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Response to Reviewers:

Dear Reviewer#1

We wish thank you for your kind suggestions - improvements for this manuscript. All modifications were made, ie: the abstract contains now more relative information and details and more recent (2012 and 2013)articles were included for comparison with the results of the present study while minor improvements were made in the general design of the "discussion" section according to your concept "not to resemble a literature review".

Dear Reviewer#2
We wish to thank you with much appreciation for your positive comments.
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Alexander Veis, DDS, PhD, Assistant Professor, Department of Dentoalveolar Surgery, Surgical Implantology & Radiology Dental School, Aristotle University of Thessaloniki, Greece

Nikolaos Dabarakis, DDS, PhD, Assistant Professor, Department of Dentoalveolar Surgery, Surgical Implantology & Radiology, Dental School, Aristotle University of Thessaloniki, Greece

Christos Koutrogiannis, DDS, MSc, Dentoalveolar Surgery, Private Practice

Irodis Barlas, DDS, Postgraduate Student, Private Practice

Elina Petsa, DDS, Postgraduate Student, Private Practice

Georgios Romanos, DDS, PhD, Prof. Dr. med.dent.

Professor and Associate Dean for Clinical Affairs, Stony Brook University, School of Dental Medicine, Stony Brook, New York, USA

Corresponding author: Alexander Veis, 5 Theochari St., 54621 Thessaloniki, Greece; Tel: +302310269079; Email: aveis@dent.auth.gr
Abstract
The aim of the present study was to evaluate histologically vertical bone regeneration outcomes after using bovine bone graft material in block and granular forms. The buccal bony plates of the outer mandibles of ten New Zealand rabbits received BioOss® blocks that were immobilized using orthopedic mini-plates and other ten received granular forms that was gently packed and stabilized into the custom-made perforated metallic cubes. The mean graft area (GA), new bone area (NBA), bone-to-graft contact (BGC) and the maximum vertical height reached by the new bone development (MVH) were histometrically evaluated and showed no significant differences between two graft types. The new bone was observed mostly close to the basal bone, and developed penetrating the trabecular scaffold in the form of seams that covered the intra-lumen surfaces of the block type graft while in the granular graft type the new bone was observed to grow between the graft particles usually interconnecting them. Either form of Bio-Oss® was capable of providing considerable vertical bone augmentation.

Key words: block graft; particulate graft; vertical augmentation
INTRODUCTION

The presence of a sufficient volume of healthy bone at recipient sites is a major prerequisite for the success of dental osseointegrated implants. Vertical bone loss after tooth extraction can compromise oral rehabilitation with dental implants. Vertical augmentation of the residual alveolar bone can be achieved using guided bone regeneration, distraction osteogenesis, and fixation of onlay bone grafts. Although autologous onlay block-grafting techniques may provide an initial adequate vertical increase of the residual ridge height, subsequent graft resorption often ensues, compromising the ultimate results. In addition, the morbidity associated with intraoral autograft harvesting and the hospitalization needed to harvest material from extraoral sites has led to the use bone-graft substitutes.

Deproteinized bovine bone mineral (DBBM) (Bio-Oss®, Geistlich Pharma AG, Wolhusen, Switzerland) in granular form has been widely used as a bone-graft substitute in implant dentistry. Numerous experimental animal studies indicate that it may be incorporated into the bone tissue and that intimate contact will be established between the biomaterial and newly formed bone. In a number of studies, granular BioOss® has been suggested for vertical bone augmentation in deficient residual ridges in conjunction with stabilized rigid reinforced membranes or titanium mesh. Bio-Oss® graft in block form has also been proposed for vertical augmentation of deficient residual ridges. The need for membrane placement over the block graft to facilitate bone healing is controversial. A recent animal study by Zecha et al showed no additional bone in-growth after the application of biodegradable membranes over onlay block grafts. Similarly, no
significant differences were found in sites treated with xenograft block material treated with and without collagen membranes.\textsuperscript{7} Findings in agreement with the previous were recorded after the application of non-resorbable barrier membranes on cortico-cancellous human block grafts.\textsuperscript{21}

The aim of the present study was to evaluate histologically and histomorphometrically vertical bone-regeneration outcomes in rabbit mandibles after using bovine bone graft (Bio-Oss\textsuperscript{®}) in block form without a membrane and in granular form covered by a customized metallic membrane.

