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ORIGINAL ARTICLE

Histological and ultrastructural evaluation of bone around Bio-Oss® particles in sinus augmentation

G Orsini[1], A Scarano[1], M Degidi[1,2], S Caputi[1], G Iezzi[1], A Piattelli[1]

[1]*Dental School, University of Chieti-Pescara, Chieti, Italy;* [2]*Private Practice, Bologna, Italy*

AIM: The aim of the present study was to evaluate histological and ultrastructural features of bone surrounding Bio-Oss® particles retrieved, in the same patient, 20 months and 7 years after sinus augmentation.

MATERIALS AND METHODS: A 54-year patient who needed sinus elevation before implant rehabilitation participated in this study. Two bone cores at two different times were harvested from a Bio-Oss® regenerated sinus and processed for examination under light and transmission electron microscopy.

RESULTS: Under light microscopy, in the 20-month specimen, most of the particles were surrounded by a thin layer of newly formed bone; in the 7-year specimen there was mainly compact bone in direct contact with the particles. Under transmission electron microscopy, it was possible to characterize the bone–biomaterial interface; in the 20-month specimen an electron-dense layer was seen, whereas, almost no electron-dense lines were seen at the interface in the 7-year specimen.

CONCLUSIONS: Bio-Oss® particles did not interfere with bone-healing processes after sinus augmentation procedures and promoted new bone formation. This study can help clinicians to understand better the morphological characteristics of bone regeneration processes using Bio-Oss® after 20 months and, most importantly, after a longer time of interaction with surrounding tissues.

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Keywords: anorganic bovine bone; bone--biomaterial interface; sinus augmentation procedures; transmission electron microscopy

Introduction

Autogenous grafts as fillers for maxillary sinus augmentation procedures are believed to be the gold standard, but their main disadvantages are a limited availability, a tendency to undergo partial resorption, the need for an additional surgery under general anaesthesia, and the associated morbidity. Among the numerous allografts and xenografts proposed by many investigators, anorganic bovine bone (Bio-Oss®) is one of the most popular biomaterials used for sinus elevation surgery (Valentini and Abensur, 1997; Froum *et al*, 1998; Haas *et al*, 1998; Valentini *et al*, 1998; Piattelli *et al*, 1999; Maiorana *et al*, 2003). The biological interactions occurring at the bone–biomaterial interface are critical for long-term clinical success (Davies, 1996). Bio-Oss® is a xenograft consisting of deproteinized, sterilized bovine bone with 75–80% porosity and a crystal size of approximately 10 μm in the form of cortical granules; it has a natural, nonantigenic porous matrix and is chemically and physically identical to the mineral phase of human bone; it has been reported to be highly osteoconductive and to show a very low resorption rate (Berglundh and Lindhe, 1997; McAllister *et al*, 1998; Piattelli *et al*, 1999; Haas *et al*, 2002; Furst *et al*, 2003; Orsini *et al*, 2005). Different studies have been published about the long-term performance of Bio-Oss® (Hammerle *et al*, 1998; Hallman *et al*, 2001). The duration of resorption and ultimate replacement of graft materials with vital bone is not completely understood (Margolin *et al*, 1998). Understanding the mechanism and rate of resorption of the different biomaterials is of relevance for clinicians (Artzi *et al*, 2000). Some studies have reported signs of resorption of the Bio-Oss® particles (Berglundh and Lindhe, 1997; Hurzeler *et al*, 1997; Hammerle *et al*, 1998; Yildirim *et al*, 2000; Karabuda *et al*, 2001; Sartori *et al*, 2003), whereas others have reported a lack of breakdown (Valentini and Abensur, 1997; Hallman *et al*, 2002; Merkx *et al*, 2003; Schlegel *et al*, 2003). Osteoclasts have been described to be present around Bio-Oss® particles (Hurzeler *et al*, 1997; Karabuda *et al*, 2001; Merkx *et al*, 2003; Stavropoulos *et al*, 2004). Other researchers did not identify osteoclasts (Artzi *et al*, 2001), while still others believe that despite the absence of osteoclastic activity the inward growth of bone could indicate a slow resorption of the xenogenic graft material (Yildirim *et al*, 2000). The potential resorption of Bio-Oss® by osteoclasts could be

Correspondence: Prof. Adriano Piattelli, M.D., D.D.S., Via F. Sciucchi 63, 66100 Chieti, (Italy). Tel: 0039-0871-3554083,
Fax: 0039-0871-3554076, E-mail: apiattelli@unich.it
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confirmed by the progressive increase in relative bone volume over a 10-year period (Sartori *et al*, 2003). Remnants of Bio-Oss® particles have been reported to be present even years after their insertion in bone (Skoglund *et al*, 1997; Piattelli *et al*, 1999; Schlegel *et al*, 2003).

