Surface structure, biocompatibility and hard tissue regeneration of nanostructured biphasic calciumphosphate cereamic and natural bovine deproteinized bone matrix. An in-vitro and in-vivo analysis.

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Summary - **Alongside the use of the patient's own bones, various xenogenic and alloplastic bone substitute materials are established for use in augmentation surgery in dental implantology. They yield results which are predictable according to the respective indications for treatment, with a simultaneous reduction of donor-site morbidity.**

The aim of this study was the scientific analysis of a new type of biphasic bone substitute material (Maxresorb [MR], Botiss Dental, GmbH Berlin) in comparision to a natural bovine bone mineral (Bio Oss Spongiosa [BOS], Geistlich Biomaterials, Wolhusen, Switzerland). To this end, scanning electron microscope analyses and cell and animal experiments were performed.

The scanning electron microscope surface analysis revealed that the MR material has nanoporous parts in addition to a highly porous macro- and microstructure. BOS showed a more smooth surface at 500x magnification. A good proliferation of bone cells was observed on MR, whereas BOS revealed some limitations on cell attachment and proliferaton in-vitro. Therefore, initial attachment as well as proliferation were significantly better on MR than BOS ($P<0.05$, Mann Whitney U-Test). In the animal experiments, following a lateral augmentation of the maxilla in eight animals, the first signs of osseous regeneration in MR were observed after a healing period of 4 weeks. After a healing period of 8 weeks, there was a complete union of the MR augmentation material with woven bone. BOS showed a more delayed bone formation, originating from the bottom of the defect. After 24 weeks of healing, the woven bone was converted into lamellar bone, with a very slow surface resorption of MR. BOS showed no resorption of the granules and a more delayed bone maturation, whereas volume stability was found excellent in both groups.

It was concluded that both MR and BOS show good biocompatibility, supporting osseointegration with marked volume stability and slow resorption.

Keywords: **bone substitute material, augmentation, bovine, bone regeneration, biphasic, augmentation**

Introduction

Implants placed in a compromised jaw require additional surgical measures to create a sufficient bone bed as a precondition for a good aesthetic result and long-term stability. Autogenous bone block grafts and the distraction osteogenesis procedure have proved their worth for extensive dental hard substance deficits and defects with vertical components. For horizontal defects, bone splitting and spreading have demonstrated predictable success, as have augmentations with the patient's own bone and/or bone substitute materials. However, the availability of
the patient's own bone is limited and entails the risk of complications at the extraction point. Bone substitute materials, on the other hand, have demonstrated a slightly increased risk of infections. They are frequently used in conjunction with barrier membranes in the field of guided tissue regeneration [1]. The membranes keep undesired soft tissue away from the defect, which has a positive effect on the regeneration result. Sinus floor elevation plays a particularly important role due to the special anatomical characteristics of the augmentation bed as a four-wall bone defect. Very good results can be obtained here with both autogenous as well as xenogenic and alloplastic materials [2].

Regarding the regeneration of bone defects, three main components are of overriding importance: bone-forming cells, a regenerative matrix and growth factors. In the case of autogenous tissue, all three components were transferred directly to the recipient bed. Here, depending on the removal method, the existing osteoblasts remain mostly vital [3]. Alternatively, osteoblasts can differentiate from ubiquitously occurring precursor cells under the influence of special growth factors [4]. If the regenerative matrix consists of a non-vital bone substitute material, the latter is incorporated into the regenerating hard tissue in an osteoconductive manner. The bone substitute material thus acts as a place holder and guide rail for the bone regeneration [5].

Alongside natural bone matrices from human (allogenic) or animal (xenogenic) donor tissue, various alloplastic materials are established in dental implantology. They are chemically synthesised based on the physicochemical properties of natural bone tissue and possess different resorption kinetics. The possibility of a transfer of pathogens is fundamentally ruled out in the case of alloplastic materials [6].

The aim of the present study was the morphological, cell culture experimental and histological study of a biphasic granular bone substitute material (Maxresorb® [MR], Botiss Dental, Berlin), which is composed of 60% hydroxylapatite and 40% ß-tricalcium phosphate ceramic (ß-TCP). A natural bovine bone matrix (Bio Oss Spongiosa [BOS], Geistlich Biomaterials, Wolhusen, Switzerland) served as control.

Material and Methods

The study can be divided into three parts: for the evaluation of the surface morphology, the individual materials were initially examined using a scanning electron microscope *(SEM analysis)*. The second part involved testing the biocompatibility. To do this, the materials were incubated with an osteoblast-like cell line and the proliferation of cells on the surface was determined after various periods of time had elapsed *(in-vitro part)*. In the animal experiment part, the bone substitute materials were applied to lateral maxillary defects and the osseous regeneration and resorption of the augmentation material was evaluated after different healing periods *(in-vivo part)*.

SEM part

Individual granules from two different bone substitute materials (Maxresorb® [MR], biphasic ß-TCP, Botiss Dental, Berlin; and BioOss® Spongiosa [BOS], natural bovine hydroxylapatite, Geistlich Biomaterials, Wolhusen, Switzerland) were mounted on carbon carriers and sputtered with gold of a layer thickness of 30 nm by means of a low-voltage cool sputter coater. The surface morphology was then evaluated using a scanning electron microscope (Zeiss DSM 950) at magnification levels 25, 50, 100, 200, 1000 and 2000.

