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Original

Osteogenic activity of β-tricalcium phosphate in a hydroxyl sulphate matrix and demineralized bone matrix: a histological study in rabbit mandible

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Abstract: Currently, in oral and maxillofacial surgery, there is a clinical need for efficient bone grafting materials, and various efforts are being made to improve materials used as bone substitutes to facilitate faster and denser bone regeneration. The purpose of this study was to evaluate *in vivo* the osteogenic potential of synthetic β-tricalcium phosphate in a hydroxyl sulphate matrix (β-TCP/HS) and human demineralized bone matrix (DBM) putty. Sixteen New Zealand White rabbits were used. In each animal, two bone defects (8 mm length × 3 mm width × 3 mm depth) were created in the left and right regions of the mandible, respectively. The defect on one side, chosen randomly, was filled with β-TCP/HS (group A) or DBM putty (group B), while the defect on the opposite side was left unfilled in order to serve as a control site. Two animals in each group were sacrificed at the end of the 1st, 3rd, 5th and 6th week after surgery, respectively, and the osteotomy sites were processed for histological evaluation. Our findings confirmed that β-TCP/HS and human DBM putty possess osteogenic activity and can support new bone formation, although at a slower rate than the spontaneous healing response, in rabbit mandibular osseous defects. (J Oral Sci 52, 377-384, 2010)

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Introduction

In the maxillofacial region, bone defects and deficits occur commonly as a result of periodontal disease, infectious disorders (osteomyelitis), trauma, removal of cysts or tumors, and after dento-alveolar, orthognathic or implant surgery. Various techniques have been developed in order to reconstruct these bone defects with the use of autogenous bone grafts, bone derivatives or bone substitutes (1).

The ideal bone graft should be a non-toxic and non-carcinogenic material that consistently induces bone formation, and is readily available, easy to use and can be supplied in sufficient quantity. Autogenous bone is considered a gold-standard bone grafting material, as it possesses osteoconductive and osteoinductive properties, while being non-allergenic and fully resorbable, eventually being replaced by the patient's own bone. Furthermore, autogenous bone is the only grafting material that contains vital osteoprogenitor cells, supporting osteogenesis, i.e. formation of new bone from surviving cells within the graft (1-3).

However, in order to avoid additional surgery, which increases cost and morbidity, and generally inconveniences the patient, the use of bone grafting substitutes derived from human, bovine and synthetic sources is an alternative

solution (4-7).

Synthetic β-TCP in a hydroxyl sulphate matrix (Fortoss® Vital, Biocomposites®, Etruria, Stoke-on-Trent, England) acts as a scaffold for bone proliferation as it is slowly resorbed by osteoclastic activity and substituted by living bone cells that grow directly in contact with the mineral. One of the most influential factors in the process of β-TCP is macro- and micro-porosity that promotes the ingrowth of blood vessels and enables osteocyte dendrites to infiltrate the micropores. The hydroxyl matrix is pyrogen-free and bacteriostatic, creating a nano-porous cell-occlusive membrane that prevents the early invasion of unwanted soft tissue cells. The product is a simple-to-use, moldable cohesive paste that sets to form a hard but resorbable osteoconductive bone graft material.

Demineralized bone matrix (DBM) putty (Grafton® Osteotech Inc., Eatontown, NJ, USA) is banked human demineralized freeze-dried bone that has been combined with glycerol. It is prepared from donated human bone, mostly cadaveric in origin, by the D-Min® process (Osteotech, Inc.; demineralization using a two-step Triton X-100/0.6 N solution), adhering to the standards set by the American Association of Tissue Banks. It has been suggested that this type of bone grafting material supports bone formation via osteoconduction and osteoinduction. The latter is associated with growth factors, in particular BMPs, insulin-like growth factor (IGF), and fibroblastic growth factor (FGF). The material used in this study was formulated into a putty form and seems to be resorbed by a cell-mediated process of natural bone remodeling, whose rate depends on the porosity of the particles, and the surface area and purity of the material (6,8-12).