**MATERIALS AND METHODS**

Twenty male New Zealand rabbits weighing between three and four kilograms were separated into two groups. Ten animals, assigned to group A, received block-form grafts, and the remaining 10 animals (group B) received granular-form grafts in conjunction with custom-made perforated metallic chambers. The surgical protocol was approved by the responsible Animal Committee of the University of Thessaloniki (protocols #119 and #237).

**Surgical Protocol**

The animals were pre-anesthetized with an intramuscular injection of diazepam (1-2mg/kg Stedon\textsuperscript{®} 10mg; Adelco, Chromatourgia Athinon, Athens, Greece). After 10 minutes, the animals were anesthetized with an intramuscular injection of ketamine (35mg/kg Imalgene\textsuperscript{®} 1000, Merial, Lyon, France) and xylazine (5mg/kg Rompun\textsuperscript{®}, Bayer AG, Leverkusen, Germany). The surgical area was shaved and disinfected using povidone iodine solution 10\% (Betadine\textsuperscript{®} 10\%, Mundipharma S.A.,
Block versus particulate Bio-Oss

Switzerland). In addition, 1.8ml of a local anesthetic (2% xylocaine/epinephrine 1:80,000, Dentsply, Sankin, Tokyo, Japan) was used to enhance the anesthetic result.

A horizontal 2-3cm-long incision was then made to the buccal aspect of the mandible. The primary incision included the skin and subcutaneous layer, which were lifted using a pair of tweezers. A second incision followed and was extended deeply through the muscles to the periosteum. The buccal bone of the outer mandible was exposed using a mucoperiosteal elevator. The bony surface was flattened using a round #2 carbide bur under copious irrigation with sterile saline solution. Then, with a drill, screw holes were made for the self-drilling screws used to immobilize the plates and metallic cubes. For the animals in Group A, cubic form block grafts (Bio-Oss® spongiosa block, Geistlich Pharma AG, Wolhusen, Switzerland) were shaped using a scalpel blade to 4x4x4 mm dimensions.

The blocks were placed in direct contact with the host-bone surface (no holes in the basal bone were created) and immobilized using orthopedic mini-plates (straight plate 4-hole, W. Lorenz Surgical, Inc., Jacksonville, FL, BIOMET® MICROFIXATION Anticipate, Innovate™) (Fig. 1 left). The plates were Π-shaped and matched the block dimensions; they were stabilized using screws (Self Drilling Screws, W. Lorenz Surgical, Inc., Jacksonville, FL, BIOMET® MICROFIXATION Anticipate, Innovate™) that were fixed in place using a specific hand screwdriver (Standard Handle και X-lock standard driver, W. Lorenz Surgical, Inc. BIOMET® MICROFIXATION Anticipate, Innovate™). For the animals in group B, the particulate bone graft material (Bio-Oss® Particulate, Geistlich Pharma AG, Wolhusen, Switzerland) was
first irrigated with sterile saline solution and then gently packed into the custom-made perforated metallic cubes (4x4x4mm internally). These were secured over the flattened lateral mandible region with the same screws used to immobilize the orthopedic plates of group A (Fig. 1 right).

The incision was then sutured in layers. The muscles and periosteum were sutured using resorbable Vicryl sutures (Ethicon, Inc., Johnson and Johnson, Somerville, NJ), and the skin was sutured using non-resorbable silk sutures (Medipac, Kilkis, Greece). Subcutaneous injection with the antibiotic enrofloxacin (5mg/kg Baytril® 5%, Bayer, Germany) was administered to the animals for three days post-operatively. The animals were left to recover from the anesthesia in a warm and quiet location and kept for two months in separate cages to heal. During this period, the cages were cleaned every three days, and the animals were fed ad libidum.

