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Bone augmentation in rabbit calvariae: comparative study between Bio-Oss® and a novel β-TCP/DCPD granulate

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Abstract

Aim: In the present in vivo study, we compare the bone regeneration capacity of a novel brushite cement synthesized in our laboratory (DTG) with Bio-Oss® using rabbits as an animal model.

Methods: The study was performed in a group of 14 adult New Zealand rabbits using the bone conduction model. Two titanium cylinders were fixed into perforated slits made on the parietal cortical bone of each rabbit. One cylinder was left empty (negative control) and the other was filled with either Bio-Oss® or brushite set-cement granules (test cylinder). Four weeks after the intervention, the animals were sacrificed and biopsies were taken. The following parameters were analysed: bone tissue augmentation, bone mineral density and biomaterial resorption. The comparison of data between the different groups was performed using the Mann–Whitney test with a significance level of $p < 0.05$.

Results: The mean bone mineral density and augmented mineral tissue inside the test cylinders were similar but higher than those of negative controls. Material resorption and bone tissue augmentation were significantly higher in the defects treated with the brushite-based set cement ($p < 0.05$).

Conclusions: Brushite cement granules were more resorbable and generated more bone tissue than Bio-Oss® inside the titanium cylinders placed in the rabbit calvaria.

Key words: Bio-Oss®; Brushite; β-tricalcium phosphate; glycolic acid; titanium bone cylinder; vertical bone augmentation

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Bone regeneration techniques constitute a valid surgical procedure for increasing bone quantity and quality in areas where insufficient bone volume prevents the stabilization of osteointegrated implants. Biomaterials for stimulating osseous regeneration should combine osteogenic, osteoinductive and osteoconductive properties. Besides, they should be resorbed and gradually replaced by newly formed bone (Giannoudis et al. 2005). The use of several bone substitutes has been described for bone regeneration but so far, only autografts (autologous bone grafts) re-unite all the mentioned properties, being the most suitable material. However, the limited availability of autografts in intra-oral areas and the post-operative morbidity associated with the use of extra-oral grafts forces the physicians to use other biomaterials in bone regeneration (Block & Kent 1997).

An alternative to autografts are allogenic biomaterials (grafts from another individual of the same species), such as human demineralized freeze-dried bone, provided by tissue banks. Unfortunately, there is some controversy regarding the osteoinductive capability of these materials (Schwartz et al. 1998); besides, they have the risk of immunological rejection and transmission of infections such as HIV and hepatitis that requires special manufacture measurements (Giannoudis et al. 2005). Currently, only bone morphogenetic proteins (BMP) seem to have osteoinductive properties but their use for dental practice is still in the experimental phase (Nevins 1996).

The osteoconductive properties offered by natural bone substitutes from animal origin, such as collagens and bovine hydroxyapatite Bio-Oss® (Geistlich Biomaterials, Wolhusen, Switzerland) overcome some of the autografts' limitations (Von Arx et al. 2001). For instance, Bio-Oss® chemical composition is very

similar to that of human bone hydroxyapatite (HA) as it contains a calcium/phosphate proportion of 1.67 identical to bone HA (Suzuki et al. 2000). Besides, its mineral matrix contains crystals of *ca.* 100 μm diameter, presenting morphological and structural properties very similar to those of the human bone (Rosen et al. 2002). Furthermore, Bio-Oss® rough topography favours osteoblastic anchorage, proliferation and synthesis of bone matrix on its surface (Acil et al. 2000), and is currently one of the most frequently used biomaterials in bone regenerative procedures. However, HA-based biomaterials are very slowly resorbed in vivo.

Third-generation biomaterials are designed with the aim to aid the body in self-healing. One desirable characteristic in bone materials is their ability to be remodelled, i.e. the biomaterial is resorbed by osteoclasts and subsequently replaced by newly formed bone through osteoblastic activity (Schilling et al. 2004). Biomaterials with a slow resorption rate, such as HA, interfere with bone growth, while biomaterials with a fast resorption rate, such as calcium sulphates, compromise the stability of the surgical site during the healing process (Stavropoulos et al. 2004, Lieberman et al. 2005). New biomaterials are designed to have resorption speeds that match bone growth rate. It is to be noted that brushite crystals have an in vivo resorption rate similar to human bone growth speed, *ca.* 20 μm/day, and this fact can be very important in stabilizing the newly formed bone. (Bohner et al. 2005).