Light microscopy (LM) provides the most important information about the presence of bone or soft tissue contact, but it does not give ultrastructural information about the organization at the interface (Linder, 1985; Ganeles *et al*, 1986; De Lange and Donath, 1989; Steflik *et al*, 1989; Davies *et al*, 1990; Van Blitterswijk *et al*, 1990; Sennerby *et al*, 1991). In transmission electron microscopy (TEM) studies, an electron-dense granular layer at the interface with hydroxyapatite (HA) and titanium has been reported (Van Blitterswijk *et al*, 1990; Sennerby *et al*, 1991). The origin and organic composition of this layer remain obscure, and it has been speculated that it may play an important role in HA–bone interactions and may have a content similar to that found in natural cementing substance (Kawaguchi *et al*, 1993). This interfacial layer comprises various bone proteins such as bone sialoprotein, α_2-HS-glycoprotein, osteocalcin, osteopontin, perhaps proteoglycans, and most probably other as yet unidentified components (De Lange *et al*, 1990; McKee and Nanci, 1996; Nanci *et al*, 1996; Ayukawa *et al*, 1998). On an ultrastructural level, in the bone–HA interface there was apparently no direct contact between bone and implant crystals because they were interconnected by a very thin non-mineralized organic bone matrix only observable by high power transmission electron microscopy (TEM) (Van Blitterswijk *et al*, 1990). Previous studies indicated that this organic layer could represent a mucopolysaccharidic film (Hofman *et al*, 1999).

The aim of the present study was an LM and TEM analysis of the bone–Bio-Oss® interface in the specimens retrieved, in the same patient, after a sinus augmentation procedure at two different time periods: 20 months and 7 years.

Materials and methods

This study was a retrospective analysis of bone specimens retrieved at two time points in a 54-year-old woman with no systemic disease. The patient signed the informed consent and the protocol was approved by the Ethics Committee of our University. At the initial visit, the patient underwent a clinical and occlusal examination, and periapical and panoramic radiographs and computerized axial tomography scans were performed. The patient underwent a unilateral maxillary sinus augmentation 7 years ago. Then medical adverse events occurred to the patient (severe heart and lung problems) and the patient was lost to follow-up; dental implants were placed after 20 months in sites 1.4 and 1.6. A fixed partial prosthesis was inserted immediately, and its function was evaluated every 6 months, with no complications reported. However, 7 years after the sinus augmentation procedure, the implant in site 1.6 was retrieved because of failure due to a fracture and a new implant was placed in this site. Moreover, this implant was immediately restored and, at the 1-year follow-up, the implant appeared to be successfully osseointegrated, as no mobility or peri-implant radiolucency was present and a correct function of the prosthesis was recorded.

Surgical protocol

Under local anaesthesia, a crestal incision slightly towards the palatal aspect throughout the entire length of the edentulous segment was performed, supplemented by buccal releasing incisions mesially and distally. Full-thickness flaps were elevated to expose the alveolar crest and the lateral wall of the maxillary sinus. Using a round bur under cold (4–5°C) sterile saline irrigation, a trap door was made in the lateral sinus wall. The door was rotated inward and upward with a top hinge to a horizontal position. The sinus membrane was elevated with curettes of different shapes, until it became completely detached from the lateral and inferior walls of the sinus.

