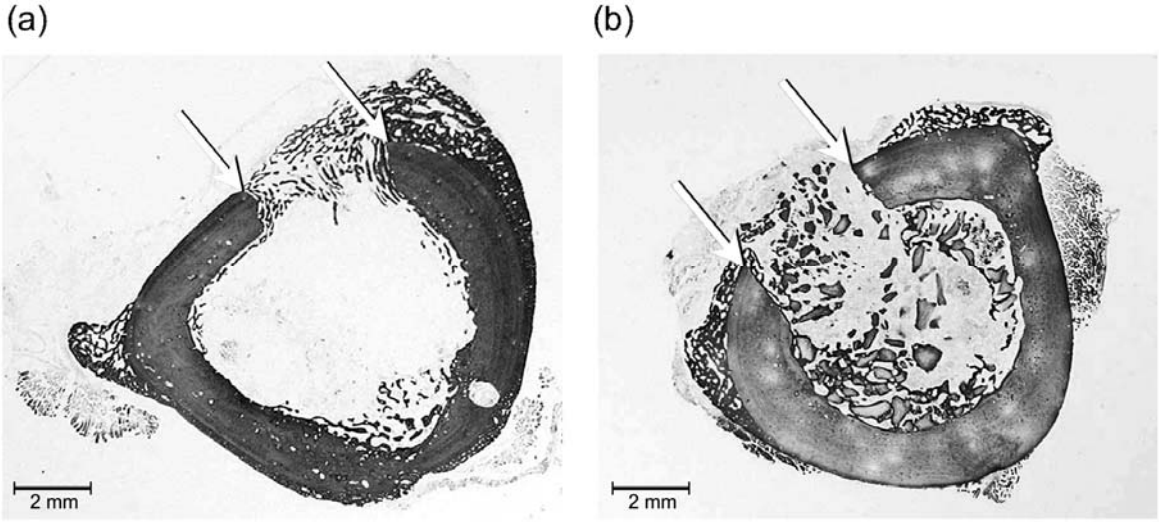

Fig. 1. Macroscopic slides of histological control (a) illustrates the bone formation induced by the Cerasorb + Bio-Oss mixture (b) stained with toluidine blue. Arrows indicate the site of the prepared monocortical bone wound

