

ORIGINAL ARTICLE

Microvessel density and vascular endothelial growth factor expression in sinus augmentation using Bio-Oss[®]

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AIM: The aim of this study was to evaluate microvessel density (MVD) and vascular endothelial growth factor (VEGF) expression in sinus augmentation with Bio-Oss[®]. **METHODS:** Twenty patients participated in this study. The sinuses were filled with 100% Bio-Oss[®]. Implants were inserted after 3 months in group A, and 6 months in group B. A trephine was used to harvest bone cores. As control, the pre-existing subantral bone was used.

RESULTS: The mean MVD in control bone was 23.6 ± 1.8 . In the sites augmented with Bio-Oss[®], at 3 months, the MVD was 23.3 ± 2.1 , while in the sites retrieved at 6 months the MVD was 29.5 ± 2.4 . The difference in MVD between the control bone and group A was not statistically significant. The difference between the control bone and group B was statistically significant ($P < 0.05$). The statistical analysis showed that the difference in MVD between group A and group B was statistically significant ($P < 0.05$).

CONCLUSIONS: Bio-Oss[®] seemed to induce an increase in MVD that reached a higher value after 6 months. The percentage of vessels positive to VEGF was higher in group A than in group B. Our data also showed a higher percentage of vessel and stromal cells positive to VEGF and higher MVD values in areas where there was newly formed bone compared with areas where maturation processes were occurring, and this fact could point to a close spatial relationship between angiogenesis and osteogenesis.

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Keywords: anorganic bovine bone; microvessel density; sinus augmentation procedures; vascular endothelial growth factor; xenogenic bone

Introduction

Bone formation is a coordinated process involving various biological factors (Huang *et al* 2005). Many growth factors/cytokines expressed during fetal skeletal development and induced in response to injury are believed to have a significant role in the repair process (Carano and Filvaroff, 2003). Included are members of the fibroblast growth factor, transforming growth factor, bone morphogenetic proteins, insulin-like growth factors, platelet-derived growth factor, and vascular endothelial growth factor (VEGF) (Carano and Filvaroff, 2003). Members of the VEGF family stimulate endothelial cell proliferation, and play an important role in bone remodeling by attracting endothelial cells and osteoclasts and by stimulating osteoblast differentiation (Eriksson *et al*, 2004). Blood vessels are an important component of bone formation and maintenance (Lakey *et al*, 2000). The process of vascular induction is termed angiogenesis and it plays a key role in all regenerative processes (Folkman, 1995; Carter *et al*, 2000; Lakey *et al*, 2000). Bone formation is closely linked to blood vessel invasion (Eckardt *et al*, 2003). Fracture healing requires an adequate blood supply (Lienau *et al*, 2005) and all tissues, except the cartilage, require microvasculature to heal without the formation of a scar (Winet, 1996). Angiogenesis is regulated through a complex interplay of molecular signals mediated by growth factors (Lakey *et al*, 2000) involving extracellular matrix remodeling, endothelial cell migration and proliferation, capillary differentiation and anastomosis (Folkman, 1995). One of the methods to evaluate the presence of blood vessels in a tissue is the counting of microvessels to evaluate microvessel density (MVD).

Vascular endothelial growth factor is an important factor in the initiation and progression of gingivitis to periodontitis, promoting the expansion of the vascular network (Bullon *et al*, 2004). VEGF has been considered as the most potent candidate for the induction of angiogenesis in tumor growth (Shang and Li, 2005). VEGF activity is essential for normal angiogenesis and appropriate callus formation and mineralization in

response to bone injury (Carano and Filvaroff, 2003). VEGF production seems to be the major mechanism in which angiogenesis and osteogenesis are tightly related during bone repair (Carano and Filvaroff, 2003).