Three grams of Bio-Oss® particles, ranging from 0.25 to 1.00 mm (Geistlich, Wohlhusen, Switzerland) were mixed with sterile saline solution in a proportion of 2:1 and carefully packed in the sinus cavity using a plugger. A collagen membrane (Biogide; Geistlich, Wohlhusen, Switzerland) was positioned against the packed sinus window. The mucoperiosteal flap was then repositioned and sutured with multiple horizontal mattress sutures. Amoxicillin (1 g two times per day) was prescribed for 1 week and analgesics as required. Sutures were removed 2 weeks after surgery. Postsurgical visits were scheduled at monthly intervals to check the course of healing. The sinus was allowed to heal and, after 20 months, at the time of implant surgery, one bone core was harvested from the regenerated site 1.6 using a 5-mm-diameter trephine under cold (4–5°C) sterile saline irrigation. After 7 years, the implant 1.6 was retrieved because of failure and a bone core was harvested from this site. The specimens were cut into two halves to be processed for both LM and TEM.

Specimen processing

LM

One half of the specimen was immediately fixed in 10% buffered formalin and processed to obtain thin ground sections with the Precise 1 Automated System (Assing, Rome, Italy). The specimens were dehydrated in ascending series of alcohol rinses and embedded in a glycolmethacrylate resin (Technovit 7200 VLC; Kulzer, Wehrheim, Germany). After polymerization, the specimens were sectioned along their longitudinal axis with the diamond disc at about 150 μm, and ground to about 30 μm with a specially designed grinding machine (Precise 1, Assing). The sections were stained with acid fuchsin and toluidine blue.

The other parts of the retrieved cores were washed in a saline solution and quickly immersed in 2.5% glutaraldehyde and 2.5% formaldehyde (prepared from fresh paraformaldehyde) buffered at pH 7.2 with 0.1 M sodium phosphate for 4 h at room temperature and left

overnight at 4°C. After washing for 1 h in the buffer alone, the specimens were decalcified using 4.13% ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, Milan, Italy). They were postfixed in 1% cacodylate-buffered osmium tetroxide for 1 h, dehydrated in graded concentrations of ethanol and embedded in LR White resin (London Resin, Berkshire, UK). These specimens were cut with glass knives on a Reichert Jung Ultracut E ultramicrotome (Leica, Milan, Italy) and stained with toluidine blue. The 1-μm-thick sections obtained were then prepared for observation under TEM.

All the semi-thin sections obtained were observed in normal transmitted light under a Leitz Laborlux Microscope (Laborlux S; Leitz, Wetzlar, Germany) connected to a high-resolution video camera (3CCD; JVC KY-F55B, Yokohama, Japan) and interfaced to a monitor and PC (Intel Pentium III 1200 MMX; Intel®, Santa Clara, CA, USA).

TEM

Selected areas of the 1-μm-thick histological sections obtained from the two retrieved specimens were then prepared for TEM evaluation. Areas randomly chosen at the Bio-Oss®–surrounding tissue interface and in the near proximity of the particles were selected and trimmed for ultra-thin sectioning. Thin sections of about 80 nm were prepared with a diamond knife, mounted on copper grids, stained with 4% uranyl acetate and lead citrate for examination in a Jeol 1010 TEM operated at 60 kV (JEOL Ltd., Tokyo, Japan). The TEM was connected with a Digital Camera MegaView III equipped with the Analysis Imaging System GmbH (Munster, Germany). A total of six regions for each specimen contacting the perimeter of Bio-Oss® particles were examined.

Results

LM

In the 20-month specimen, most of the particles were surrounded by a thin layer of newly formed bone, which mainly presented features of woven bone (Figures 1 and 2). A few regions of the particles were in contact with biologic fluids and marrow spaces. In some fields, osteoblasts were observed in the process of apposing bone directly on the particle surface (Figure 1). No gaps were present at the bone–particle interface, and the bone seemed to be always in close contact with the particles. No acute inflammatory cell infiltrate was present around the particles or at the interface with bone.

In the 7-year specimen, the Bio-Oss® particles seemed smaller compared with the 20-month specimen. Numerous Bio-Oss® particles appeared to be cemented by bone, which presented features of mature bone (Figures 3 and 4). Very few areas were in contact with marrow spaces. At high magnification, the bone near the Bio-Oss® particles presented numerous osteocytes. The Haversian canals appeared to be colonized by capillaries and cells: in some of the Haversian canals it was possible to observe the presence of acid fuchsin-positive, not yet mineralized, material lining their inner surface (inset of Figure 3). Bio-Oss® particles presented a lower staining affinity than the host bone.