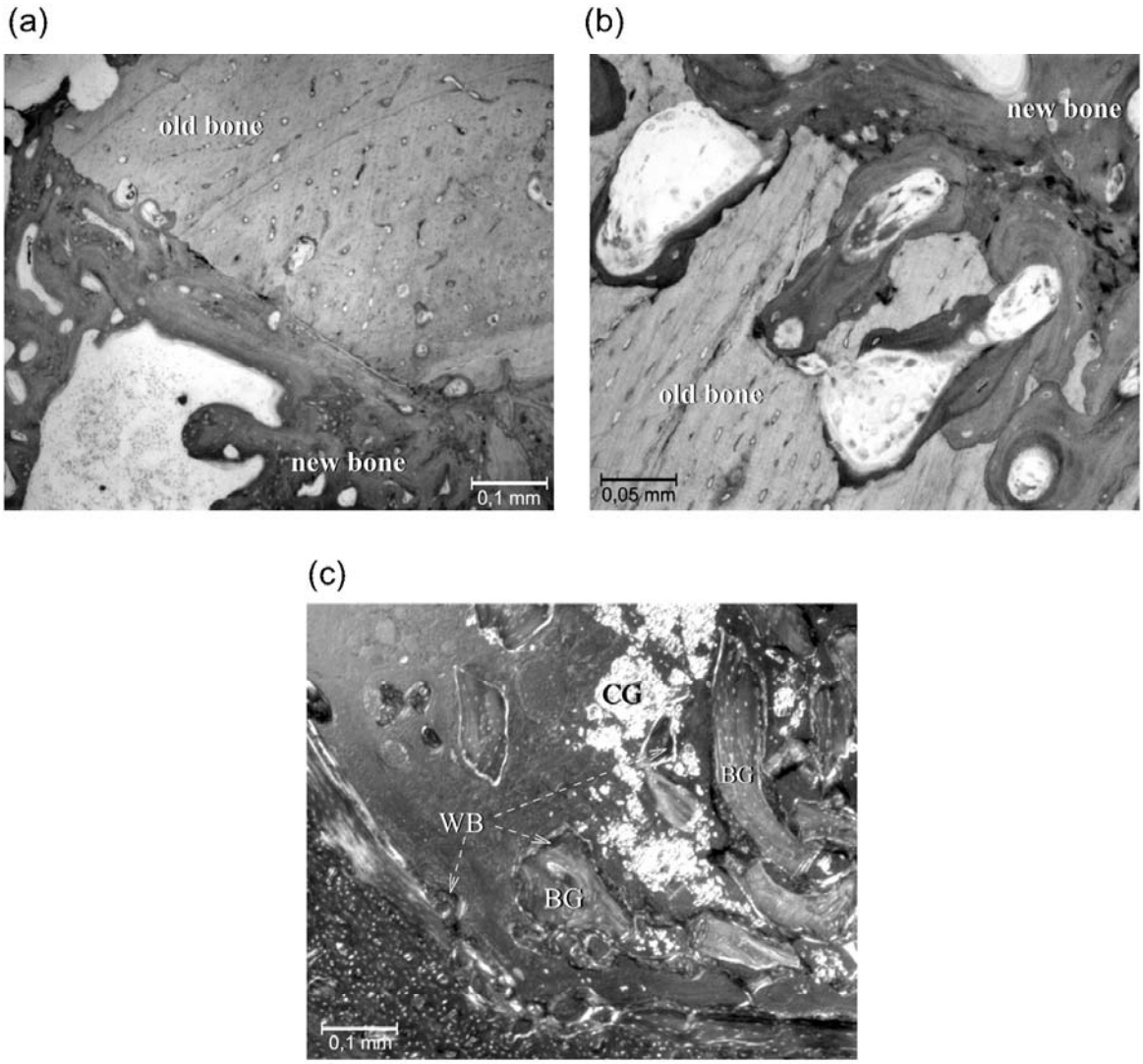

Fig. 2. Picture of new bone formation in the control sample. (a) Bright field (×10 magnification); Picture of the bone formation induced by the Cerasorb+Bio-Oss mixture. (b) Bright field (×40 magnification); (c) polarized light microscopic image (×20 magnification); CG – Cerasorb granules, BG – Bio-Oss particles, WB – woven bone

In Figure 2a, the border of the old and new bone can be clearly differentiated.

In the woven bone, an osteoblast line indicated the formation of bone and primary osteons in the lamellar bone (Fig. 2a, b). Both Bio-Oss (BG) and Cerasorb (CG) granules were visible (Fig. 2c), as a sign that the resorption of the material had not yet finished. At the same time, an osteoid bone (woven bone – WB) network could be discerned around the granules (Fig. 2c).

Histomorphometric measurements

In the Cerasorb + Bio-Oss mixture, the extent of induced osteogenesis was increased almost 1.5-fold, as compared with the control side, where the areal density of the newly-formed bone was 33.9%. For the Cerasorb + Bio-Oss mixture the areal density of the newly-formed bone was 48.7%.

DISCUSSION

In modern dental implantology, the need for bone augmentation techniques demands adequate osteoinductive and osteoconductive effects from the bone substitutes. It is very important that the original anatomical form of the given bone (e.g. the alveolar process as dental implantology concerns) should be reconstructed and also that an appropriate bone structure should be achieved as soon as possible [6].

A variety of biological and synthetic materials are available for reconstruction with augmentative surgical methods of alveolar bone defects. The available bone substitutes exhibit different abilities in guided bone regeneration as regards their biological function in the new bone formation.

Bio-Oss is a highly osteoconductive xenograft material certified for the regeneration of bone defects. It displays very low resorbability and acts as an inert scaffold onto which bone-forming cells and blood vessels creep, forming the new bone [7, 10].