In-vitro part

The quantities of MR and BOS just covering the well floor were filled into the wells (n=8 wells per KM and time point) of a 96-well plate (Costar®, ultra low attachment, No 3474, Corning Incorp., Schipol-Rijk, Holland) and cultivated with $1x10^4$ SaOs-2 cells per well in DMEM, 10% foetal calf serum and 1% penicillin/streptomycin. After two hours, three days and seven days cultural experiment duration, the LDH concentration in the well was photometrically determined using a special LDH assay (CytoTox 96® non-radioactive cytotoxicity assay, Promega, Mannheim). To this end, the plates were centrifugated at 650 g for 8 minutes and the cell culture supernatants discarded. After a further washing stage, the remaining samples, comprising cells and bone substitute material, were cooled to -80°C to release the LDH and the LDH determination was carried out. A medium replacement took place on the third day of the seven-day procedure. After 2 hours, 3 and 7 days some granules were rinsed with DMEM solution and fixed in 10% glutaraldehyde solution to allow for morphological analysis of the cell proliferation.

In-vivo part

Three months after tooth extraction and defect setting in the lateral anterior maxilla, a mucoperiosteal flap was formed on both sides in eight dogs under endotracheal anaesthesia after crestal incision. 0.5 ccm MR or BOS with granule size 0.5–1 mm was applied laterally to the alveolar ridge following a split mouth design. MR was covered with a native pericardium membrane (Jason® pericardium collagen membrane, Botiss) rehydrated in NaCl in the context of guided bone regeneration. BOS was covered using the collagen membrane (BioGide, Geistlich Biomaterials) of the same manufacturer as BioOss. The flap was readapted after periosteal incision, and the opening was sealed against saliva using a resorbable suture (PGA Resorba®, Resorba, Nuremberg). After a healing period of four, eight and twelve and 24 weeks, the respective jaw sections were removed for further histological examination. The tissues were fixed in formalin, dehydrated in a graded alcohol series and prepared using a hard tissue histological method [7]. The evaluation of the histological sections took place after toluidine blue staining under a transmitted-light microscope (BX50, Olympus, Hamburg, Germany) in conjunction with a video attachment (SIS Color View2, Soft Imaging System GmbH, Münster, Germany). A special software (SIS analySIS Auto Software 3.2, Soft Imaging System GmbH, Münster, Germany) was used for the analysis.

Results

The scanning electron microscope analysis of MR at low magnification revealed spherically configured particles of different sizes, which were characterised by a large quantity of micro- and macrostructural pores on the surface. Cavities were clearly recognisable in many places within the individual constructs (Fig. 1). At higher magnifications, the surface revealed a large quantity of individual nanocrystalline components. The size of the smallest particles on the surface was approx. 50 nm. These were partly fused with each other and had intermittent cavities, which created an overall nanoporous appearance. (Fig. 2). BOS, which is also spherically configured, (Fig. 3). At higher magnification, a smoother, laminated surface with only sporadically interspersed pores was identified (Fig. 4).

The results of the in-vitro analyses can be found in Fig. 5. For MR, a significant increase in the number of cells over the period ($P < 0.05$) was observed. For BOS, the number of cells decreased significantly from two hours to seven days ($P < 0.05$). Comparison between groups revealed a higher cell attachment and proliferation on MR than on BOS ($P > 0.05$, Mann Whitney U-Test).

In the animal test, proper wound healing was observed for all animals. No wound dehiscences or infections occurred.
Histologically, inspecting overview magnifications, an initial new bone formation (blue) arising from the basal bone bed (lilac) was observed for the 4-week specimens (Fig. 6). For the blue-stained BOS, just a minimal bone formation was found on the bottom of the defect (Fig. 7).

After 8 weeks, entire MR graft showed bone formation on and in between the individual particles. For BOS, a more delayed bone formation spreading out from the defect area was visible. No changes in bone volume but further bone maturation was found after 12 weeks for MR. In the BOS group, bone matrix was found on and in between the basal granule layer but not reaching the peripheral part of the graft. After 24 weeks, MR-grafted area revealed a mature bone within the entire augmented area and good volume maintenance (Fig. 8). BOS showed a more basal bone formation and good volume maintenance, however the rate of mineralized vital bone tissue within the graft seemed to be lower than that of MR.