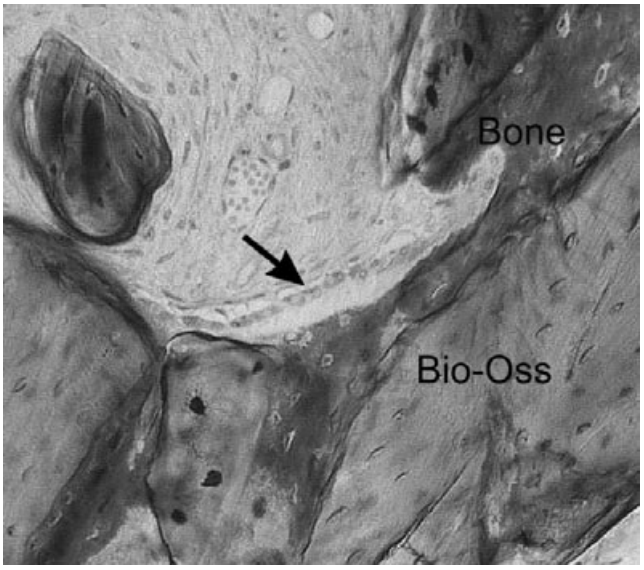

Figure 1 Light micrograph of the undecalcified part of the specimen retrieved after 20 months. Newly formed bone is observed around Bio-Oss and there are numerous osteoblasts in the process of apposing bone directly on the particle surface (arrow) (acid fuchsin and toluidine blue; magn 40×)

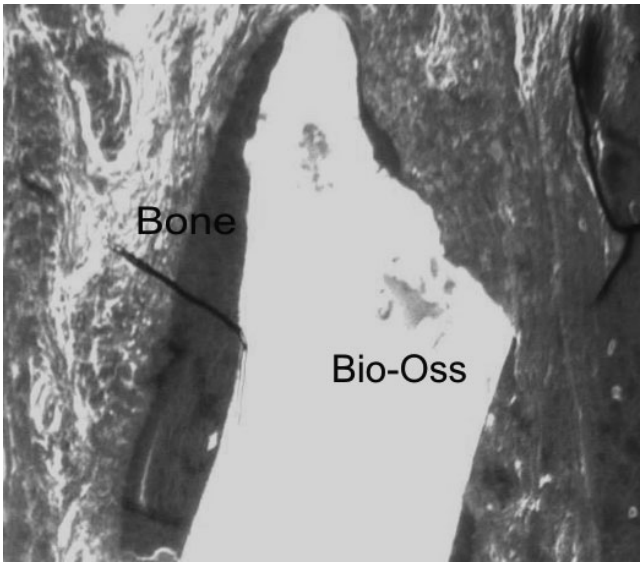

Figure 2 Light micrograph from a decalcified histological slide of 1 μm of thickness. There is a portion of a Bio-Oss particle after 20 months, which is in part surrounded by a thin layer of bone and in part is in contact with unmineralized matrix (toluidine blue; magn 10×)

TEM

The majority of the Bio-Oss® particles were surrounded by newly formed bone. Qualitative estimation of the bone tissue surrounding the particles, in the 20-month specimen, showed all the phases of the bone-healing processes. In some areas there were collagen fibres randomly disposed and newly formed bone tissue

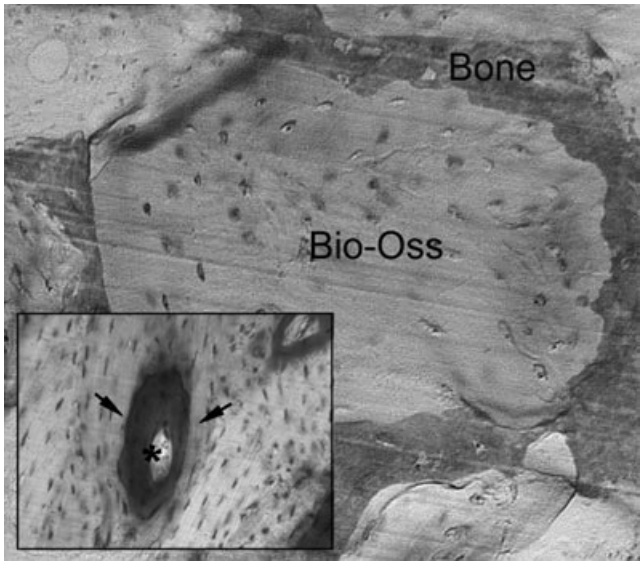

Figure 3 Light micrograph of the undecalcified part of the specimen retrieved after 7 years. The Bio-Oss particle is completely surrounded by bone (acid fuchsin and toluidine blue; magn 20×). At high magnification (inset) it is possible to observe that bone near the biomaterial presents numerous osteocytes and Haversian canals colonized by capillaries and cells (*), lined by a not yet mineralized matrix, highly positive to acid fuchsin (arrows; magn 100×)