Bio-Oss[®] (Geistlich, Wohlhusen, Switzerland) is a deproteinized sterilized bovine bone with 75–80% porosity and a crystal size of approximately 10 μm in the form of cortical granules (Valentini and Abensur, 1997; Froum *et al*, 1998; Piattelli *et al*, 1999; Landi *et al*, 2000; Schwartz *et al*, 2000; Hallman *et al*, 2001; Artzi *et al*, 2002; Haas *et al*, 2002; Maiorana *et al*, 2003; Merckx *et al*, 2003; Rodriguez *et al*, 2003; Sartori *et al*, 2003; Tadjodin *et al*, 2003). It has been reported that Bio-Oss[®] is osteoinductive (Schwartz *et al*, 2000) and shows very low resorbability (Piattelli *et al*, 1999; Sartori *et al*, 2003). Bio-Oss[®] is often used for maxillary sinus floor elevation (Valentini and Abensur, 1997; Froum *et al*, 1998; Piattelli *et al*, 1999; Landi *et al*, 2000; Hallman *et al*, 2001; Artzi *et al*, 2002; Haas *et al*, 2002; Maiorana *et al*, 2003; Rodriguez *et al*, 2003; Sartori *et al*, 2003; Tadjodin *et al*, 2003).

The aim of the present study was to conduct a comparative immunohistochemical evaluation of MVD and VEGF in sinus augmentation procedures using anorganic bovine bone (Bio-Oss[®]) after 3 and 6 months.

Patients and methods

Study design

Twenty patients (13 men and seven women, mean age 55 years, range 39–68) participated in this study. All patients gave their written informed consent and the protocol of the study was approved by the Ethics Committee of the University. All patients presented a maxillary partial unilateral edentulism involving the premolar/molar areas, with a residual alveolar ridge height of about 3–7 mm. In 10 patients, the residual subantral bone was at least 5 mm; in these cases the amount of graft material used was smaller and implants were inserted after a period of 3 months (group A). In the other 10 patients the residual subantral bone was less than 4 mm, and implants were inserted after 6 months (group B). All patients were no-smokers, they did not have systemic diseases or maxillary sinus pathology, or presented recent extractions in the involved area. All patients had a good oral hygiene. At the initial visit the patients received a clinical and occlusal examination, and periapical and panoramic radiographs and computerized axial tomography (CAT) scans were performed to evaluate possible intrasinus pathologies as well as bony wall morphology.

Surgical protocol

Under local anesthesia, a crestal incision, slightly toward 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. Bio-Oss[®] was mixed with sterile saline solution and carefully placed in the sinus cavity. A membrane (Biogide; Geistlich) was positioned against the packed sinus window. The mucoperiosteal flap was then replaced and sutured with multiple horizontal mattress sutures. Amoxicillin (1 g b.i.d.) was prescribed for 1 week and analgesics as required. Sutures were removed 2 weeks after surgery. A second CAT scan was performed; the dimension of the graft was evaluated and, by means of a surgical template, the implants were placed. At the time of implant surgery, after 3 months in group A and after 6 months in group B, bone cores were harvested using a 3.5 \times 10 mm diameter trephine under a cold (4–5°C) sterile saline solution irrigation.

The bone cores were retrieved through a transcresal way at a minimum distance of 5 mm from the nearest teeth; the dimension of the bone cores was 3 \times 8 mm.

Implants were then inserted, and the second stage surgery was carried out after an additional healing period of 6 months. A total of 60 implants (42 XiVe and 18 Frialit2; DENTSPLY-Friadent, Mannheim, Germany) were inserted. A total of 20 bone cores, 10 at 3 months and 10 at 6 months, were retrieved. As controls, the portions of pre-existing subantral bone were used.

Histologic analysis

Bone cores were processed for light microscopy and for immunohistochemistry. All specimens were sectioned in half parallel to the long axis; one half was processed to obtain thin ground sections with the Precise 1 Automated System (Assing, Rome, Italy). The specimens were dehydrated in an 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 a high-precision diamond disk at about 150 μm , and ground down to about 30 μm with a specially designed grinding machine. The slides were stained with acid fuchsin, and toluidine blue. The slides were observed in normal transmitted light under a Leitz Laborlux Microscope (Leitz, Wetzlar, Germany).