Inspecing the individual granules at higher magnifications, MR particles (grey) appeared surrounded by blood vessels (blue) and embedded in a loose regenerative matrix after 4 weeks. Sporadic initial accumulations of osteoid (dark blue) could be detected alongside osteoblasts, including within the augmented area (Fig. 9). BOS showed no bone formation on the surface, but an initial bone apposition originating from the defect bottom could be distinguished (Fig. 10). After a healing period of eight weeks (Fig. 11), the entire MR graft was dominated by newly formed hard tissue. Individual MR granules (grey) appeared largely encapsulated in woven bone or joined to each other in a kind of network via hard tissue bridges (Fig. 12). The transition between the bone substitute material and the woven bone was very accentuated. A sporadic initial growing of hard substance into the porous parts of the granules could be seen. A partly moniliform arrangement of osteoblasts could be identified on the surface of the trabeculae. Actual bone marrow with fat cells and blood vessels was identified in the non-mineralised parts. An inflammatory reaction could not be detected. BOS showed a more delayed bone formation (Fig. 13), with initial bone apposition on the BOS surfaces. but much lower newly formed bone area.
A healing period of twelve weeks revealed a similar picture to the 8-week histologies, although the bone structure alongside MR appeared increasingly mature (Fig. 14). In the regenerated hard tissue, scattered resorption lacunae could be seen alongside small mineralised, newly-formed bones in the sense of an osseous remodelling. The transition between MR and surrounding bone tissue appeared considerably less pronounced in some areas. Here, the surface parts of the granules seemed to degrade into smaller mineralisation nuclei. BOS revealed increasing bone formation in direct contact to the granule surfaces, but also in between the individual particles. High number of blood vessels could be identified in the non-calcified areas (Fig 15).
After 24 weeks (Fig. 16), the augmentation materials appeared for the most part regenerated with lamellar bone tissue. Whereas most MR and BOS particles could still be clearly distinguished from the surrounding hard tissue, some MR granules showed only rudimentary borders with the vital bone tissue. In many places, clear indications of functional transformation in the sense of bone resorption and apposition could be seen. BOS revealed no resorption lacunae but a very good osseous integration within the newly formed bone matrix.

DISCUSSION

In this work, the morphology, biocompatibility and osseous regeneration of the biphasic calcium phosphate ceramic Maxresorb (MR) were investigated and compared to the well-established bovine bone substitute material BioOss (BOS). With high porosity, MR showed a significant proliferation of osteoblasts on the surface, which were considerably superior to that on the comparison product BOS. Histologically, an increasing osseous organisation of the augmentation materials over time was detected. In this regard, MR appeared fully encapsulated in woven bone for the first time after only eight weeks. For BOS, a healing period of 12 weeks was found to be necessary for this stage of bone apposition. After a maximum healing period of 24 weeks, the original woven bone had transformed into lamellar bone. Both MR and BOS were not resorbed at this point. However, there was a sporadic slight superficial osseous remodellation of the MR granules.

Scanning electron microscope analysis revealed that the surface of MR - in contrast to BOS - possessed a clear porosity on both the macro-level and the micro-level. Individual particles and interstices were considerably smaller than 1 µm and thus also attained a certain nanoporosity. This provided support to the better adhesion and proliferation of cells observed in the in-vitro part. In the case of cells which grow adherently such as osteoblasts and fibroblasts, a direct union of the cell membrane with the surface is an indispensable condition for proliferation. The process of cell adhesion to the surface of bone substitute materials occurs in different stages:

1. Absorption of serum proteins on the surface of the bone substitute materials
2. Contact of the rounded osteoblasts with the substrate
3. Attachment of the cells to the substrate
4. Spread of the cells on the bone substitute material [8]

Here, surface roughness plays a considerable role as regards the attachment of cells on bone substitute materials. Deligianni [9] observed a better cell attachment and proliferation of bone cells when rough hydroxylapatite (HA 180) was used as opposed to fine hydroxylapatite (HA1200). The comparison of the present in-vitro results with other studies shows that BOS yielded very similar results including with other cell lines involved in bone regeneration. In a recent study by Herten et al., a drop in the vitality of primary osteoblasts and bone marrow stem cells was also observed after incubation with BOS [10]. A synthetic, pure ß-TCP, on the other hand, revealed a significant increase in the number of cells over time. The ß-TCP component in MR

may thus also have positively affected the biocompatibility of the material in the present work.

Mesenchymal progenitor cells from the bone marrow play an important role in in-vivo bone regeneration. Kassem et al. described the formation of primary osteoblasts from bone marrow stem cell cultures in 1991 [11]. Also, in a study by Wiedmann-Al-Ahmad [12], BOS and a modification of BOS coated with collagen showed the lowest proliferation of human osteoblast-like cells in direct comparison with 14 other bone substitute materials. This effect could be due in part to the initial attachment of the cells being impeded by the smooth surface of BOS observed in the SEM. In the study by Herten et al., however, a non-adherent cell line (U937) showed a clear proliferation even where BOS was present [10]. Deligianni et al. pointed out that an improved attachment of fibronectin also meant that a stronger union could form between hydroxylapatite and the cells. These effects, which can only be examined to a very limited extent in a cell culture, play a considerable role in in-vivo bone regeneration. The surface properties of bone substitute materials change substantially where there is direct contact with blood proteins or extracellular matrix proteins. That BOS yielded considerably better results in-vivo than in-vitro [13] can, in part, be ascribed to such effects. In other studies, the proliferation on BOS could also be improved in-vitro through use of a vacuum pump for granule deaeration or by using perfusion technology [14]. In the case of the ß-TCP granules, no difference to the positive control (polystyrene) could be detected even without further measures [15].