The purpose of this study was to evaluate *in vivo* the osteogenic potential of two commercially available bone grafting materials, a synthetic graft substitute (Fortoss® Vital) and a human bone derivative (Grafton® DBM putty), in relation to natural bone healing in surgically prepared mandibular osseous defects in rabbits.

Materials and Methods

Animals used

Sixteen adult male New Zealand White rabbits, each weighing 3 kg (± 250 g), were used in this study with the approval of the Institutional Animal Care and Use Committee of the Veterinary Department of Athens Prefecture (permit no K/765.2003). The animals were fed a balanced rabbit diet and caged individually in a standard manner at the animal research facility "N. S. Christeas", Medical School, University of Athens. All animals were allowed seven days from their arrival at the facility for acclimation to their new environment.

Surgical protocol

After acclimation, the surgical protocol was initiated. Anesthesia, surgical procedures, post-surgical care, and animal sacrifice were performed in the animal research facility under standard aseptic conditions. The animals were divided according to the implanted material into two groups, group A (β-TCP/HS) and group B (human DBM Putty), with 8 animals in each group.

The animals were sedated with an intramuscular injection of 25 mg/kg body weight ketamine hydrochloride (Imalgene® 100 mg/ml) plus 5 mg/kg body weight xylazine hydrochloride (Rompun® 20 mg/ml) and subsequently intubated. General anesthesia was induced with Propofol® 25 mg/kg given intravenously, under standard monitoring. Postoperatively and for 2 days, all the animals were given an intramuscular injection of long-acting antibiotics (Zinacef® 750 mg) and an analgesic suppository of Paracetamol.

The edentulous alveolar ridges of the body of the mandible between the incisor and the first molar on both sides of each animal were selected for the surgical procedure. Full mucoperiosteal flaps were raised intraorally on both sides of the mandible in each animal. Osseous defects measuring 8 mm length × 3 mm width × 3 mm depth were prepared in the edentulous left and right regions of the alveolar ridges of the mandible, using a sterile round burr 3 mm in diameter under water cooling (Fig. 1). One randomly selected osseous defect in each animal was washed out with sterile saline and left unfilled, while the defect on the opposite side was filled with β-TCP/HS in group A and with human DBM putty in group B (Figs. 2a, b). The wounds were closed primarily with resorbable 4-0 suture (Vicryl, Ethicon). Each grafting material was

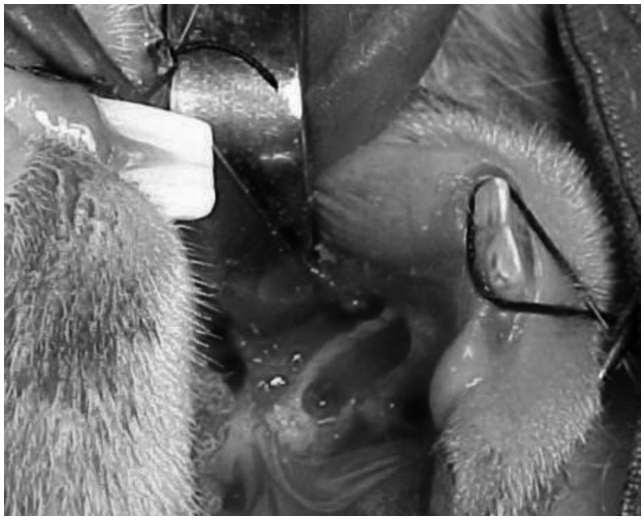


Fig. 1 Clinical view of the surgically prepared osseous defect.