The other half of the specimen was decalcified with the following technique: 37.22 g of the sodium salt of ethylenediaminetetraacetic acid was dissolved in 1 l of distilled water. Thereafter 70 ml of concentrated HCl was added. After filtration, the specimens were placed in this solution for not more than 2–4 h. The specimens were then placed in 70% alcohol solution, dehydrated and embedded in paraffin. All hematoxylin–eosin-stained sections were reviewed, the quality of the slides was checked, and the slides for quantitative evaluation were selected. Immunostaining for CD34 was performed using the alkaline phosphatase-anti-alkaline phosphatase method (APAAP) with a rabbit polyclonal antibody (Santa Cruz Technology, Santa Cruz, CA,

USA). Sections of 4 μm were cut and mounted on poly-L-lysine-coated slides.

Paraffin sections were dewaxed with xylene and rehydrated with a graded alcohol series. Endogenous peroxidase was blocked with incubation for 5 min in 3% H_2O_2 . Microwave pretreatment for 20 min at 750 W, with citrate buffer pH 6 was used for antigen retrieval. The sections were cooled for 20 min at room temperature and incubated with anti-CD34 monoclonal antibody (BI-3C5; 1:100, Dako, Glostrup, Denmark) for 12 h. Immunolabeling of CD34 was detected using an LSAB-positive peroxidase kit (Dako) applied for 20 min. 3-3 diaminobenzidine was used as chromogen and the sections counterstained with Mayer's hematoxylin.

The antibody against human CD 34-related antigen was used to highlight blood microvessels; all morphologic structures with a lumen surrounded by CD 34-positive endothelial cells were considered as blood microvessels. Microvessels were counted using an IBAS-AT image analyzer (Kontron, Munich, Germany); for evaluation, a 400 \times magnification was used and the individual microvessel profiles were circled to prevent the duplication or omission of microvessel count. For each case, 10 high power fields, corresponding to 1.1 mm^2 each, were selected randomly and measurements were performed. The values were expressed as number of microvessels per square millimeter (MVD).

The immunohistochemical staining of VEGF was performed using the strep-ABC (streptavidin-biotin-peroxidase) method. Sections (3 μm) were cut and mounted on poly-L-lysine-coated slides. Paraffin sections were dewaxed by xylene, rehydrated and finally washed in phosphate-buffered saline (pH 7.4) for 10 min. In order to unmask the antigens, a microwave oven and a 2.1% content of citric acid related to VEGF antibody were used. It was not necessary to submit the sections to prior treatment. Subsequent steps were optimized by automatic staining (Optimax; BioGenex, San Ramon, CA, USA). Sections were incubated with primary antibody for 30 min at room temperature. Slides were rinsed in buffer, and immunoreaction was completed with the strep-ABC method, applying the 'Super sensitive immunodetection' kit by BioGenex and utilizing a multi-link as a secondary biotinylated antibody. After incubation with a chromogen employing 'liquid DAB substrate pack' (BioGenex), the specimens were counterstained with Mayer's hematoxylin and coverslipped. VEGF was evaluated in vessels and cells of the inflammatory infiltrate (mainly lymphocytes, plasma cells, and neutrophils) as well as in stromal cells with fibroblastic morphology. Semiquantitative analysis was performed for VEGF.

The value was considered low (+) when < 10% of the cells were positive for this factor, as intermediate (++) when the percentage of positive cells was between 10% and 50%, and as high (+++) when the percentage of positive cells was > 50%. VEGF was evaluated by using a light microscope (Laborlux S; Leitz) connected to a high-resolution video camera and interfaced to a monitor and PC. This optical system was linked to a digitizing pad and a histometry software package with

image capturing capacity (Image-Pro Plus 4.5; Media Cybernetics Inc., Immagine & Computer, Milan, Italy). The assessment was carried out at the level of the endothelial cells lining the vessels. Five random fields were chosen for each specimen.

Histomorphometry of newly formed bone, Bio-Oss, marrow spaces, area occupied by osteoblasts and area occupied by osteoclasts was carried out using a light microscope (Laborlux S; Leitz) connected to a high-resolution video camera (3CCD, JVC KY-F55B; JVC, Yokohama, Japan) and interfaced to a monitor and PC (Intel Pentium III 1200 MMX; Intel[®], Santa Clara, CA, USA). This optical system was linked to a digitizing pad (Matrix Vision GmbH, Oppenweiler, Germany) and a histometry software package with image capturing capabilities (Image-Pro Plus 4.5; Media Cybernetics Inc.).