In the animal experiment part, MR and BOS showed different staining characteristics after processing the specinens following the standard protocol for hard tissue histology. Whereas BOS was stained intensively blue, MR granules did nod show any uptake of the toluidine blue dye and therefore remained grey. This observation can be explained by the fact that MR as a synthetic material manufactured at 1500° Celsius does not contain any proteins or hydrate water and therefore, as known for ß-TCP as well, is not able to attach the toluidin molecules. BOS as a natural bone mineral is harvested from bovine bone – in addition to a low-temperature processing (the manufacturer gives no information about the particular temperature), the bone matrix seems to preserve the same dying characteristics as natural bone tissue. However, it has to be mentioned that dying characteristics of a bone substitute material represent an in-vitro effect of the histological preparation and does ot give any information about the quality of a substitute.

After a healing period of 4 weeks, a large number of small blood vessels could be seen alongside an initial formation of osteoid. This can be interpreted as a predecessor stage for osseous regeneration. An animal experiment showed that the formation of capillaries stood in direct spatial and temporal correlation to extraskeletal new bone formation [16]. This observation can be explained with the aid of the principle of bone formation: osteogenic cells develop from undifferentiated mesenchymal progenitor cells, which are either located in the connective tissue of the bone marrow or develop from pericytes of the adjacent connective tissue of small capillaries [11, 17]. Thus, the invasion of blood vessels when using bone substitute materials represents an important prerequisite for osseous regeneration. In this in-vivo experiment, the augmentation material was already fully osseously organised after eight weeks; over the further course of the process, there was additional physiological bone maturation.

In dental implantology, it is primarily hydroxyapatite and the alpha and beta phases of tricalcium phosphate which are established as alloplastic materials. ß-tricalcium phosphate is seen as completely and quickly resorbable [18]. A good pressure stability and better volume constancy are cited as advantages of hydroxylapatite [19]. A mixture of both as in the studied biphasic calcium phosphate seems—as observed histologically—on the one hand to induce a quick osseous integration via the ß-TCP part, while the hydroxylapatite part ensures a long-term stability with only very delayed resorption. Thus, a homogenous osseous organisation of the entire augmentation material could be determined within 8 weeks, whereas after 24 weeks considerable parts of the augmentation material were still dominated by unresorbed bone substitute material in spite of demonstrated high porosity. A loss in volume of the augmentation material, as familiar from pure ß-TCP [19], was neither detected for MR nor BOS. This could be of importance in the context of avoiding overaugmentation, particularly for the sinus floor elevation application. In addition, an admixture of MR to autogenous material such as known for xenogenic materials [20] could provide protection against resorptions.

As a limit of the present study it has to be mentioned that the groups MR and BOS were covered with different collagen membranes to keep the manufacturer the same of both membrane and bone substitute material (MR: Jason membrane, BOS: BioGide). In a present study which will be published at the end of 2011 [21], both membranes were identified as native porcine collagen membranes revealing high biocompatibility, no inflammatory biodegradation and early vascularization. However, Jason membrane was identified to have a longer barrier function than BG, which in turn could positively influence the total regeneration outcome. Therefore, from a clinical point of view, the delayed bone formation of BOS could be based on both longer barrier function of the membrane but also surface and osteoconductive characteristics of the respective bone substitute material.

What the long-term period is in which MR is fully resorbed and transformed into actual bone cannot be estimated from the present data. It will be considerably longer than that of pure ß-TCP, however, but shorter than for BOS. Further clinical studies and animal experiments

are necessary to be able to make statements about the long-term remodelling and a comparative statement about other bone substitute materials.