Synthetic materials, such as β-tricalcium phosphate (β-TCP) and dicalcium phosphate dehydrate (brushite or DCPD) raise great interest as they are cheap, do not present immunologic or infectious problems (Giannoudis et al. 2005) and have a higher resorption rate in vivo than HA materials, allowing bone formation simultaneously with material resorption (Chow et al. 2003, Trisi et al. 2003).

In 1987, Mirtchi and Lemaitre introduced the first cement made from DCDP and β-TCP obtained from the reaction of a base (β-TCP) and an acid (monocalcium phosphate). The set cement composition presented a mixture of two minerals: β-TCP and DCDP (Mirtchi & Lemaitre 1989). The significance of this cement lies in its capacity to decompose in physiological environments and be resorbed by the body. Investigations performed on this cement showed that DCDP is resorbed up to three times faster than HA or β-TCP (Chow et al. 2003). This property seems to prove its feasibility in accelerating the substitution of the biomaterial by newly formed bone. Surprisingly, this biomaterial has barely been studied for dental implantology purposes, although it has proved to be useful in orthopaedic applications for stabilizing fractures or filling defects (Bohner et al. 2005).

Most investigations that evaluate the bone-forming capacity of biomaterials are performed in critical size defect (CSD) models (see Fig. 1a). However, in daily clinical practice, bone regeneration is often needed to grow vertically from the surface of the native bone, i.e. a vertical bone augmentation. The evaluation of bone-regeneration is better performed with the bone conduction model (Fig. 1b), introducing barriers in the bone defect that prevent lateral bone formation and allow bone growth only in the vertical direction (Lundgren et al. 1999, 2000).

The purpose of this study was to evaluate the bone-regenerative capacity of a novel brushite/β-TCP pre-set cement in granular form (DTG) and to compare its behaviour with the commercial bovine bone Bio-Oss®. The two biomaterials were implanted in rabbits using a calvaria, a titanium cylinder bone conduction model, and new bone tissue formation was analysed 4 weeks after the intervention.

Material and Methods

Before beginning the ''in vivo'' animal study, the protocol was approved by the ethical committee for animal experiments of the Complutense University of Madrid (UCM). Experiments were conducted in accordance with the guidelines laid down by the European Communities Council Directive of 24 November 1986 (86/609/EEC), and adequate measurements were taken to minimize pain and discomfort in the animals.

The preparation of the new biomaterial is explained elsewhere (Tamimi et al. 2005) but herein a brief description of the synthesis is given (Tamimi et al. 2005). The new biomaterial (DTG) was made from a brushite cement composed of monocalcium phosphate (0.8 g) and β-TCP (1.4 g) that sets in a glycolic acid aqueous solution (1 M) using a powder to liquid ratio of 1.7 g/l. The cement was left to set ex vivo into a hard material that was milled with a mortar and sieved to obtain granules with diameter ranging between 0.2 and 1.0 mm. The final composition of the granules was 87% DCPD and 17% β-TCP (Tamimi et al. 2005). Bovine bone regeneration biomaterial Bio-Oss® was acquired from Geistlich Biomaterials (Baden, Germany). The in vivo study was performed in a rabbit experimental model using a titanium bone conduction cylinder. The titanium cylinders (Apositos Sanitarios Aragoneses, Huesca, Spain) had an inner rough surface with the following dimensions: 4 mm height, 0.5 mm thickness and 9 mm inner diameter.

Fig. 1. Schematic representation of two possible methods for evaluating bone regeneration in vivo: (a) critical-size defect model in which bone regeneration occurs with undefined direction; and (b) bone-conduction chamber model for evaluating true vertical bone augmentation. Bone tissue is represented as the grey background, the directions of growth by the white arrows and the obstacles used for limiting bone growth are shown by the black stripes.