After a two-month healing period, the animals were anesthetized and sacrificed using intravenous injection of 10% sodium chloride (DEMO S.A., Athens, Greece). The augmentation sites were retrieved from the mandibles using a mechanical micro-saw. The stabilization plates were unscrewed and removed from the block-graft samples, while the metallic chambers from the group B animals were left in place during the histological preparation procedures.

Histological Preparation
All samples were immersed in formaldehyde solution successively (in 30% for an hour and in 10% for two days) and prepared for non-decalcified histological sections as described by Veis et al.24 The procedure included dehydration of the samples in
ascending grades of alcohol (50%-100%) for six days and successive immersion in ascending series of resin solution (50-100%) (Technovit® 7200, Heraeus Kulzer GmbH, Wehrheim, Germany). The final 100% resin with the embedded specimens was polymerized for 12 hours under 430nm light. A high-precision microtome (Accutom II®, Struers, Copenhagen, Denmark) and grinding devise (DAP-V®, Struers, Copenhagen, Denmark) were used to obtain 50-80μm-thin sections glued on glass slides using a light-cure resin (3M® ceramic primer and Kulzer Technovit® 7210 VLC adhesive). Two central sections were taken from each specimen and stained with Sanderson’s RBS and Trichrome stain.

The histomorphometrical evaluation was made using a light microscope (Axiostar Plus®, Zeiss, Gottingen, Germany). The resulting images were digitized using a digital camera (AxioCam ICc3, Carl Zeiss, Jena, Germany) and a frame grabber and analyzed by the use of appropriate computer software (AxioVision v4.6.3, Carl Zeiss Imaging Systems, Jena, Germany). In each specimen, the percentages of the new bone area (NBA), graft area (GA), bone-to-graft contact (BGC), and maximum vertical height of new bone (MVH) were measured and analyzed statistically. The MVH was expressed as the percentage of maximum coronal extent of new bone and the total vertical length of the augmentation area. The Mann-Whitney U-Test was used for BGC and NBA, where the values did not follow normal distribution and the Independent Samples t-Test was used for GA and MVH with normal distribution values. The statistical level of significance was set to 0.05; the statistical analyses were made using the SPSS 12.0 software (SPSS Inc., Chicago, IL, USA).
RESULTS

All the surgical sites healed uneventfully without exhibiting any kind of dehiscence.

Histological evaluation

Both the block and granular forms of Bio-Oss® were integrated with the host bone. They maintained their original dimensions, and no signs of osteolysis or necrosis within the augmentation area were observed. New bone regeneration could be found in all the histological samples. It was encountered mostly close to the basal bone as well as in the middle of the augmentation area. Different bone-development patterns were observed in the two groups.

In group A (block form; Fig. 2) the new bone derived from the basal native bone (Fig. 3 left: magnification of rectangle A in Fig. 2 left) and was developed penetrating the trabecular scaffold in the form of seams that covered the intra-lumen surfaces of the graft (Fig 3 right: magnification of rectangle in Fig. 2 right) In Group B (granular form; Fig. 4) the new bone was developed as rods and plates interspersed among the graft particles. Osteoblast layers in high activity were developed around the new bone bridges. Osteoid apposition could be seen as reddish layers in contact either with the newly formed bone or directly with the graft material (Fig. 5 left: magnification of Fig. 4 left rectangle). In the specimens from group B, the new bone apposition interconnected the graft particles, as compared with the “flowing” pattern of new bone development on the walls of the lumens within the scaffold of Bio-Oss® block (group A). However, it was not rare even in the group A specimens to observe a “bridging” effect by new bone growth within the graft spaces (Fig. 6 right arrows).
Generally, the closer the proximity to the basal bone, the higher the graft coverage was by new bone. This was notable in both groups.