Cerasorb has good bioresorptive properties and as a bone substitute it maintains biological support during healing and is gradually replaced by the newly-formed bone [12]. Its clinical indication differs from that of Bio-Oss due to its excellent biodegradation capacity [5]. For instance, Cerasorb is advantageous for sinus elevation procedures as the floor of the maxillary sinus undergoes rapid regeneration by virtue of its multiple blood vessel supply [12]. Its application has the clinical advantage that it resorbs quite quickly (in some weeks) and totally. However, vertical augmentation of the atrophied alveolar crest, or the regeneration of other load-bearing bone sites, requires a bone substitute with lower resorbability and high stability, such as Bio-Oss.

The goal of this pilot investigation was to study the artificial bone growth-supporting effect of a Cerasorb and Bio-Oss mixture on osteogenesis in order to determine the most advantageous characteristics of these bone substitutes. The two materials were mixed on the supposition that their mixture would provide a qualitatively new effect, i.e. a long-term form-maintaining function with relatively small foreign material imbibition in the bone. In spite of its poor resorbability, Bio-Oss provides an appropriate scaffold. Cerasorb is a good bone-developing material, but in some cases it does not serve long enough as a scaffold for the new bone bridges, as it resorbs too early. With the combination of Bio-Oss and Cerasorb, the most advantageous properties of the bone substitutes could be utilized.

Furthermore, the combination of such osteoconductive bone substitutes (in this case the mixture of Cerasorb and Bio-Oss) with an osteoinductive agent offers promising perspectives for reconstructive bone surgery [14].

ACKNOWLEDGEMENTS

We are grateful to the Department of Experimental Surgery (University of Szeged) for their help in the animal experiments and the excellent handling of the animals; to Balázs Gaál (veterinarian at the University of Szeged), Beáta Péva (Department of Anatomy, Faculty of Medicine) and László Bene (Department of Mechanical and Process Engineering, Faculty of Engineering) for valuable help and discussions. This work was supported by the GVOP-3.2.1.-2004-04-0408/3.0 Project funded by the EC and the Hungarian Ministry of Economy and the ETT project (434/2006) supported by the Hungarian Ministry of Health.

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IV.

Dr. Matusovits Danica, dr. Suba Zsuzsa, dr. Takács Dániel, dr. Donath Karl, dr. Turzó Kinga és prof. dr. Fazekas András

Bio-Oss és Cerasorb keverék csontképzést serkentő hatásának vizsgálata állatkísérletben

Bevezetés

Közismert, hogy a Bio-Oss csontsebbe helyezve kiváló vázat képez a gyógyulás során. Stabil kristályos szerkezete miatt in vivo körülmények között a felszívódása nagyon lassú. Ez előnyös a csont regenerációjában a formamegtartást tekintve, hátrányt jelenthet azonban a későbbi műtéti beavatkozások – például implantáció – alkalmával, az új csont ugyanis inhomogén szerkezetű lesz. Egy másik széles körben alkalmazott osszeokonduktív anyag a Cerasorb néven ismert tiszta béta-trikalcium-foszfát. A csontújraképződéskor ez az anyag – lévén prekurzor a remineralizáció biokémiai folyamatában – gyorsan felszívódik. Ez előnyös abból a szempontból, hogy a gyógyulás befejeződése után a csont biológiailag a keményszöveti struktúrának megfelelő homogén szerkezetűvé válik. Az azonban, hogy felszívódása megelőzheti a teljes gyógyulást, a csont formai regenerációja szempontjából hátrányt jelenthet.

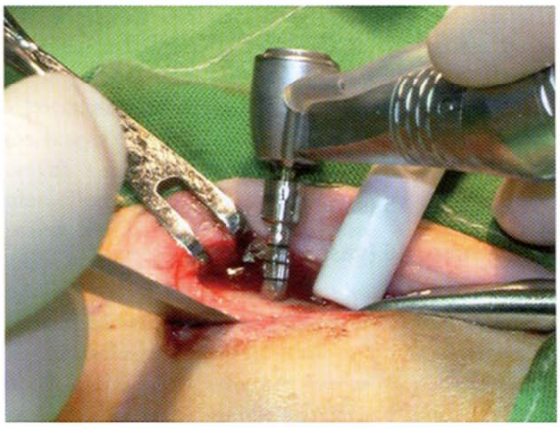
Célkitűzés

Vizsgálatainknak célja az volt, hogy kiderítsük, a két csontpótló anyag együttes alkalmazásával lehetséges-e a különálló alkalmazás esetén mutatkozó hátrányok kiküszöbölése, és ugyanakkor az előnyös hatások érvényesítése.