Fourteen healthy 6-month-old female New Zealand rabbits weighing between 3.9 and 4.4 kg were used as experimental animals in order to compare in vivo the bone augmentation capacity of Bio-Oss® with DTG (Tamimi et al. 2005). The animals were accommodated in the official stable for animal assays of the UCM at 22–24°C with 55–70% humidity, light cycles of 12 h, and air renewal 15 times/h. The rabbits were fed with a Panlab® (Panlab S.L., Barcelona, Spain) diet while drinking was permitted *ad libitum*. The rabbits were divided into two groups of seven each, the first group was to be treated with Bio-Oss® (group 1) and the second group with DTG (group 2).

Surgical procedure

The rabbits were anaesthetized with an intra-muscular dose of 0.75 mg/kg ketamine (Imalgene 1000®, Rhone, Merieux, France) and 0.25 mg/kg xilacine (Rompun®, Bayer, Leverkusen, Germany). Animals were placed in *sternal recumbency*, the head was shaved and the cutaneous surface was disinfected with a povidone iod solution before the operation. The calvaria bone was exposed through a skin incision

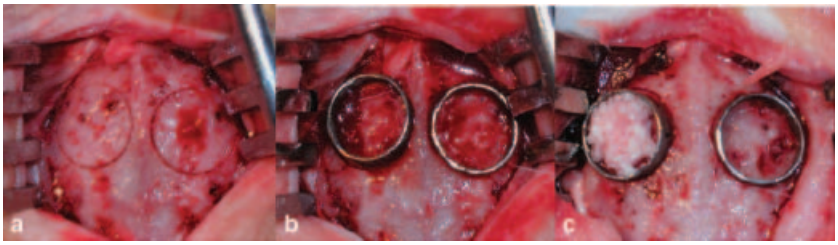

Fig. 2. (a): Photograph of rabbit calvaria with two slits for fixing the titanium cylinders. (b): photograph showing the fixation of the titanium cylinders on the slits. (c): photograph showing the right chamber grafted with Bio-Oss® while the left one was kept un-grafted for negative control.

approximately 4 cm in length over the *linea media*. A pair of tweezers was used to lift the skin before the periostium was also incised in the same place. A periosteal elevator was used for separating the periosteum from the bone surface. Two circular slits (0.5 mm thick × 9 mm inner diameter × 0.5 mm deep) were made in the parietal bone using a trephine on a slow-speed electric handpiece by applying 0.9% physiologic saline irrigation. The slits were made on each side of the median sagittal suture without crossing it. A titanium cylinder barrier was created by mechanically fixing the titanium cylinder on each slit applying slight pressure on it. The bone surface surrounded by the ring was slightly roughened with a rounded burr to promote bleeding (see Fig. 2). The tested biomaterials are stabilized inside the cylinders, and on each rabbit the right cylinder was grafted with 0.25 g of experimental biomaterial (Group 1: Bio-Oss® and group 2: DTG), while the contra-lateral chamber was filled with autologous blood clot as negative control (group 1: control 1 and group 2: control 2). Closure of periosteum and subcutaneous tissues was performed with resorbable Dexon® 3/0 sutures (North Haven, CT, USA), while the skin was relocated with (3/0) silk continuous sutures (Apositos Sanitarios Aragoneses, Huesca, Spain). Post-operative antibiotics were administered, Terramicina® (Pfizer, Madrid, Spain), in water for 7 days. The animals were sacrificed 4 weeks after the intervention with an overdose of sodium pentobarbital IV (Dolethal®; Vetoquinol, Lure, France).

Post-mortem, a surgical burr attached to a slow-speed electrical handpiece was used to harvest the bone blocks containing the titanium conduction cylinders from the animal's calvariae. Samples were then preserved fixed in formaldehyde 10% buffer solution at pH 7.0 before further analysis.