REFERENCES

1. Dahlin, C., A. Linde, J. Gottlow, et al.: Healing of bone defects by guided tissue regeneration. Plast Reconstr Surg. 81(5): 672-6 (1988)
2. ten Bruggenkate, C.M. and J.P. van den Bergh: Maxillary sinus floor elevation: a valuable pre-prosthetic procedure. Periodontol 2000. 17: 176-82 (1998)
3. Chiriac, G., M. Herten, F. Schwarz, et al.: Autogenous bone chips: influence of a new piezoelectric device (Piezosurgery) on chip morphology, cell viability and differentiation. J Clin Periodontol. 32(9): 994-9 (2005)
4. Reilly, T.M., R. Seldes, W. Luchetti, et al.: Similarities in the phenotypic expression of pericytes and bone cells. Clin Orthop Relat Res, (346): 95-103 (1998)
5. LeGeros, R.Z.: Properties of osteoconductive biomaterials: calcium phosphates. Clin Orthop Relat Res, (395): 81-98 (2002)
6. Pappalardo, S., S. Puzzo, V. Carlino, et al.: Bone substitutes in oral surgery. Minerva Stomatol. 56(10): 541-57 (2007)
7. Donath, K.: The diagnostic value of the new method for the study of undecalcified bones and teeth with attached soft tissue (Sage-Schliff (sawing and grinding) technique). Pathol Res Pract. 179(6): 631-3 (1985)
8. Grinell, F.: Cellular adhesiveness and extracellular substrate. Int Rev Cytol. 53: 65-129 (1978)
9. Deligianni, D., P. Korovessis, M.C. Porte-Derrieu, et al.: Fibronectin preadsorbed on hydroxyapatite together with rough surface structure increases osteoblasts' adhesion "in vitro": the theoretical usefulness of fibronectin preadsorption on hydroxyapatite to increase permanent stability. J Spinal Disord Tech. 18(3): 257-62 (2005)
10. Herten, M., D. Rothamel, F. Schwarz, et al.: Surface- and nonsurface-dependent in vitro effects of bone substitutes on cell viability. Clin Oral Investig, (2008)
11. Kassem, M., L. Risteli, L. Mosekilde, et al.: Formation of osteoblast-like cells from human mononuclear bone marrow cultures. Apmis. 99(3): 269-74 (1991)
12. Wiedmann-Al-Ahmad, M., R. Gutwald, N.C. Gellrich, et al.: Search for ideal biomaterials to cultivate human osteoblast-like cells for reconstructive surgery. J Mater Sci Mater Med. 16(1): 57-66 (2005)
13. Zitzmann, N.U., P. Scharer, C.P. Marinello, et al.: Alveolar ridge augmentation with Bio-Oss: a histologic study in humans. Int J Periodontics Restorative Dent. 21(3): 288-95 (2001)
14. Deligianni, D.D., N.D. Katsala, P.G. Koutsoukos, et al.: Effect of surface roughness of hydroxyapatite on human bone marrow cell adhesion, proliferation, differentiation and detachment strength. Biomaterials. 22(1): 87-96 (2001)
15. Aybar, B., A. Bilir, H. Akcakaya, et al.: Effects of tricalcium phosphate bone graft materials on primary cultures of osteoblast cells in vitro. Clin Oral Implants Res. 15(1): 119-25 (2004)
16. Schmid, J., B. Wallkamm, C.H. Hammerle, et al.: The significance of angiogenesis in guided bone regeneration. A case report of a rabbit experiment. Clin Oral Implants Res. 8(3): 244-8 (1997)
17. Rickard, D.J., M. Kassem, T.E. Hefferan, et al.: Isolation and characterization of osteoblast precursor cells from human bone marrow. J Bone Miner Res. 11(3): 312-24 (1996)
18. Wiltfang, J., H.A. Merten, K.A. Schlegel, et al.: Degradation characteristics of alpha and beta tri-calcium-phosphate (TCP) in minipigs. J Biomed Mater Res. 63(2): 115-21 (2002)
19. Fujita, R., A. Yokoyama, T. Kawasaki, et al.: Bone augmentation osteogenesis using hydroxyapatite and beta-tricalcium phosphate blocks. J Oral Maxillofac Surg. 61(9): 1045-53 (2003)
20. Simion, M., F. Fontana, G. Rasperini, et al.: Vertical ridge augmentation by expanded-polytetrafluoroethylene membrane and a combination of intraoral autogenous bone graft and deproteinized anorganic bovine bone (Bio Oss). Clin Oral Implants Res. 18(5): 620-9 (2007)
21. Rothamel D., Smeets R., Fienitz T., Happe A, Dreiseidler T., Ritter L., Zöller J. Biocompatibility and biodegradation of a native, porcine pericardium membrane. Results from in vitro/in vivo examination. Int J Oral Maxillofac Surg, 2011 (in press)