Polarized light was used to differentiate between woven and lamellar bone. Finally, a statistical analysis was performed, and ANOVA was used to evaluate the presence of statistically significant differences.

Results

Control bone

The bone cores obtained from pre-existing bone under the sinus floor showed normal compact bone with the presence of regularly distributed vascular structures of small and average-sized dimensions located in marrow spaces and in Haversian canals. Bone trabeculae of varying dimensions were present. Osteocytes were present inside the osteocyte lacunae (Figure 1). In some areas a few osteoblasts were observed secreting osteoid matrix. Marrow spaces showed the presence of adipocytes, sinusoids and capillaries of different sizes. Hematopoietic cells in different stages of maturation were also observed.

The mean value of the MVD in control bone was 23.6 ± 1.8 , distributed in the peripheral and central portions of the lacunae. VEGF stained 15% of all

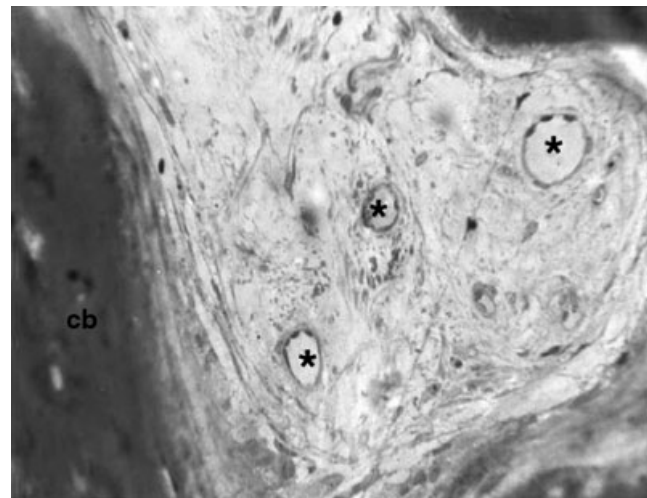


Figure 1 Control bone showing the presence of compact bone (cb) with the presence of regularly distributed vascular structures (*) of small and average-sized dimensions located in marrow spaces (H&E $\times 160$)

vessels within the newly formed connective tissue found inside the marrow spaces and around the newly formed bone.

Sites regenerated with Bio-Oss® at 3 months

Most of the Bio-Oss® particles were surrounded by newly formed mature, compact bone with well-organized osteons. In some fields, osteoblasts were observed in secreting bone directly on the particle surface. No gaps were present at the bone–particle interface, and the bone was always in close contact with the particles. No inflammatory cell infiltrate was present around the particles or at the bone–particle interface. Some of the particles appeared to be cemented by this newly formed bone. At higher magnification the bone presented wide osteocytic lacunae (Figure 2a). In almost all particles the Haversian canals appeared to be colonized by capillaries and cells: in some of the Haversian canals acid fuchsin-positive, non-mineralized material was present lining the inner surface. The most peripheral osteocytic lacunae present in the Bio-Oss® appeared to be always filled by osteocytes, while the most central ones appeared to be filled by small cells with morphologic and staining features different from the osteocytes. Only in a few cases the osteocytic lacunae were empty. The Bio-Oss® particles presented marked staining differences from the host bone and had a lower affinity for the stains. Only in a few areas was it possible to see multinucleated giant cells.

The vascularity showed mainly a microvessel pattern, with the presence of small newly formed blood vessels located mainly around and within the connective tissue inside the marrow spaces (Figure 2b). Marrow spaces showed the presence of collagen, fibroblasts and microvessels that were located within the connective tissue and in the most peripheral portions of the marrow spaces in close contact with the newly formed bone. No vessels were present between the Bio-Oss® particles and the bone. In the most apical area osteoblasts represented about 10% of the surface area, while osteoclasts accounted for 3%. In the most coronal portion, osteoblasts accounted for 15–20%, while osteoclasts accounted for 5%.

The mean value of the MVD was 23.3 ± 2.1 . This value was 32 ± 1 in the areas surrounding newly formed bone, while the MVD decreased to 10 ± 1.5 in the areas of more mature, compact bone.