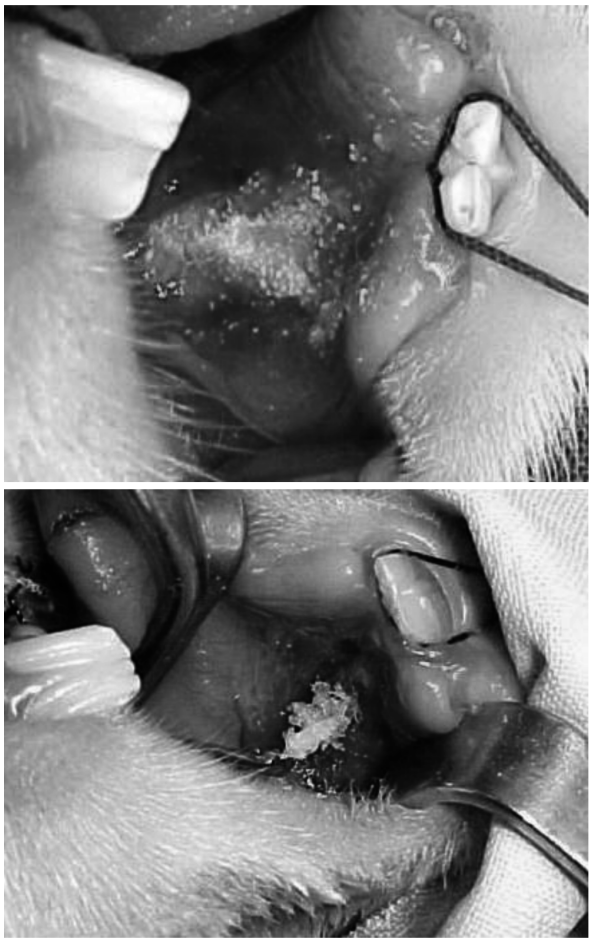
used to fill 8 bone cavities in different animals, resulting in 16 active osseous cavities, while 16 unfilled cavities comprised the control site group.

Two animals from each group were allocated to subgroups of different healing periods: 1, 3, 5 and 6 weeks after the surgery. At the end of the observation period, the animals were sedated as described above, and then sacrificed by an overdose of sodium thiopental (Pentothal®).

Histological analysis

At sacrifice, the mandible of each animal was removed and blocks of bone were taken, containing the graft sites and the control osteotomy sites. All specimens were labeled and fixed in 10% buffered formalin for 24 hours.

After fixation, specimens were decalcified in Biodeck-R rapid decalcifying solution (Bio-Optica, Milan, Italy), embedded in a paraffin wax and cut into slices 5 μm thick using a rotary microtome. The sections were stained with hematoxylin and eosin, and all were examined histologically by light microscopy in a blinded manner.