FIGURES

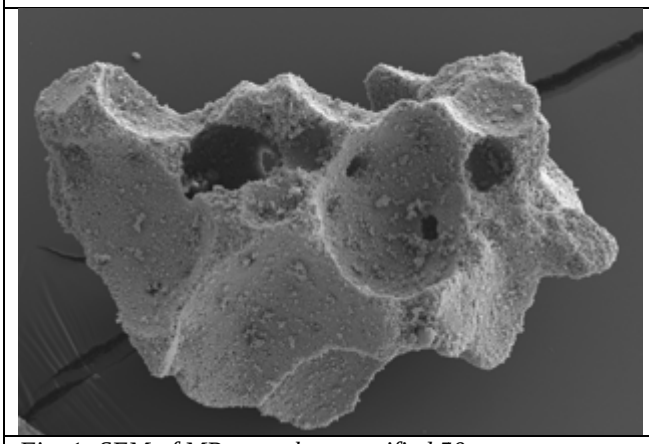

Fig. 1: SEM of MR granule, magnified 50x

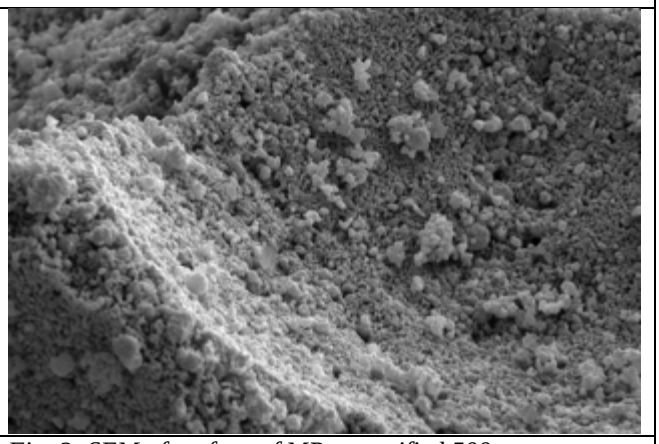

Fig. 2: SEM of surface of MR, magnified 500x

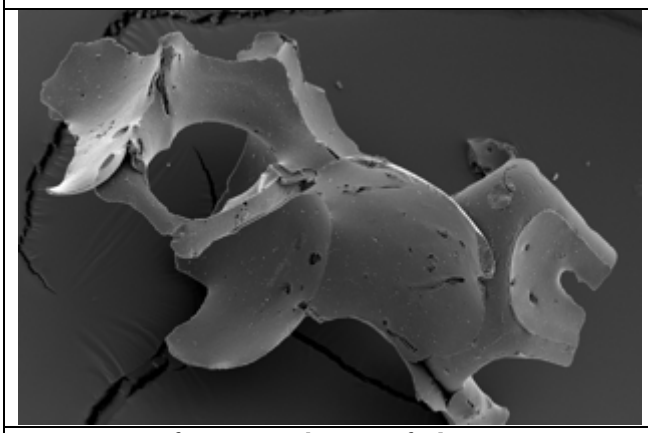

Fig. 3: SEM of BOS granule, magnified 50x

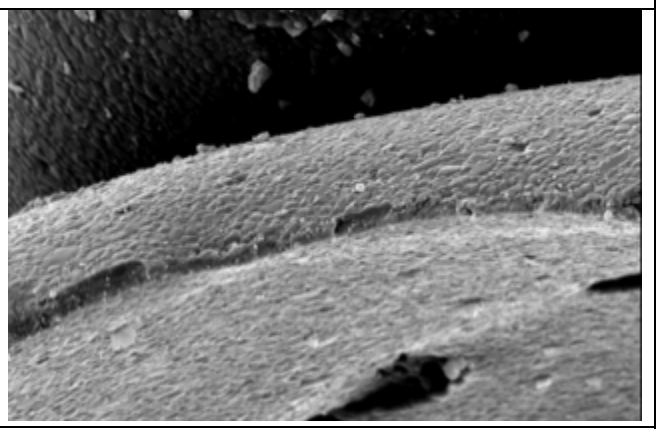

Fig. 4: SEM of surface of BOS, magnified 500x

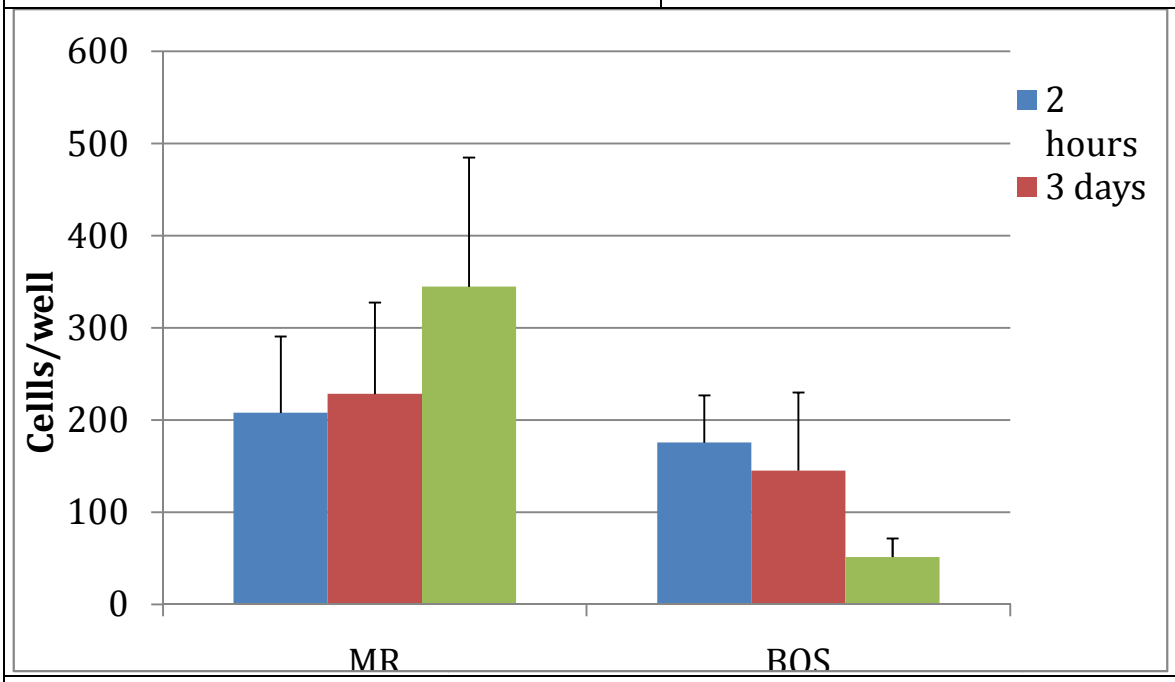

Fig. 5: Number of cells on MR and BOS after an incubation period of 2 hours, 3 days and 7 days