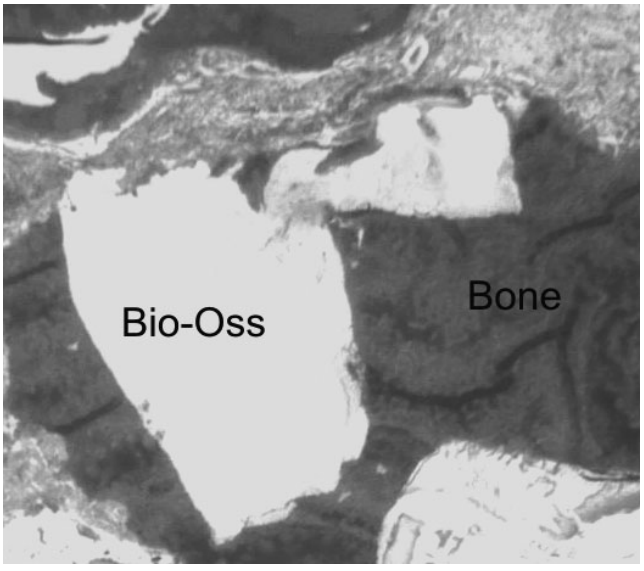

Figure 4 Light micrograph from a histological slide of 1 μm of thickness. There is one small Bio-Oss particle after 7 years presenting its internal portion with very low affinity for the staining and completely surrounded by compact bone (toluidine blue; magn 10×)

deposed by osteoblasts (Figure 5). In the collagen-rich mineralized areas, it was possible to observe the characteristic periodicity of the collagen fibrils. Only in a few areas, generally at a certain distance from the particles, compact bone was seen. The perimeter of Bio-Oss® particles sometimes showed an electron-dense layer similar to 'cement lines' and 'laminae limitantes' (Figure 6). This layer had a variable morphology. In some parts it was interrupted, while in other regions, the

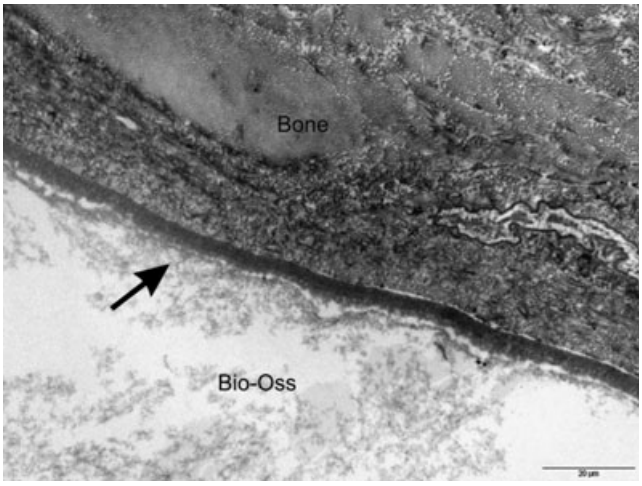

Figure 5 Micrograph of the 20-month specimen presenting the Bio-Oss particle in contact with bone. There is an evident electron-dense band at the interface (arrow), while the internal portion of the Bio-Oss particle appears amorphous (bar: 50 μm)