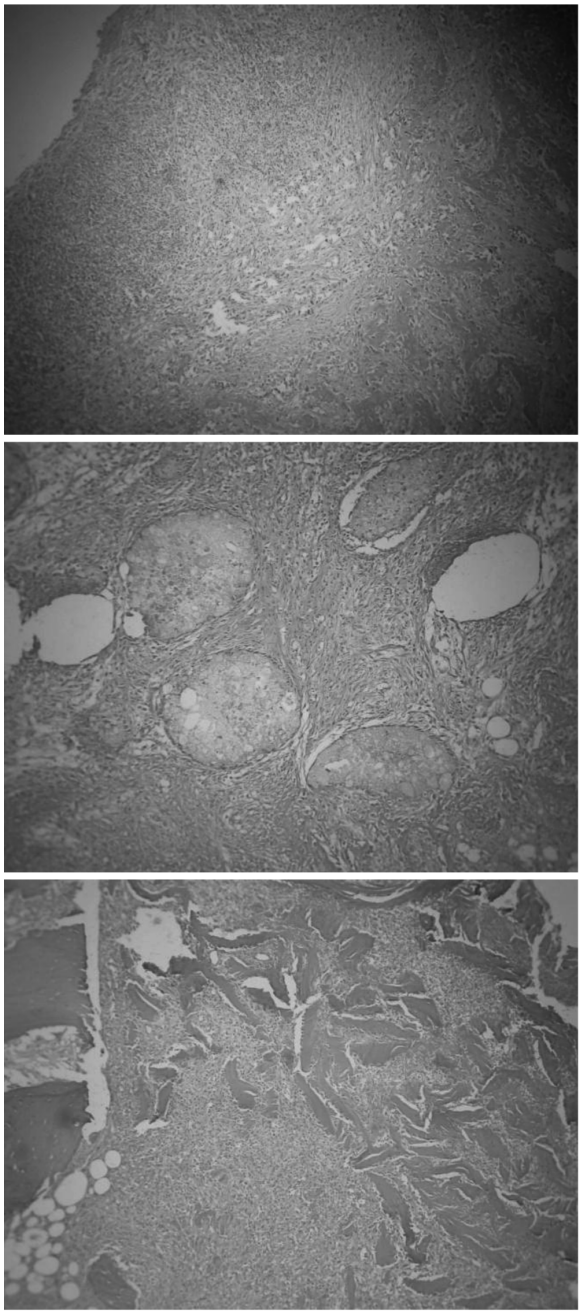


(a) **(b)**

Fig. 2 (a) An osseous defect grafted with Fortoss® Vital. (b) An osseous defect grafted with Grafton® DBM putty.

Results

At the end of the first week, the osseous defects in all groups were filled with granulation tissue, i.e. cellular and vascular connective tissue with dense inflammatory infiltrates and hemorrhagic foci (Figs. 3a, b, c). In addition,



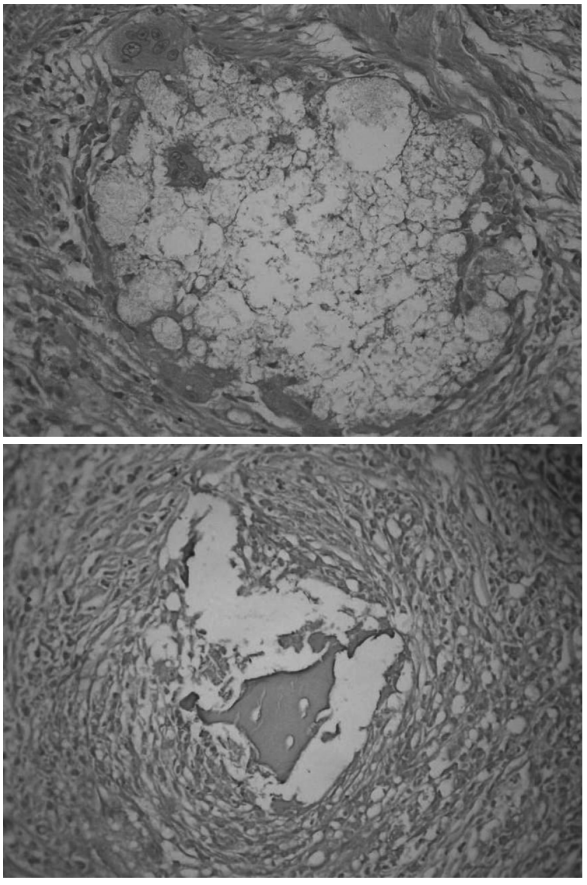
(a) **(b)** **(c)**

Fig. 3 First week: the osseous defects are filled with granulation tissue. (a) Control group. (b) Group A (Fortoss® Vital): grafting material appears as lightly stained basophilic masses. (c) Group B (Grafton® DBM Putty): grafting material appears as acellular bone fragments (hematoxylin and eosin, original magnification ×100).

the grafting material was evident in the form of lightly stained basophilic masses in group A (β-TCP/HS), and as bone fragments in group B (DBM putty). The grafting material was surrounded by epithelioid and giant cells in group A (Fig. 4a) and by granulation tissue heavily infiltrated by lympho-plasmacytes in group B (Fig. 4b). These reactions were a constant feature at all stages of the experiment.

At the end of the 3rd week, woven and mature bone trabeculae filled the osseous cavities in the control group. In group A, woven bone formation was evident in the connective tissue filling the cavities, along with grafting material. In group B, however, the bone cavities were filled with vascular connective tissue, grafting material, small trabeculae of osteoid, and focal inflammatory infiltrates (Figs. 5a, b, c).