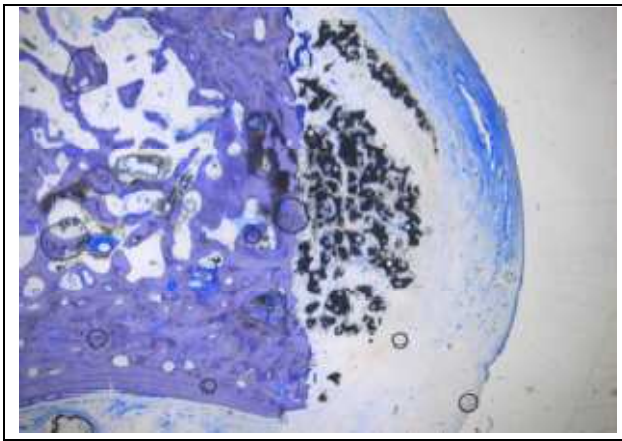

Fig. 8: MR + JM, healing period 4 weeks, magn. 12,5x

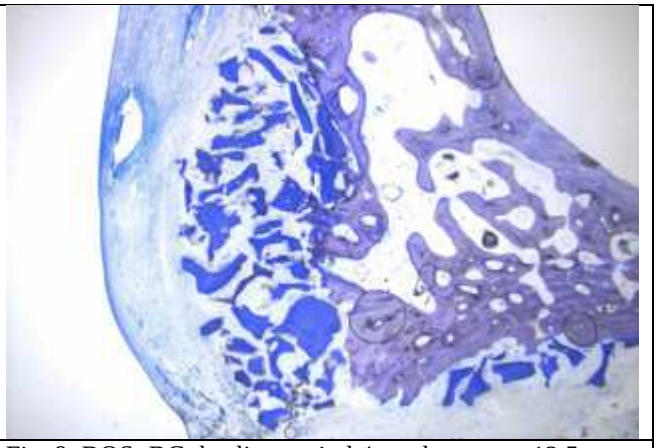

Fig. 9: BOS+BG, healing period 4 weeks, magn. 12,5x

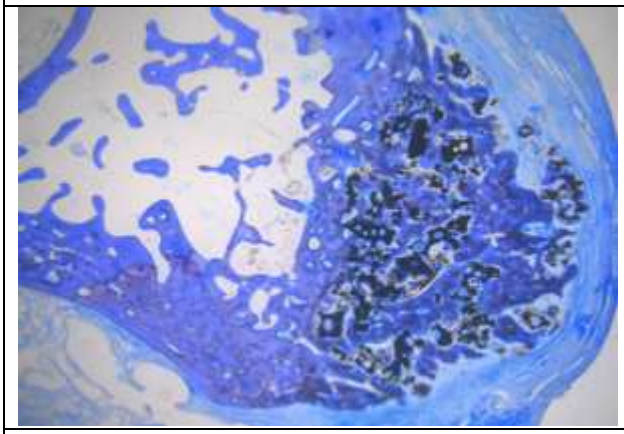

Fig. 10: MR + JM, healing period 24 weeks, magn. 12,5x

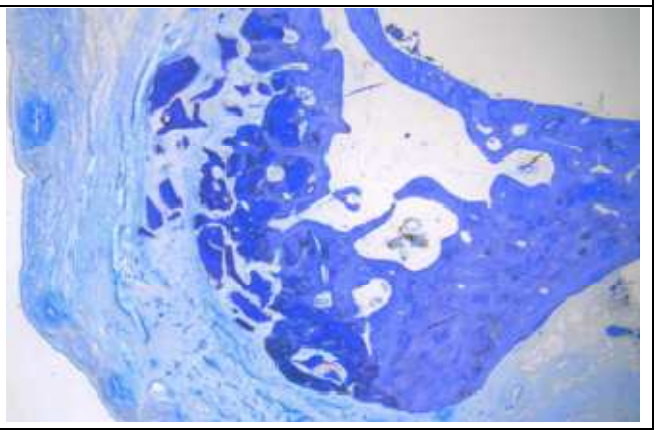

Fig. 11: BOS+BG, healing period 24 weeks, magn. 12,5x

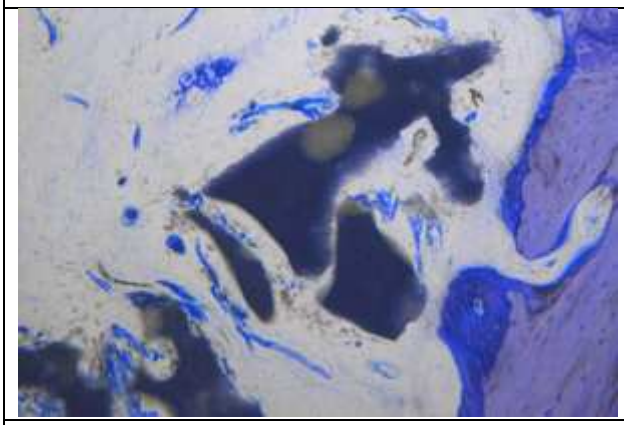

Fig. 12: MR + JM, healing period 4 weeks, magn. 200x

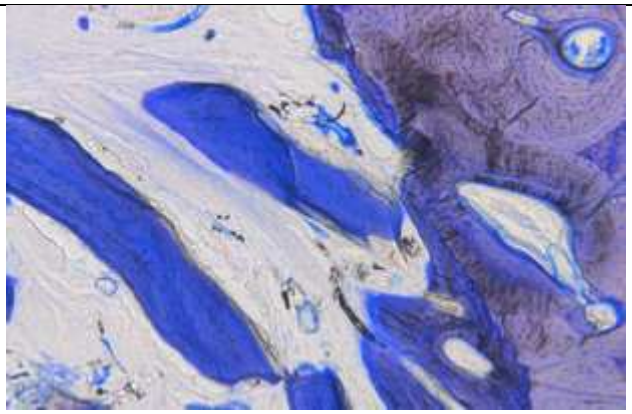

Fig. 13: BOS+BG, healing period 4 weeks, magn. 200x