Vascular endothelial growth factor expression was higher in small and average-sized vessels (Figure 2c). VEGF stained 85% of all vessels within the newly formed connective tissue found inside the marrow spaces and around the newly formed bone tissue, while the percentage of vessels positive to VEGF decreased to 40% in the areas of mature, compact bone. The intensity of VEGF expression, evaluated in a semiquantitative fashion, was + + +. Between 10% and 15% out of 100 stromal cells were VEGF positive in the areas adjacent to newly formed bone, while the number of VEGF-positive cells decreased to 2–3% in the areas adjacent to mature, compact bone. Collagen connective tissue represented about 32% of the total area of the marrow

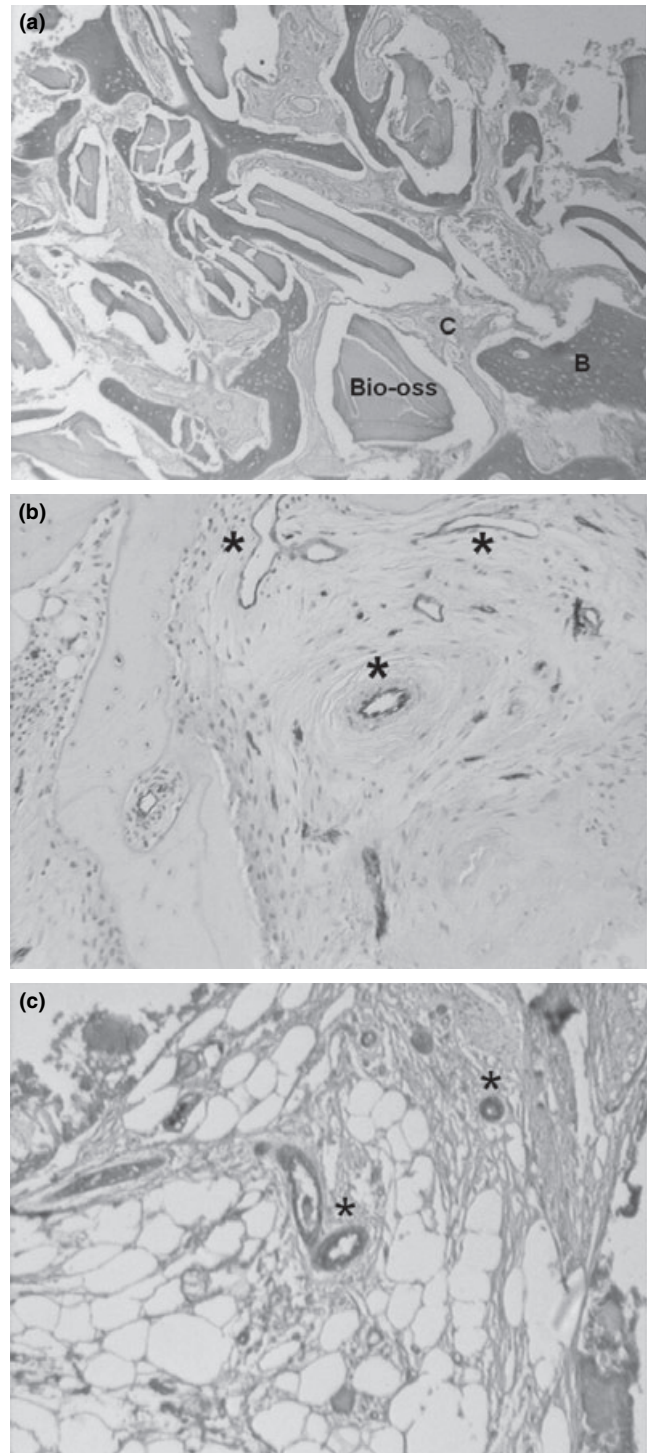


Figure 2 (a) Bio-Oss at 3 months. The bone presents wide osteocytic lacunae. The most peripheral osteocytic lacunae present in the Bio-Oss® appears to be always filled by osteocytes. The Bio-Oss® particles (Bio-oss) present marked staining differences from the host bone (B) and show a lower affinity for stains. Vascularity shows mainly a microvessel pattern. Marrow spaces show the presence of collagen (C). H&E $\times 200$. (b) Bio-Oss at 3 months. Marrow spaces show CD34+ microvessels (*), located predominantly in the connective tissue. CD34 staining (APAAP) $\times 200$. (c) Bio-Oss at 3 months. VEGF has a high intense expression in small vessels (*). VEGF staining (APAAP) $\times 200$

spaces. Newly formed bone accounted for 12% of the total bone tissue, and of this 60% was lamellar and 40% was woven. Residual Bio-Oss® particles represented 35% of the total bone tissue.