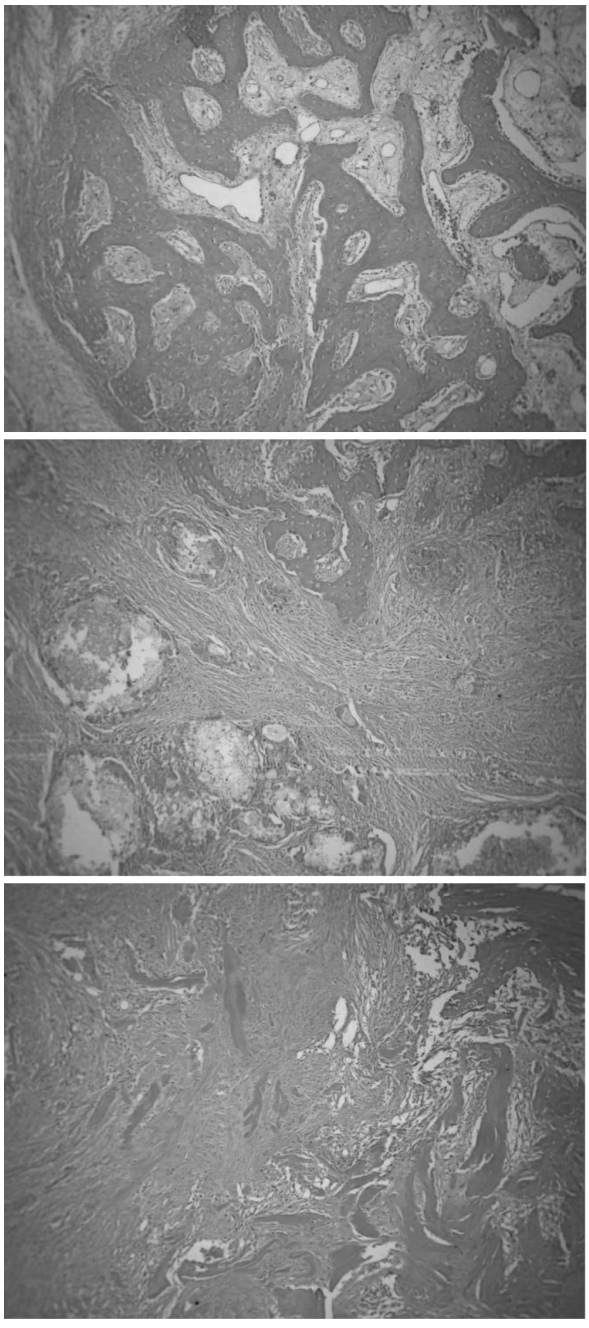
At the end of the 5th week, the osseous defects in the control group were filled with mature cellular bone and adipose marrow. The histological features in the healing areas were identical to those of adjacent bone not affected by the surgical procedure. In group A, the defects were filled with newly formed and mature bone surrounding scattered granules of the material (Fig. 6). In group B, the



(a)

(b)

Fig. 4 (a) Fortoss® Vital grafting material is surrounded by epithelioid and giant cells. (b) Grafton® DBM putty is surrounded by granulation tissue heavily infiltrated by lympho-plasmacytes (hematoxylin and eosin, original magnification ×400).



(a)

(b)

(c)

Fig. 5 Third week: (a) Control group: woven and mature bone trabeculae fill the osseous cavity. (b) Group A: new bone formation is evident in the connective tissue filling the cavities, along with the grafting material. (c) Group B: the bone cavities are filled with vascular connective tissue. Grafting material, small trabeculae of osteoid and focal inflammatory infiltrates are evident (hematoxylin and eosin, original magnification ×100).

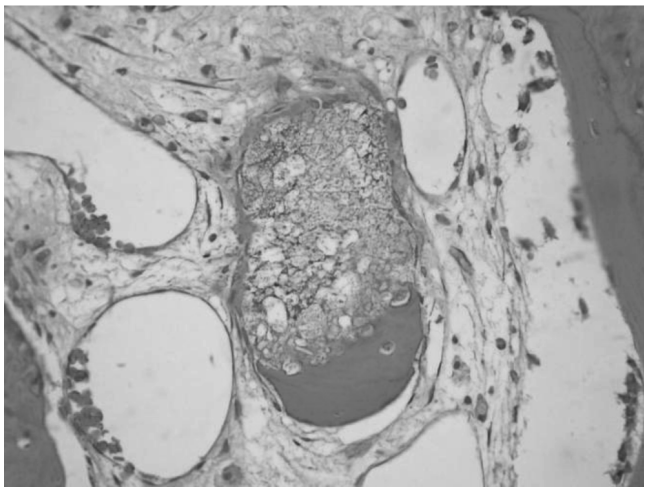


Fig. 6 Newly formed and mature bone surrounding scattered granules of Fortoss® Vital grafting material (hematoxylin and eosin, original magnification ×200).