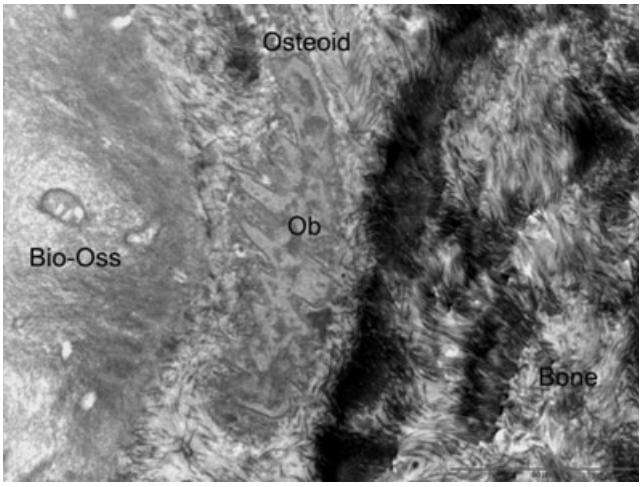

Figure 6 Transmission electron micrograph of the 20-month specimen: there is an osteoblast (Ob) producing osteoid, interposed between the Bio-Oss particle and the woven, not yet well-mineralized bone (bar: 50 μm)

Bio-Oss outermost layer seemed less electron-dense than the rest of the particle (Figure 7). Internally, the Bio-Oss® particles showed the presence of a not well-organized amorphous tissue, which seemed to dissolve in the central area, while presenting a more structured layer at the periphery. Where the bone was in close contact with the particles, an initial 'lamellar' organization was observed (Figure 8).

The 7-year specimens, on the other hand, showed that almost all the particles had a direct contact with mature bone (Figure 9). Cement lines-like structures were not clearly visible, as almost no electron-dense layers were seen at the interface with compact bone (Figure 9). At low magnification, it was possible to recognize that the bone presented features of well-organized lamellar bone (Figure 10) with osteocytes that had small cytoplasmatic

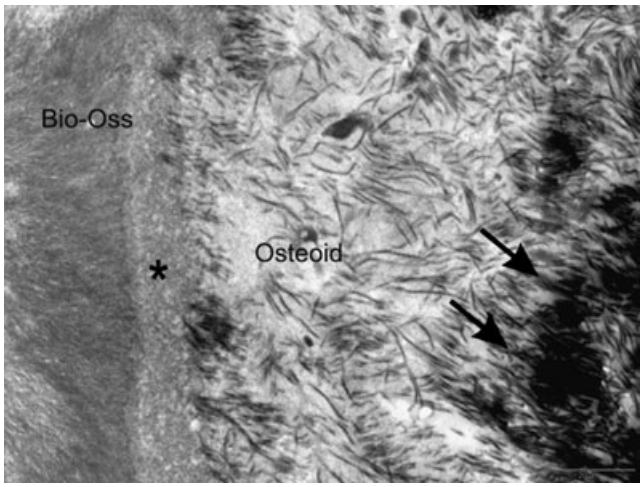

Figure 7 Transmission electron micrograph of the 20-month specimen: Bio-Oss particle is in close contact with osteoid which is starting to mineralize (arrows). The outermost layer of the Bio-Oss particle seems less electron-dense (*) (bar: 20 μm)

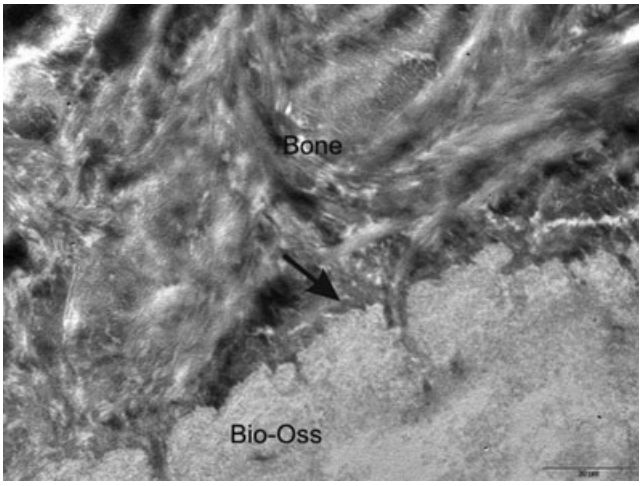

Figure 9 Transmission electron micrograph of the Bio-Oss–bone interface after 7 years. There is close contact between the particle and the bone, which presents features of mature bone (arrow). No evident electron-dense layer is present (bar: 20 μm)