Sites regenerated with Bio-Oss® at 6 months

Largely vital lamellar bone with some woven bone and a cell-rich fibrous bone marrow was present (Figure 3a). The bone tissue contained clearly distinguishable osteocytes in normally appearing osteocyte lacunae. Many trabeculae demonstrated active bone remodeling with abundant osteoblasts and a thick layer of osteoid and the presence of resorption lacunae. In the most apical area osteoblasts accounted for 8% of the surface area, while osteoclasts accounted for 7%. In the most coronal portion, osteoblasts accounted for 10–15%, while osteoclasts accounted for 4%.

Microvessels were mainly located in close contact with the bone, while vessels of greater dimensions were found inside the more mature marrow spaces (Figure 3b). A higher quantity of large-sized vessels was observed; these vessels had an appearance similar to those found inside pre-existing marrow spaces.

The mean value of the MVD was 29.5 ± 2.4 . This value was 35 ± 2.2 in the areas surrounding newly formed bone, while the MVD decreased to 21 ± 1.8 in the areas of more mature, compact bone.

There was a reduction in VEGF expression because there was an increase in the number of large-sized vessels that showed a lower positivity to VEGF, with a concomitant decrease in the positivity of stromal cells and connective tissue (Figure 3c). VEGF stained 65% of all vessels within the newly formed connective tissue found inside the marrow spaces and around the newly formed bone, while the percentage of vessels positive to VEGF decreased to 30% in the areas of the mature, compact bone. The newly formed bone tissue was stained but its staining affinity was lower than that of the microvessels. Bio-Oss® particles were not stained by VEGF. The intensity of the VEGF expression, evaluated in a semiquantitative fashion, was +/+++. Twenty of 100 stromal cells were VEGF positive in the areas adjacent to newly formed bone, while the number of VEGF-positive cells decreased to 2% in the areas adjacent to the mature, compact bone. Collagen connective tissue accounted for 4% of the total area of the marrow spaces. Newly formed bone accounted for 35% of the total bone tissue, and of this 80% was lamellar and 20% was woven. Residual Bio-Oss® particles accounted for 30% of the total bone tissue. In addition, some inflammatory cells were VEGF positive.

Statistical analysis

The difference in MVD between control bone and Bio-Oss® at 3 months was not statistically significant. The difference between the control bone and sites regenerated with Bio-Oss® at 6 months was statistically significant ($P < 0.05$). The difference in MVD between sites regenerated with Bio-Oss® at 3 and 6 months was statistically significant ($P < 0.05$).