The new bone patterns developed in various maturation stages. In the early stages, it was found as primary slender spongiosa with large osteocytes (Fig. 5 right and Fig. 6 left arrows) and as woven bone with osteoblasts, osteoclasts, and osteons in the active remodeling phases. In some areas in both groups, the trabecular bridges consisted of woven bone in the center with lamellar development at the periphery, resembling composite bone and revealing the tendency for early maturation of the newly formed bone (Fig. 6 right from group A).

New bone growth was not evident only in the space within the grafted area. In both groups, bony projections were observed starting from the basal bone laterally to the seating borders of the block graft and climbing up to the middle of the block height (Fig. 2 right, arrows and Fig. 4 right, arrows). In some specimens from group B, these bony projections invaded the augmentation area through the lateral holes of the metallic chambers, sometimes in close contact with the metallic walls (Fig. 4 right, arrows). The basal host bone appeared to have a tendency to incorporate the block graft and/or the metallic chamber.

The coronal part of both graft forms was invaded by fibrovascular connective tissue. Although no signs of inflammation were observed, new bone formation in this area was either slender or nonexistent. Only in two samples from group A was new bone formation observed in the coronal region (Fig. 7: magnification of Fig. 2 left, rectangle C). In a few samples from group B, new bone was observed in the coronal
region, but interestingly, it was in contact with the walls of the metallic membrane rather than around the proximal graft particles (Fig. 2 right, arrowheads).

**Histomorphometrical analysis**

The mean graft area (GA) in group A was 37.08 ± 12.96%. It was relatively lower but not statistically significant in group B: 31.74 ± 6.26% ($p=0.212; >0.05$). The mean new bone area (NBA) in group A was 9.68 ± 6.22%, whereas in group B the mean NBA was lower, 5.71 ± 2.83%. The difference was marginally not statistically significant (Mann-Whitney $p=0.065; >0.05$). Following the same pattern, the mean bone-to-graft contact (BGC) in group A was 35.13 ± 28.12%, while in group B it was slightly higher (39.22 ± 22.79%) but still without a statistically insignificant difference (Mann-Whitney $p=0.525; >0.05$). The last measurement was the mean value of the maximum vertical height reached by the new bone development (MVH). In group A, it was 78.78 ± 13.28%. It was slightly higher in group B (83.22 ± 9.28%). This difference was not statistically significant ($p=0.353; >0.05$).

**DISCUSSION**

Rabbit tibia$^{22-24}$ and calvaria$^{21,25}$ have been used as experimental models in numerous studies for testing biomaterials and surgical techniques. However, to approximate conditions in the oral cavity, an experimental rabbit mandible model was used in the present study. A similar experimental model was used in another recent study.$^8$ Although surgical access to the lateral mandible is not as convenient as it is to the tibia and calvaria, after the cortical surface was flattened, it presented a beneficial base for stabilizing materials for further evaluation of the vertical bone
augmentation outcomes. In the present study, the surgical area was covered by the periosteum, muscle layers, and skin, and despite the relative functional pressure during mastication, all the surgical sites healed uneventfully. The instability of the particulate grafting material limits its use for vertical bone augmentation; it requires protection by biological barriers or the presence of bone walls. A solid metallic chamber thus was used in this study to achieve both accommodation of the particulate material and comparable dimensions with the block grafts. The block grafts were stabilized using Π-shaped orthopedic plates instead of placing screws in small size block grafts. The brittle consistency of the block grafts has been shown to compromise stability when bone screws are used.

Standard parameters (new bone area (NBA), graft area (GA), bone-to-graft contact (BGC), and maximum vertical height (MVH)) were used to assess the augmentation outcomes and differences between the two graft forms. The experimental protocols were not similar, and no studies were found using the protocol employed in the present study. Thus, the results of the present study were compared with other studies in which the two Bio-Oss® graft forms or other bone grafts were used for vertical or lateral bone augmentation without the immediate placement of dental implants.