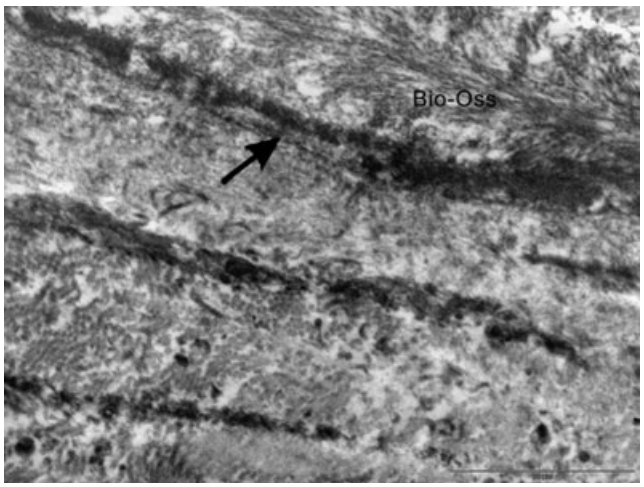

Figure 8 Transmission electron micrograph of the 20-months' specimen: detail of the Bio-Oss/bone interface characterized by an electron-dense layer (arrow). The bone is starting to present a lamellar organization, that also appears electron-dense (bar: 20 μm)

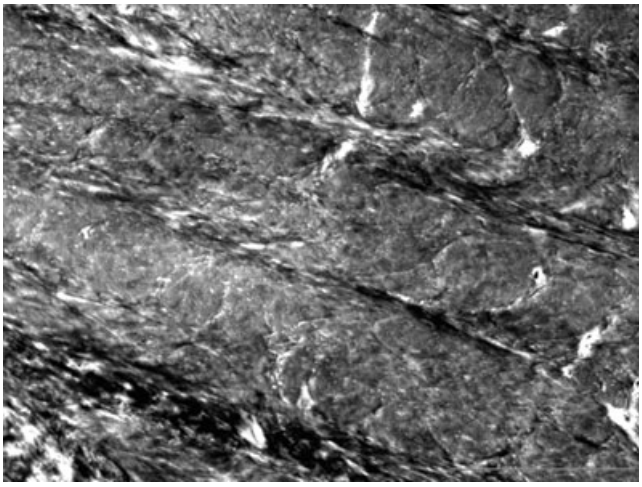

Figure 10 Transmission electron micrograph of the 7-year specimen showing that the bone of about 100 μm from the Bio-Oss particle presents a well-organized lamellar structure (bar: 100 μm)

processes (Figure 11). Only in a very few areas was there osteoid matrix presenting numerous dispersed collagen fibrils (Figure 12).

Discussion

Histological data about Bio-Oss® in humans are limited in number (Landi *et al*, 2000). Understanding the mechanism and rate of resorption, particularly in xenografts, is of special interest (Artzi *et al*, 2000).

The duration of resorption of Bio-Oss® *in vivo* has been reported to be 2–3 years (Froum *et al*, 1998) and Tadjoedin *et al* (2003) reported a decrease of Bio-Oss® of about 10% per year by osteoclast activity. Schlegel *et al* (2003) reported a loss of 15% in Bio-Oss® after 90 and 180 days. Wallace *et al* (1996) reported that the amount of Bio-Oss® gradually decreased over time and was completely absent in the 20-month sample. Another study showed that Bio-Oss® particles were still present after 4.5 years without any evident signs of resorption (Ewers *et al*, 2004).

Long-term data from humans are mandatory to elucidate whether the presence of the grafted particles would interfere eventually with the longevity of functional implants in this osseous composition (Artzi *et al*, 2000). It is believed that the absence of Bio-Oss® resorption will not jeopardize the osseointegration of dental implants as no contact between the graft particles and the implant surface is present (Valentini and Abensur, 1997). It has been reported that the long-lasting presence of Bio-Oss® particles, completely incorporated into the bone, might strengthen the bone tissue mass, creating a dense cancellous network, thus