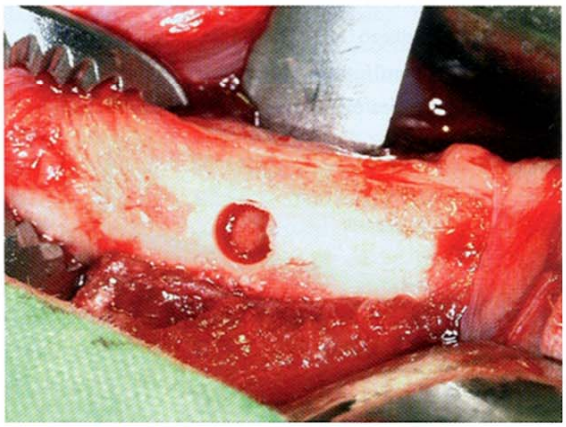
Módszerek és anyagok

Kísérleteink során 1-2 milliméteres szemcseméretű Bio-Oss granulátumot (Geistlich Pharma AG) és 1-2 milliméteres finomságú Cerasorb M (Curasan, Kleinostheim) csontpótlót használtunk a sebek feltöltésére, amelyeket új-zélandi fehér nyulak femurjában alakítottunk ki. A műtéteket az állatkísérletekre vonatkozó nemzetközi etikai és állattartási szabályoknak megfelelően végeztük, és az állatokat elaltattuk a beavatkozáshoz. A narkózis bevezetéséhez 5,0 mg/testsúlykilogramm xylazin (Xylazin 2% injekciós oldat formájában), 40,0 mg/testsúlykilogramm ketamin (Ketavet injekciós oldat formájában) és 0,8 mg/testsúlykilogramm acepromazine (Vetranquil injekciós oldat formájában) koktélt alkalmaztunk, amelyet intramuszkulárisan fecskendeztünk be. A műtétek során a kísérleti állatok folyamatosan kaptak Ringer-laktátot, 0,3 ml/perc sebességű infúzió formájában, egy fülvénán keresztül. A kiválasztott műtéti hely fertőtlenítése és izolálása után bőrmetszést ejtve kipreparáltuk a fascia latát. A femur csontfelszínéhez a m. tensor fasciae latea és a m. abductor cruris cranialis szétválasztásával jutottunk el. A csontseb alakításának első lépéseként sebészi fúrómotort (Elcomed, W&H) használva 2 mm átmérőjű gömbfúróval kijelöltük a tervezett sebzési helyet a femur proximális harmadában, a trochanter maiortól 2 cm-rel disztálisan. Ezután csontfészek-alakító sebészi fúróval monokortikális furatot képeztünk a nyúl combcsontjában. Annak érdekében, hogy valamennyi seb azonos lehessen, minden furat elkészítéséhez a Camlog Implantációs Rendszer (Camlog Biotechnologies) sebészi készletébe tartozó 3,3 mm átmérőjű csontfészek-alakítót használtuk, a fúró egyidejű folyamatos belső és külső hűtésével *(1. ábra)*.