Densitometry

Post-mortem bone mineral density analyses were performed on the calvaria blocks using the XR-26 Norland® Densitometer (Norland Corp.; Fort Atkinson, WI, USA). In each calvaria block, a bone area of $0.5 \times 0.5\,cm^2$ was analysed inside both the experimental (Bio-Oss and DTG) and control (control 1 and control 2) chambers. The exploring resolution was $1.0 \times 1.0\,mm^2$, measuring resolution at $0.5 \times 0.5\,mm^2$ and exploration speed at 40 mm/s. The bone mineral content (BMC) values for the experimental and control samples were obtained from the densitometry analysis and the bone mineral density (BMD) was calculated using the formula:

$$BMD = \frac{BMC}{Area}$$

where the area is always $0.25\,cm^2$, BMC values are expressed in grams and BMD values in g/cm^2.

Histology and histomorphometry

Bone samples were dehydrated in ascending series of alcohol (60–100%), embedded in 2-hidroxy-ethyl-methacrylate, and then photopolymerized 6 h with UV light, 2 h with white light and 6 h with blue light into ready-to-cut sample blocks. A saw microtome Exakt® (Exact GmBH, Norderstedt, Germany) was used to cut coronal sections from the cylinders as described elsewhere (Donath & Breuner 1982, Slotte et al. 2003) 200 μm thick. The sections were then ground to a total thickness of 50–80 μm by means of a grinder Exakt® (Norderstedt) in order to achieve better histological visualization without risking the loss of the samples. Afterwards, surface staining was performed with haematoxylin & eosin (HE) and toluidine blue (TB; Donath & Breuner 1982). The histological evaluation of bone neoformation was carried out by means of optical microscopy. To perform the histomorphometric analysis, light micrographs (at magnification × 6) of the biopsy slices were captured with a digital camera and analysed with the histomorphometry software MIP-4 (Digital Image System, Barcelona, Spain). Six randomly selected slices were analysed for each biopsy. In each section, the area inside the cylinder was included for histomorphometric evaluation while the original cortical bone and the area outside the cylinder were excluded. The already existing bone was lamellar while the regenerated bone was woven and grew inside the cylinder so both types of bone could be easily differentiated in the histological observations.

The following measurements were taken from each cylinder: total sample volume, newly formed bone and remaining graft volume. These data permit to calculate the following parameters: average augmented bone volume formed in the cylinder (BV), volume of the remaining graft material (RG) and augmented mineralized tissue (AMT).

$$BV(\%) = \frac{\text{Newly formed bone volume}}{\text{total sample volume}} \times 100$$

$$RG(\%) = \frac{\text{Remaining graft volume}}{\text{total sample volume}} \times 100$$

$$AMT = BV + RG$$

Statistical analysis

A statistical software package (SPSS 7.0, Chicago, IL, USA) was used to analyse the histomorphometric and densitometry measurements by the Mann–Whitney test. Significance for the analysis was set at $p < 0.05$.

Results

There were no surgical complications during the preparation of the bone conduction cylinder and its filling with the experimental biomaterials. After the intervention, the animals recovered without post-operative signs of infection and no animals were lost during the study.

Densitometry

As shown in Table 1, the chambers grafted with Bio-Oss® and with DTG

had similar BMD values, which are significantly higher than those of the ungrafted negative controls (controls 1 and 2).

Histology

The observation of the histological sections taken from the different groups of bone conduction cylinders showed no inflammatory reaction. In the ungrafted chambers (controls 1 and 2) scarce bone formation activity was observed and only a few short and isolated trabeculae could be distinguished on the external surface of the cortical (Fig. 3). Neither osteoblasts nor osteoclasts were found in both control chambers.

In the chambers grafted with Bio-Oss® (Figs 4 and 5), implanted granules were observed distributed over the whole specimen area. Bio-Oss® was dyed red by HE and greyish with TB. Bio-Oss® particles presented lacunae free from osteocytes due to their animal origin; nevertheless, their artificial shape differentiate them from the bone trabecula growing around them. Bio-Oss® granules were mostly surrounded by a thin layer of fibrous tissue at the medium and upper tiers, and by bone trabeculae at the lower tier (corresponding to the area in contact with the calvaria). Bone regeneration was observed from the external surface of the cortical to approximately 1/3 of the height of the cylinder. However, signs of biomaterial resorption such as phagocytic cells forming Howship's lacunae on its surface, etching, pits or resorptive trail formation could not be identified on Bio-Oss® granules. From this observation, we infer that 4-weeks after implantation in rabbits' calvariae, Bio-Oss® showed osteoconductive properties but resorption did not take place.