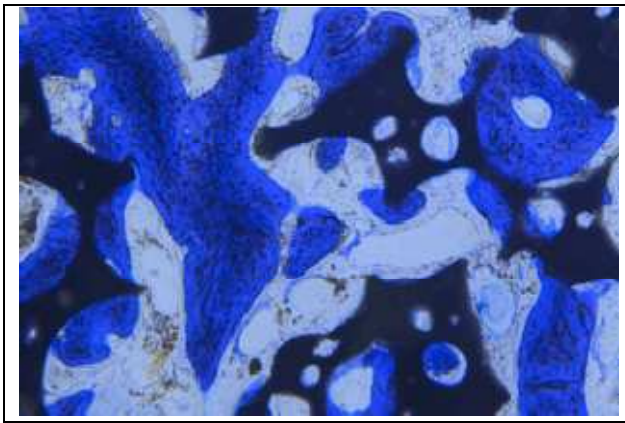

Fig. 14: MR + JM, healing period 8 weeks, magn. 200x

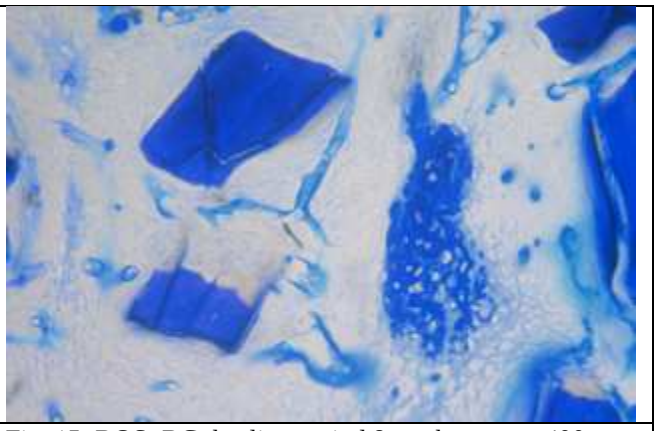

Fig. 15: BOS+BG, healing period 8 weeks, magn. 400x

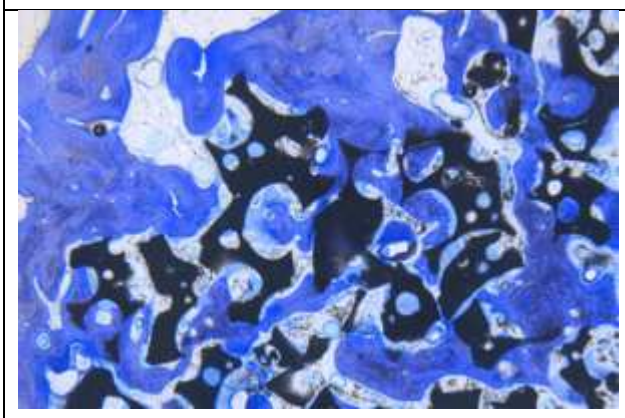

Fig. 16: MR + JM, healing period 12 weeks, magn. 100x

Fig. 17: BOS+BG, healing period 12 weeks, magn. 200x

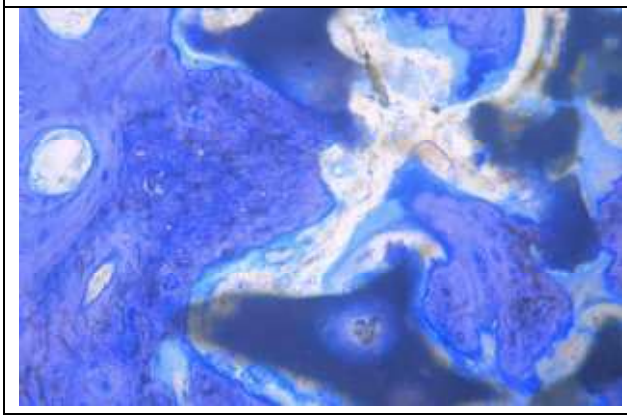

Fig. 18: MR + JM, healing period 24 weeks, magn. 400x

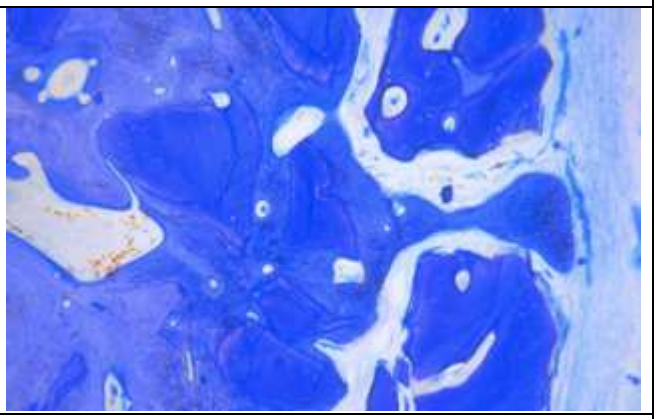

Fig. 19: BOS+BG, healing period 24 weeks, magn. 200x