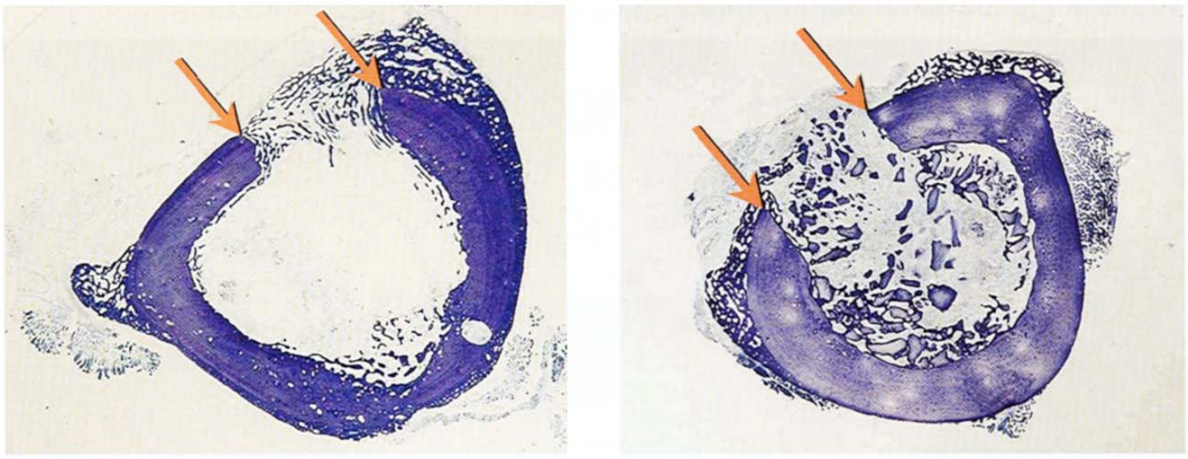
A kísérleti protokoll szerint minden kísérleti állat jobb femurja szolgált vizsgálati oldalként, és az állatok bal combcsontja volt a kontroll. A csontsebet a tesztoldalon, a Bio-Oss/Cerasorb szemcséket térfogatilag egy-egy arányban az adott nyúl vérével összekeverve töltöttük fel úgy, hogy az a furatot teljesen kitöltse. A kontrolloldali sebbe csupán pufferelt fiziológiás sóoldat került. A sebeket, mindkét oldalon egyformán, Surgicellel (Johnson and Johnson) fedtük, amelyet Histoacryllal (Braun) rögzítettünk a furat körül. Ezután rétegesen (fascia lata, szubkután szövetek, cutis) zártuk a lágyrész-sebrészt. A posztoperatív időszakban az állatok 4 napon keresztül naponta kaptak 4,0 mg/testsúlykilogramm caprofent, szubkután (Rimadyl injekciós oldatban) és antibiotikumot, 15



1. a ábra: A monokortikális csontseb preparálása Camlog 3,3 milliméter átmérőjű csontfészek-alakító fúróval...



1. b ábra: ...és a frissen preparált csontseb a nyúl femurjában.



2. a–b ábrák: A négyhetes gyógyulási időszak után vett minták szövettani preparátumai, toluidinkék festéssel (a nyilak a csontsebek határát jelzik).

mg/testsúlykilogramm enrofloxacint, szubkután (Enroxil injekciós) oldatban. A gyógyszeres utókezelést 5 napon át folytattuk. A kísérleti állatok mindegyike egészséges maradt. A műtétet 4 hetes gyógyulási időszak követte. Ezután került sor a minták kivételére a kísérleti állatokból. Ehhez az állatokat intravénás ketamintúladagolással narkózisban áldoztuk fel. Az altatás első szakaszában, még meglévő keringés mellett, általános fixációs perfúziót alkalmaztunk, 4%-os pufferolt formalinoldattal. A femur kivétele után minden kivágott kísérleti és kontrollsebet tartalmazó csontrészletet, további immerziós fixációra, 4%-os pufferolt formalinoldatban tároltunk. A szövettani feldolgozáshoz először röntgenfelvételt készítettünk a mintákról annak érdekében, hogy a beágyazás a csontseb megfelelő orientációjában történhessen. Ezután a kivett minták dehidrálása következett. A beágyazáshoz Technovit 7200VLC műgyantát (Heraeus Kulzer) használtunk. A metszeteket (csiszolatokat) dekalcinálás nélkül, Exact szövettani csiszolatkészítő berendezéssel (Donath & Breuner) állítottuk elő. A metszeteket, amelyeknek vastagsága 8–10 mikrométer volt, toluidinkékkel festettük meg. A hisztológiai és hisztomorfometriai vizsgálatot optikai mikroszkóppal (Nikon Eclipse 80i) végeztük, a felvételeket pedig Evolution MP 5.1 megapixel FireWire Digital CCD Color Camera Kit (Media Cybernetics Inc.) készítettük. A hisztomorfometriai mérésekhez Image-Pro Plus 5.1.1 képanalizáló szoftvert (Media Cybernetics Inc.) használtunk. Az eredmények értékelése a „perarea measurement" módszerrel történt (*Parfitt* et al. 1987; *Schaffler* et al. 1997), amellyel az újonnan képződött csontnak a teljes vizsgált csonthoz viszonyított mennyisége határozható meg. Így kvantitatív módon végezhető el az újonnan képződött csont mennyiségi összevetése az egyes mintákban.