bone cavities were filled with woven and mature cellular bone with fibrous and adipose marrow, while traces of the material were not evident (Figs. 7a, b, c).

At the end of the 6th week, the osseous defects in all groups were filled with mature bone with fibrous and adipose marrow. Residual granules of the grafting material were still apparent in group A (Figs. 8a, b, c).

Discussion

The purpose of this study was to investigate the osteogenic activity of two bone graft substitutes of different sources; a synthetic β-TCP in a hydroxyl sulphate matrix, and a human DBM putty. The healing course of osseous defects prepared in the mandible of rabbits filled with these materials was evaluated by histological examination (H&E) in relation to the natural bone healing of similar osseous defects.

As this experiment was conducted in an osteogenic environment, it was not possible to confirm whether any of the materials used had osteoinductive potential. Therefore, we were unable to conclude whether the new bone formation occurred as a direct result of the osteoinductive effect of β-TCP/HS and DBM putty, or whether these bone grafting materials promoted osteogenesis merely by functioning as a passive osteoconductive scaffold. The process of osteoinduction, described by Urist 40 years ago (13), can be proven only at extraskeletal sites without surrounding bone (3,6).

In our study, the osseous defects that were left untreated (control group) had become completely filled with mature bone as soon as the end of the 3rd week, earlier than in