In the cylinders grafted with DTG (Figs 6–9), the remaining granules were observed over the whole specimen area. DTG granules were intensely dark brown dyed by HE, while in TB the granules had a light grey colour. DTG particles presented a rounded morphology invaded by neoformed bone trabeculae growing from the external surface of the parietal bone, especially at the lower and medium tiers of the cylinder, and no inflammatory foreign body reaction was observed. Moreover, the surface of the biomaterial showed signs of resorption such as multinuclear/mononuclear phagocytic cells forming Howship's lacunae, surface pitting, resorption trail formation, and the remaining granules being perforated by the new bone (see Fig. 6). This material

Table 1. BMD values of each group

Group	BMD (g/cm^2)
Bio-Oss®	0.32 ± 0.05*
Control 1	0.15 ± 0.02
DTG	0.29 ± 0.07*
Control 2	0.14 ± 0.01

All data presented are mean values (± standard deviation).
*Significantly higher than all control groups ($p < 0.05$).
BMD; Bone mineral density values.

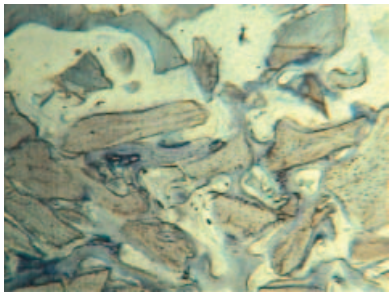

Fig. 4. Light microscope photograph of a toluidine blue-stained section from a bone conduction chamber grafted with Bio-Oss®. The photograph shows newly formed bone growing on the surfaces of Bio-Oss® granules (original magnification × 10).

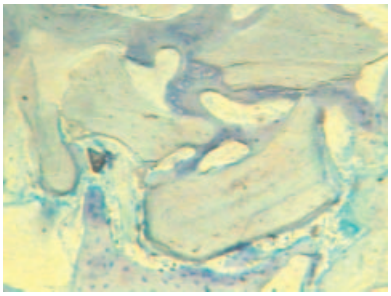

Fig. 5. Light microscope photograph of a toluidine blue-stained section from a bone conduction chamber grafted with Bio-Oss®. The photograph shows bone forming over the Bio-Oss® surface but no resorption pitting is observed on the granules, and their edges remain sharp (original magnification × 20).

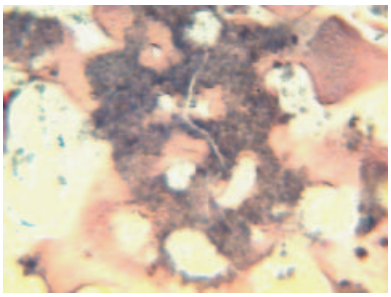

Fig. 6. Light microscope photograph of an haematoxylin & eosin-stained section from rabbit calvaria grafted with DTG. The micrograph shows a DTG granule (black) surrounded and perforated by the newly formed bone (red) (original magnification × 20).

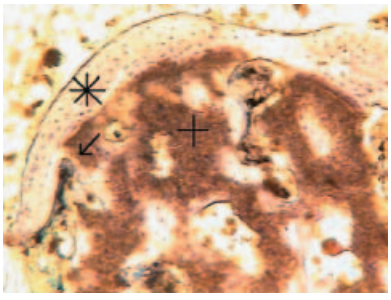

Fig. 7. Light microscope photograph of an haematoxylin & eosin-stained section from rabbit calvaria grafted with DTG. The biomaterial (+) is surrounded by bone tissue (*). Pitting resorption trail formation (arrow), Hawshipapos;s lacunae and rounding of the surface can be observed while the newly formed woven bone grows into the resorbing structure of the graft (original magnification × 20).