Eredmények

A szövettani vizsgálatkor a szöveti kép azt mutatta, hogy a kontrollmintákban a csontseb majdnem teljesen záródott (*2. a ábra*). A sebszéleken a csonton reszorpciós lakúnák és újonnan képződött csont egyaránt látható volt, mely utóbbi fiatal, éretlen, centrifugális elrendezettségű lamellás csontszövet képét mutatta, helyenként rostos jelleggel, keresztirányban futó kollagénrostokkal. A tesztseb, amelyet Bio-Oss/Cerasorb keverékkel töltöttünk fel a műtét alkalmával, nem záródott teljes egészében (*2. b ábra*).

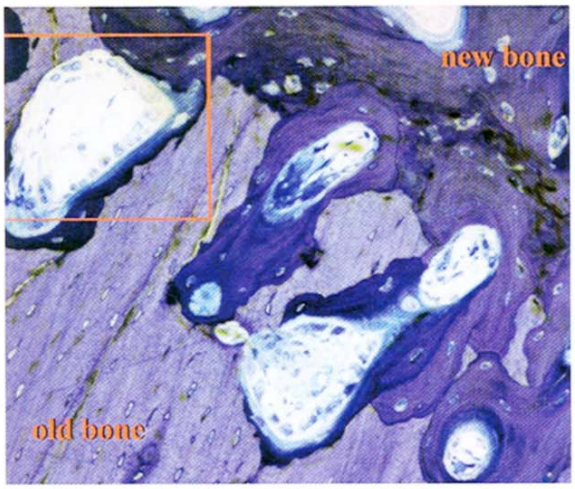
A csontpótló anyag szemcsei körül csontképződés látható, ami egyrészt körülveszi a szemcséket, másrészt a szemcsék között hidat képez. Ez a csont fiatal, éretlen rostos csont képét mutatja, kifejezett oszteoblaszt-aktivitással a szemcsék körül. A kísérleti minták nagyobb nagyítású szövettani képein (*3. a–c ábra*), amelyeken a négy hét alatt újonnan képződött csont jellegzetességei vizsgálhatók, az látszik, hogy mind a rostos csont, mind az oszteonba rendeződő lamelláris csont jelen van a gyógyulásnak ebben a szakaszában, és jól kivehetők a csontképző oszteoblasztok is.

Az is látszik, hogy a csontpótló granulumok reszorpciója még messze nem fejeződött be, de egyes szemcsék körül egyértelmű az oszteoid szöveti struktúra (rostos csont) jelenléte, ami az osszeokonduktív hatást tükrözi. A hisztomorfometriai mérések érdekes eredményt hoztak. Kitűnt, hogy a kísérleti mintákban, annak ellenére, hogy a seb a széleken nem záródott, az újonnan képződött csont mennyisége a „perarea measurement" értékek szerint 48,7% volt, szemben a kontrollmintákban mért 33,9%-os értékkel. Vagyis a csontpótló granulátummal feltöltött sebben a csontképződés intenzitását 1,5-szer nagyobbnak találtuk, ha azt a kontrollmintákban talált értékekkel vetettük össze, amely utóbbiakban egyébként a széleken a csontsebzáródás kifejezettebb képe látszott.