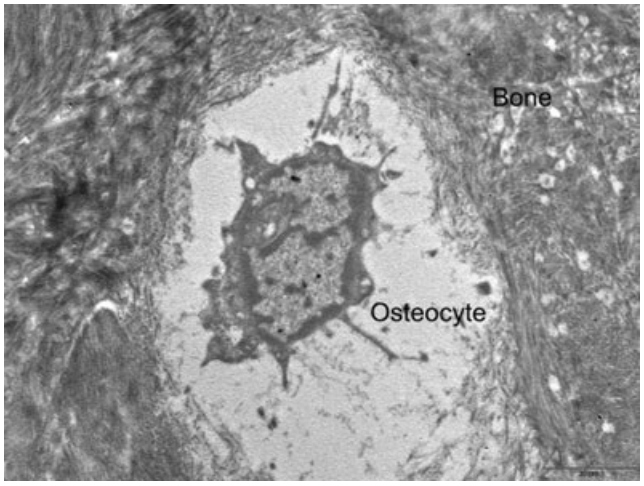

Figure 11 Micrograph of the 7-year specimen showing an osteocyte with small cytoplasmic processes trapped in the mineralized osseous matrix (bar: 20 μm)

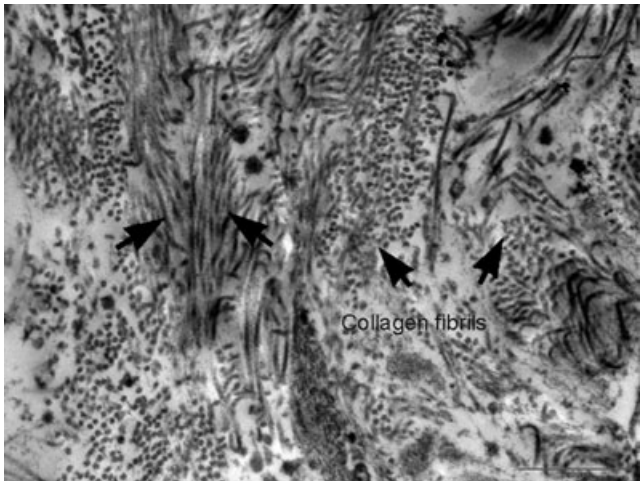

Figure 12 Transmission electron micrograph of a small region far from the Bio-Oss particles of the 7-year specimen where osteoid matrix is present. There are numerous dispersed collagen fibrils with different orientation (arrows) (bar: 10 μm)

improving its ability to withstand loading forces transmitted by implants (Artzi *et al*, 2004).

In a previous study we found that, after 6 months, Bio-Oss® did not show signs of resorption and was well integrated in the host tissues; in addition, the newly formed bone presented features similar to pre-existing osseous tissue, thus indicating the good osteoconductive properties of Bio-Oss (Orsini *et al*, 2005).

In the present specimens, the bone had grown in direct contact with the Bio-Oss® particles, which were almost all totally incorporated in the bone. Only rarely, in the 20-month retrieved specimen, osteoblast-like cells were found near the graft particles.

As already reported by Yildirim *et al* (2000), the inward growth of the bone may indicate a slow resorption of the Bio-Oss®. In our specimens, there was an inward growth of the bone in some fields; however, as only one individual was considered in this study and no stereologic measurement of the remaining grafted material was performed, we cannot conclude that a resorption of the biomaterial occurred.

Hallman *et al* (2001) believed that the mechanical properties of the bone formed on the Bio-Oss® particles would show an improvement with time because of remodelling and replacement of woven bone by lamellar bone. Our TEM results showed that after a long period of time, the bone at the interface with the biomaterial was mature and presented lamellar structures; however, further analyses are needed to see whether this osseous tissue is mechanically improved.

Moreover, our ultrastructural findings confirmed the osteoconductivity of Bio-Oss® particles because most of these particles were surrounded by the bone in different remodelling stages rather than marrow spaces (Artzi *et al*, 2002). These positive results might be dependent on the microstructure and the fine porous morphology that seem to enhance the osteopromotion of this material (Rosen *et al*, 2002; Tapety *et al*, 2004).

In conclusion, favourable long-term tissue response to the Bio-Oss® particles was found in our specimens, with mainly woven, immature bone found at the interface of Bio-Oss® after 20 months; this bone was, however, replaced by lamellar bone with time. Our results may increase the scientific knowledge of the clinician in understanding the biological interactions occurring in close proximity with Bio-Oss®. This is, according to our knowledge, the first study that presents data on TEM of Bio-Oss® after 7 years and that compares features of the bone surrounding the particles in specimens retrieved at two distant different times. Further studies should be carried out to characterize immunocytochemically the bone–Bio-Oss® interface, to elucidate whether it contains determinants of the mineralization front and which are the major proteins involved in this process.

Acknowledgements

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