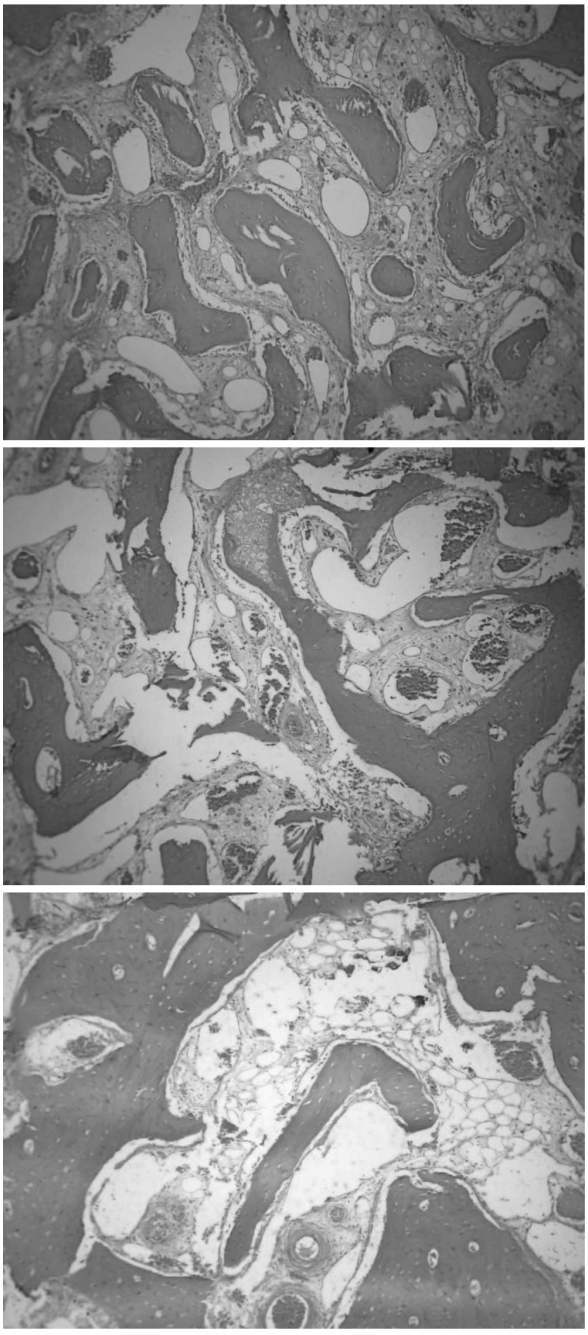


Fig. 7 Fifth week: (a) Control group: osseous defects are filled with mature bone along with fibrous and adipose marrow. (b) Group A: woven and mature bone. Scattered granules of the material are evident. (c) Group B: woven and mature cellular bone with fibrous and adipose marrow (hematoxylin and eosin, original magnification ×100).

the β-TCP/HS and DBM groups. This observation may have been attributable to the limited dimensions of the prepared osseous cavities ($8 \times 3 \times 3$ mm) which could not be considered as "critical size" defects (14-16). The

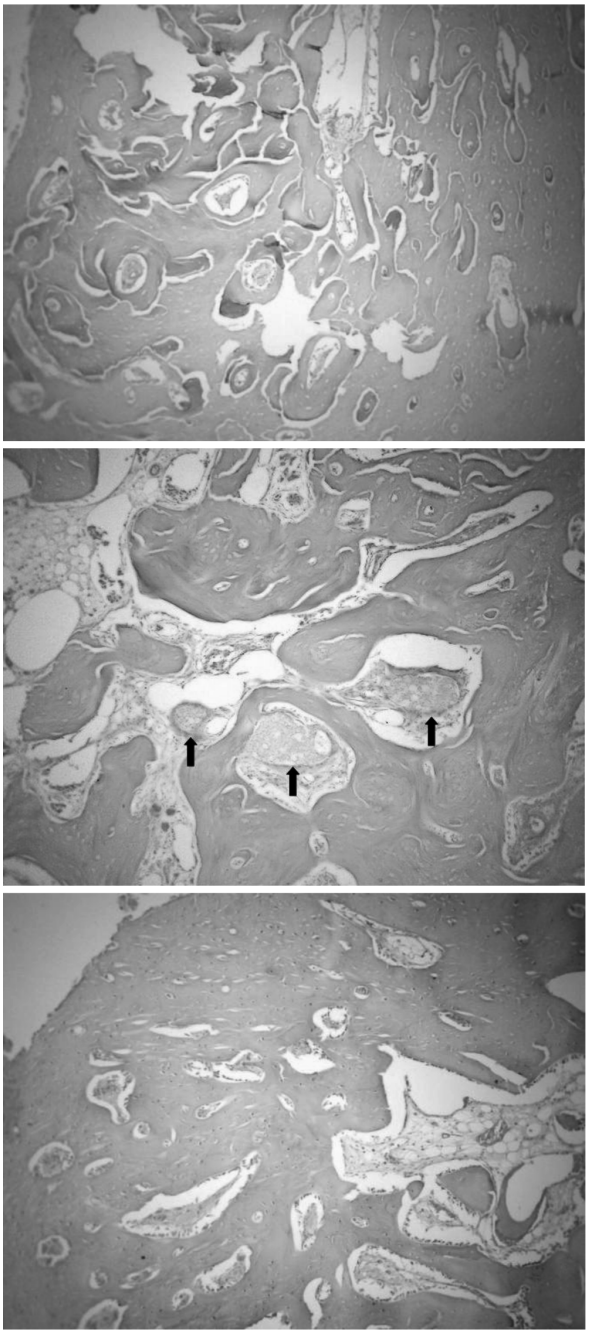


Fig. 8 Sixth week: osseous defects in all groups are filled with mature bone along with fibrous and adipose marrow. (a) Control group. (b) Group A. Residual granules of the material are still apparent (arrows). (c) Group B (hematoxylin and eosin, original magnification ×100).

anatomical topography and the limited size of the rabbit mandible rendered the preparation of "critical size" defects impossible. Despite this limitation, we chose this experimental site because the rabbit mandible has never been used in an experimental setting for the bone grafting materials we tested, and the intra-oral surgical access employed in this surgical protocol simulates, to some extent, conditions encountered in clinical practice. Furthermore, the rapid course of healing in the control group may have partly reflected the higher regeneration rate of physiologic bone in this rabbit experimental model and the slow resorption rate of the grafting materials, which are designed to act mainly as a slowly degrading biological scaffold in human osseous defects (5,6).