A. Comparison with studies using block type grafts.
Schwarz et al. compared similar Bio-Oss® graft forms in dogs either alone or in conjunction with rhBMP-2 and rhDGF-5 growth factors, however, the authors attempted lateral instead of vertical bone augmentation and their measurements were reported in absolute mm² instead of percentages. Their results nonetheless
agree with the present study in that comparable osteogenic outcomes were found for the two graft forms. As in the present study, they showed trabeculae of woven bone were found deriving from open marrow spaces of the adjacent alveolar basal bone but no obvious differences were reported with respect to the bone-regeneration patterns for the two graft forms. In contrast, the present study clearly showed that new bone development interconnected the interspersed granular graft particles, as compared with the flowing pattern of new bone covering the walls of the lumens and resembling a seam within the scaffold of Bio-Oss® block graft.

Kim et al\textsuperscript{21} used 4mm-high Bio-Oss® collagen block graft stabilized with bone screws for vertical bone augmentation in rabbit calvaria. They found lower values for the vertical bone gain (1.88mm) and percentage of new bone fill (4.89%), as compared with the present study where the maximum gain in height was about 3mm and the percentage of new bone fill was 9.68 ± 6.22% in group A. However, the two studies differed in the region augmented (calvaria instead of mandible), the graft stabilization method (screws instead of plates), and the consistency of the block grafts (Bio-Oss® collagen instead of cancellous Bio-Oss® alone).

Araujo et al\textsuperscript{18} compared Bio-Oss® block grafts with similar autologous bone blocks for lateral ridge augmentation in dogs. The blocks were cylinder-shaped, 8mm in diameter and 3mm thick. Their observations agreed with those in the present study in that the dimensions of the Bio-Oss® block graft remained unchanged, and the coronal region was filled only with small amounts of spots of new bone and basically connective tissue mentioning that the major quantity of new bone was observed close to the basal host bone. In addition, they found higher new bone development (23%) within the graft framework, but this difference can be attributed to the higher
observation period (6 months), the standardized defects, the location where the blocks were placed, and the use of dogs instead of rabbits as experimental animals. In agreement with the present study were the results published recently by Schmitt et al\textsuperscript{30} who tested vertical bone augmentation in sheeps using Bio-Oss block type grafts alone or in combination with specific growth factors. They found 10.02%±5.43 new bone formation in Bio-Oss alone group as compared to 9.68 ± 6.22% in the present study and the interesting point was that the addition of growth factors had no any promotional effect in new bone formation.

B. Comparison with studies using granular type grafts.

The coverage of a membrane is normally needed to contain granular form Bio-Oss or other granular bone grafts\textsuperscript{31} in clinical studies\textsuperscript{13,15,16}. However, in animal studies, metallic cylinders, chambers, domes or other rigid materials were used as membranes to accommodate particulate bone grafts instead of membranes\textsuperscript{25,32,33}. Torres et al\textsuperscript{25} used metallic cylinders 4mm in height in rabbit calvarias to evaluate vertical bone augmentation with particulate Anorganic Bovine Bone (ABB) alone or in combination with PRP. Their findings for the ABB-only group were similar to those of the present study. In a recent study Dung SZ and Tu YK\textsuperscript{31} used caps to cover granular alloplastic HA in rabbit calvaria and found lower mean vertical new bone gain (from 75% to 50%) in comparison to the mean 83.22 ± 9.28% of relative measurement found in the present study indicating the higher osteogenic capability of Bio-Oss bone graft. Another similar research by Zigdon et al\textsuperscript{33} using gold domes secured on rat calvaria showed different results presenting the Bio-Oss Collagen being significantly less osteogenic as compared either with β-TCP, or the granular Bio-Oss in the present study. It is interesting to notice that in two studies both
Block versus particulate Bio-Oss