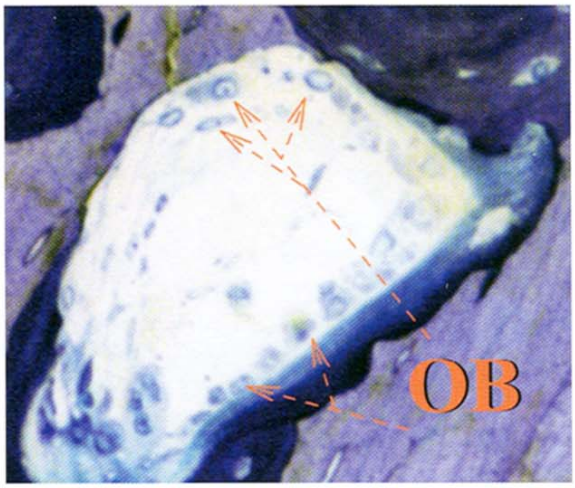
Megbeszélés és következtetések

A korszerű implantológiában a sokszor elengedhetetlen csontaugmentációs technikák megkövetelik az osszeokonduktív és/vagy osszeoinduktív anyagok alkalmazását. Fontos, hogy a kívánt csontmorfológia a lehető legbiztonságosabban, leggyorsabban és hosszú távú fennmaradást garantálva valósuljon meg. Az irányított csontregenerációban használatos anyagok kínálata ma már igen nagy. Még mindig hiányzik azonban az ideális alloplasztikus „csontpótló", legyen az tömb vagy granulátum formátumú. Vizsgálatainknak célja az volt, hogy keressük a fenti kívánalmaknak megfelelő anyagot, és két olyan alapanyagot „ötvözzünk", amelyek együttes alkalmazásával megközelíthetővé válhat az ideális csontpótló anyag. Kísérletünk eredményei szerint az ilyenfajta, csontújraképződést serkentő anyagkombinációk ígéretesek a klinikai gyakorlat szá-

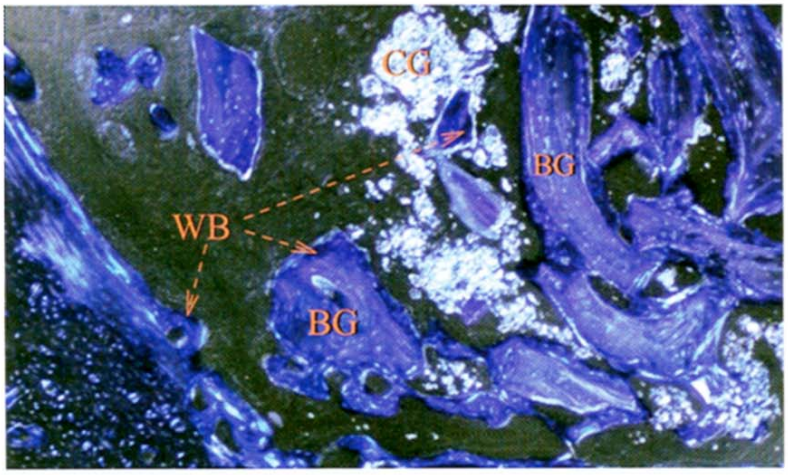
3. a–b–c ábrák: A Bio-Oss/Cerasorb keverékkel kitöltött csontseb négyhetes gyógyulás után kivett mintájának szövettani képe.



3. a ábra: A „régi" és az „új" csont határzónájának egy részlete 40x-es nagyításban. OLD BONE – az eredeti („régi") csont NEW BONE – az újonnan képződött („új") csont



3. b ábra: Az „a" ábrán látható kép bekeretezett részének kinagyított részlete. OB – oszteoblasztok sora



3. c ábra: Polarizált fényben, 20x-os nagyítással készített felvétel. CG – Cerasorb szemcsék (granulumok), BG – Bio-Oss részecskék (granulumok) WB – (woven bone) újonnan képződött rostos csont

mára. Összeoinduktív anyag hozzáadása a bizonyítottan osszeokonduktív alloplasztikus bioanyagokhoz közelebb viheti az alkalmazókat az elérni kívánt eredményhez. A gyakorlatban/klinikumban való alkalmazáshoz azonban további preklinikai vizsgálatok szükségesek.

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