Few experimental data are available for Fortoss® Vital (β-TCP in a hydroxyl sulphate matrix). Podaropoulos et al. (17) showed that Fortoss® Vital produced significantly more vital new bone with preserved bone dimensions in comparison with β-TCP alone, when implanted in surgically created osseous defects on the iliac crest of dogs. Fujita et al. (18) implanted β-TCP in the parietal bone of rats and found that new bone was produced in contact with the surface of the material. The β-TCP block has a characteristic basophilic reticular structure in which the material becomes gradually dissolved. Chazono et al. (19) implanted β-TCP into cavities drilled in rabbit femoral condyles, and found that β-TCP supports bone ingrowth, during gradual bioresorption of the material. Kondo et al. (20) examined the chronological histology associated with highly purified β-TCP implanted in rat femoral condyles and found that the material had good biocompatibility, since both bioresorption and bone formation started at an early stage after implantation. In addition, Reinhardt and Kreusser (21) and Szabó et al. (22), using β-TCP for sinus lifting, found that the material was completely resorbed and replaced by new bone, whereas Zijderveld et al. (23) in a similar study observed various amounts of remnant β-TCP in grafts after 6 months.

Our histological findings demonstrated that in group A (β-TCP/HS) by the end of the 5th week the osseous cavities were occupied mostly by newly formed woven bone and in some areas by mature lamellar bone. At this stage masses of the material were still apparent. These granules seemed to be gradually replaced by ingrowth of bone tissue. By the end of the 6-week observation period, only few residual traces of β-TCP/HS were detectable, and most of the material had been replaced by creeping lamellar mature bone. The constant presence of scattered deposits of β-TCP/HS by the end of the observation period may be attributable to the properties of the material. In general, the rate of β-TCP resorption varies and appears to depend greatly on the material's chemical structure, porosity and particle size (18,19).

With regard to DBM, Chesmel et al. (24) showed that human DBM putty performed as well as an autogenous graft in critical size cranial defects in athymic rats. Garcia and Barbosa (25) evaluated the bone repair process of

DBM histologically in rabbit calvaria surgical defects, and found that repair was enhanced in cavities filled with bovine DBM relative to cavities that were left untreated. Torricelli et al. (26) prepared bone defects in the distal femoral condyles of Wistar rats and demonstrated histologically a significant increase in the formation of newly mineralized bone when DBM was employed, in comparison to control sites.

In our experiment, by the end of the 3rd week, masses of human DBM putty were still present, surrounded by woven and lamellar bone, while at the end of 5th week the material seemed to have been totally resorbed, in contrast to β-TCP/HS, which persisted until the end of the experimental period. At the end of the 6th week, mature bone tissue completely occupied the defects. A noticeable difference from β-TCP/HS was that an inflammatory reaction was still present at the end of the 3rd week.

The resorption of human DBM putty after 5 weeks could be explained by the rapid phagocytosis of the collagenous matrix of the material, while the mineral scaffold in combination with the hydroxyl sulphate matrix of β-TCP/HS obviously requires more time to be resorbed by phagocytosis and enzymatic hydrolysis. However, the slower and gradual resorption of β-TCP/HS secures the course of bone healing, as the material acts as a barrier to unwanted ingrowth of soft tissues into the osseous defects. This could be beneficial for large "critical size" bone defects, which do not have the potential to heal spontaneously when left untreated (14,15,27,28).

In conclusion, this study has confirmed that Fortoss® Vital and Grafton® DBM putty bone graft substitutes possess osteogenic activity and can support new bone formation in surgically prepared rabbit mandibular osseous defects measuring $8 \times 3 \times 3$ mm, in relation to the chemical and physical characteristics of each material, although at a slower rate than the spontaneous healing observed in untreated control defects.

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