block\textsuperscript{21} and granular\textsuperscript{33} types of Collagen Bio-Oss present lower osteogenic capability as compared to normal Bio-Oss graft types evaluated in the present study. As in the previous studies, in this study a specific cribiform metallic cube-shaped chamber was used as a membrane to accommodate the particulate graft. On the contrary, the block-form bone grafts were placed without membrane coverage since its use is controversial. It has been claimed that suturing of the thin mucoperiosteal flap on top of the membrane over the block graft may compromise the vascular supply development both into the graft mass and in the tissue flaps, preventing them from attaching to the underlying bone graft during healing.\textsuperscript{28,29} Evaluation of bone-augmentation outcomes after use of the same block graft in conjunction with a membrane presents a topic for further research.

CONCLUSIONS

Within the limits of this study, the use of both block and particulate de-proteinized bovine bone mineral graft resulted in considerable vertical bone augmentation outcomes. The mean values of GA (graft area) and NBA (new bone area) were slightly higher in the block-form group, while the mean values for the MVH (maximum vertical height) and BGC (bone-to-graft contact) were slightly higher in the particulate group. However, no statistically significant differences were found between the two groups.

ACKNOWLEDGMENTS

The study was self-supported, but Arriani Pharmaceuticals S.A. provided materials (Bio-Oss block and granular grafting material and straight plate 4-hole BIOMET\textsuperscript{®} MICROFIXATION Anticipate, Innovate\textsuperscript{TM}) that were used in the study.
REFERENCES


Figure captions

Fig. 1 left: The block-form bone graft was placed in direct contact with the bone surface and was immobilized using Π-shaped orthopedic mini-plates and bone screws.

Fig. 1 right: The particulate-form bone graft was in the custom-made perforated metallic cubes secured with bone screws.
Fig. 2: Histological samples of group A. General view of the new bone regeneration. It was derived from the basal native bone (left) and encountered mostly close to the basal bone as well as in the middle of the augmentation area (right) in the form of flowing patterns of new bone covering the walls of the lumens within the scaffold of Bio-Oss® block. (Original magnification X 10 and stained with Sanderson’s RBS.)

Fig. 3 left: In this magnification of rectangle A in Fig. 2, new bone derived from the basal native bone and extending coronally within the framework of the block graft can be seen.

Fig. 3 right: Magnification of the rectangle in the right section of Fig. 2. The current view is located in the middle region. New woven bone can be seen in close contact with the intra-lumen spaces of the Bio-Oss® scaffold. (Original magnification X 40 and stained with Sanderson’s RBS.)
Fig. 4: Histological samples of group B, which used the granular form of Bio-Oss® bone graft. New bone was developed as rods and plates among the interspersed graft particles. (Original magnification X 10. Trichrome stain on the left and Sanderson’s RBS on the right.)

Fig. 5 left: Magnification of group B samples. This magnification of the rectangle in the middle region of the left part of Fig. 4 reveals new bone growth interconnecting the graft particles. Osteoblast layers in high activity were developed around the new bone bridges (black arrows). Osteoid apposition can be seen as reddish layers in
contact either with the newly formed bone or directly with the graft material. (Original magnification X 40, Trichrome stain left.)

Fig. 5 right: Slender spongiosa with large size osteocytes characterize woven bone in the early maturation stage. (Original magnification X 40, Sanderson’s RBS.)

Fig. 6 left: In histological samples from group A (block form bone graft), a “bridging” effect of the new bone growth could be seen within the graft trabeculae. A clear bridge consisting of composite new bone was developed interconnecting two proximal edges of the graft scaffold.

Fig. 6 right: Woven bone with numerous large size osteocytes in early development stage can be seen among proximal graft trabeculae. (Original magnification X 40, stained with Sanderson’s RBS.)
Fig. 7: Magnification of rectangle C in the coronal region of Fig. 2 left. A rare observation of new bone growth at the coronal region of a block form graft consisting of composite bone i.e. areas of lamellar and woven bone in close contact with the trabeculae walls of the block graft framework. (Original magnification X 40, stained with Sanderson's RBS.)