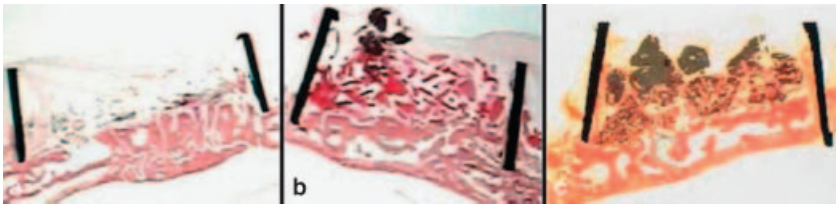

Fig. 3. Light microscope photograph of an haematoxylin & eosin-stained section from rabbit calvaria with titanium cylinders fixed on it (black strips). (a): unfilled cylinder (negative control), (b): cylinders grafted with Bio-Oss® granules. (c): cylinder grafted with DTG granules. The photograph shows newly formed bone growing into the Bio-Oss® and DTG granules, while no bone grows into the unfilled cylinder (original magnification × 2).

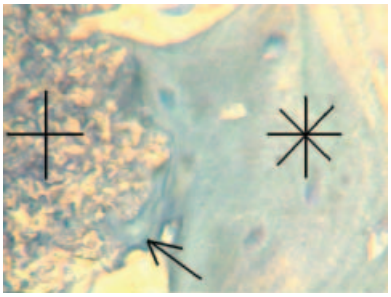

Fig. 8. Light microscope photograph of a toluidine blue-stained section from a bone conduction chamber grafted with the novel biomaterial (DTG). The photograph shows newly formed bone (*) in direct contact with the remaining monoclinic dicalcium phosphate dehydrate crystals (+). The interface between the biomaterial and the bone tissue is rough and a Howshipapos;s lacuna can be observed (arrow; original magnification × 40).

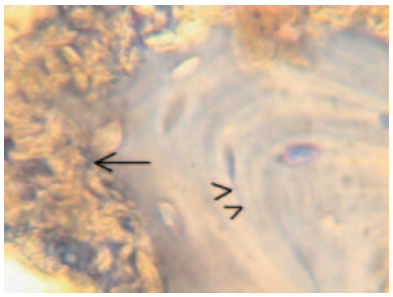

Fig. 9. Light microscope photograph of a toluidine blue-stained section from a bone conduction chamber grafted with the novel biomaterial (DTG). The photograph shows newly formed bone growing in a lamellar orientation (arrow heads) in direct contact with the remaining monoclinic dicalcium phosphate dedydrate crystals (arrow; original magnification × 40).

can be considered osteoconductive and bioresorblable.

Histomorphometry

The data obtained from histomorphometry analysis are shown in Table 2. The BV values are significantly higher for DTG-grafted cylinders, followed by Bio-Oss® cylinders and the negative controls. The RG values revealed that the chambers filled with Bio-Oss® presented less graft resorption than those filled with DTG. AMT values were similar for both Bio-Oss® and DTG cylinders, while negative controls had lower AMT.

Table 2. Results of the histomorphometry measurements

Group	AB (%)	RG (%)	AMT (%)
Bio-Oss®	11.7 ± 2.4*	37.2 ± 5.5	48.9 ± 10.0§
Control 1	5.7 ± 1.0	–	5.7 ± 1.0
DTG	16.7 ± 4.9†	22.4 ± 8.5‡	39.5 ± 4.9
Control 2	5.1 ± 1.1	–	5.1 ± 1.1

All data presented are mean values (± standard deviation).
*Significantly higher than all control groups ($p<0.05$).
†Significantly higher than all the other groups ($p<0.05$).
‡Significantly higher than Bio-Oss® group ($p<0.05$).
§Significantly higher than DTG group ($p<0.05$).
AB, augmented bone tissue; RG, Remaining unresorbed biomaterial; AMT, augmented mineral tissue

Discussion

Several authors consider that a 4-week period of implantation is enough time to observe angiogenesis and bone formation in several animal models, including rabbits, where experimental biomaterials are grafted into bone defects (Schmid et al. 1997, Boo et al. 2002, Herron et al. 2003). In our experiment, the histological changes that occurred in the grafted areas during the 4-weeks of implantation were pronounced and allow to evaluate the differences in bone regeneration capacity of both assayed biomaterials.

BMD values of the Bio-Oss® and DTG grafted cylinders were similar but significantly higher than the BMD values of the negative controls. Even though the densitometry analysis could be biased by the remaining unresorbed granules, the histomorphometric study revealed that in the control groups, no regenerated bone is formed. By contrast, both the Bio-Oss® and DTG groups offered significantly higher neoformed bone percentages.

In this study, there was no need to use histological apposition markers for identifying the newly formed bone because with the titanium cylinder model, the edges of the bone defect were clearly limited by the walls of the titanium cylinder (Slotte et al. 2003). On the other hand, the critical size defect model presents problems in identifying the original edges of the defects and apposition markers, such as tetracycline, are needed for recognizing the newly formed bone (Pautke et al. 2005).

The BV values obtained in samples treated with Bio-Oss® were comparable with that found in the literature (Slotte & Lundgren 1999) where Bio-Oss® was grafted into silicone cylinders on rats' calvariae (18.1%) or in titanium cylinders on rabbitsapos; calvariae (19.9%; Slotte et al. 2003). Nevertheless, the BV value obtained with the DTG granules was significantly higher than that achieved with Bio-Oss®. As the use of Bio-Oss® is widely spread in oral surgery, the result obtained with the DTG granules seems promising and worth continuous investigation (Tamimi et al. 2005).

Bio-Oss® is considered a non-resorbable material because it needs several years (3–6 years) of implantation before showing some slow in vivo resorption through osteoclast activity (Taylor et al. 2002). The presence of unresorbed granules within the newly formed bone is undesirable because it interferes with new bone growth and compromises the properties of the resulting tissue, affecting its osteointegration capacity for dental implants (Duda &Pajak 2004, Stavropoulos et al. 2004, De Boever & De Boever 2005, Zaffe et al. 2005). Other authors claim that biomaterials with slow in vivo resorption can interfere bone growth instead of enhancing it; however, we could not observe this effect in our study because the ungrafted negative control samples always had much less bone augmentation than the samples grafted with the experimental biomaterial (Stavropoulos et al. 2004).

In our study, Bio-Oss® showed no signs of graft resorption, and the biomaterial still occupied the whole area of the cylinder 4 weeks after the intervention. On the other hand, DTG-implanted granules were heavily penetrated by the newly formed bone through pitting and resorption trails confirming its bioresorption properties. This observation was supported by the RG values, which were significantly lower for the DTG grafted granules.

We attribute the high bioresorption of DTG to the presence of DCPD and β-TCP in its composition. β-TCP is moderately resorbable in vivo and needs only 12 weeks to be totally resorbed from bone defects created in animal models such as dogs, and 6–8 months when implanted in humans (Wiltfang et al. 2003, Suba et al. 2004). Furthermore, DCPD can be resorbed in vivo even faster than β-TCP because it is more soluble in water (Chow et al 2003, Herron et al. 2003, Tas & Bhaduri 2004). The combination of these two materials in the form of granules allows the diffusion of the ioninc species as well as the nutrients that would enhance the resorption of the material and the formation of new bone tissue. Bone growth was observed in the interior of our novel biomaterial inside the spaces where brushite resorption had already taken place; DTG appeared to be drilled by new bone formation while in the Bio-Oss® grafts, bone formation took place only around the granules.

This survey showed that both Bio-Oss® and DTG present good osteoconductive properties, achieving acceptable bone augmentation. However, the use of DTG offers faster in vivo resorption and increased bone neoformation when compared with Bio-Oss®.

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Clinical Relevance

Scientific rationale: Bio-Oss® granulate is an osteoconductive material that has been extensively used in bone regeneration despite its low resorption rate in vivo. In this study, we compare the bone augmentation capacity of Bio-Oss® with a brushite-based osteoconductive and bioresorbable material.

Principal findings: The novel biomaterial described in this study produces more vertical bone augmentation than Bio-Oss® and shows bioresortpion already at 4 weeks after implantation while Bio-Oss® remains practically unresorbed.

Practical implications: The novel brushite cement granules can be used for bone regeneration as an alternative to Bio-Oss®.