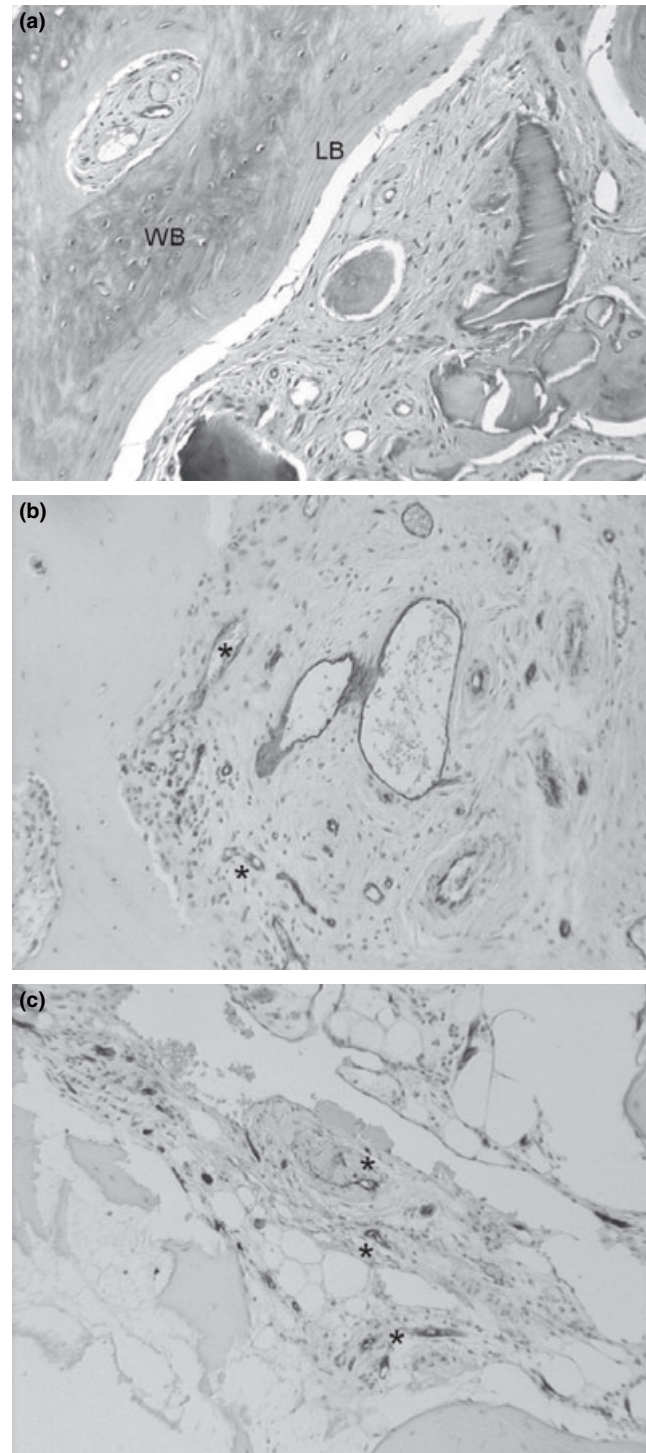


Figure 3 (a) Bio-Oss at 6 months. Lamellar bone (LB) with some woven bone (WB) and a cell-rich fibrous bone marrow is present. Microvessels are mainly located in close contact with the bone, while vessels of greater dimensions are found inside more mature marrow spaces. A higher quantity of vessels with a larger size can be observed. H&E $\times 160$. (b) Bio-Oss at 6 months. The vessels positive for CD34 (*) are in close contact with the bone. CD34 staining (APAAP) $\times 200$. (c) Bio-Oss at 6 months. VEGF stained vessels (*) within the newly formed connective tissue found inside the marrow spaces and around the newly formed bone. VEGF staining (APAAP) $\times 200$

Discussion

The interactions between bone formation and angiogenesis, and physiologic angiogenesis during bone remodeling need further study (Yao *et al*, 2004). It is well known that angiogenesis is essential for the replacement of cartilage by bone during skeletal growth and regeneration (Aoyama *et al*, 2004).

Angiogenesis is defined as the process by which new blood vessels are produced by sprouting from established vessels (Johnson *et al*, 1999). Inflamed tissues have evidence of enhanced expression of inflammatory mediators, many of which can promote angiogenesis (Johnson *et al*, 1999). Formation of an appropriate vascular bed is necessary to support the metabolic needs of the forming tissue mass (Huang *et al*, 2005). In areas with poor vascularity, the undifferentiated pluripotential cells are routed into a chondrogenic rather than an osteogenic pathway (Carter *et al*, 2000). The key to the success of any bone graft is primarily determined by the degree of vascularization (Artzi *et al*, 2005).

Bio-Oss[®] is a xenogeneic bone graft material of bovine origin. This material has been demonstrated to be osteoconductive and it acts as a scaffold for the formation of new bone (Schwartz *et al*, 2000; Hallman *et al*, 2001; Haas *et al*, 2002).

In our specimens we found that in the initial (3 months) phase of bone formation after sinus augmentation procedures using anorganic bovine bone the values of MVD were roughly similar to those of pre-existing subantral bone, while, at a later period (6 months), there was a higher and statistically significant quantity of vessels. At 6 months there was also an increase in the number of vessels of larger dimensions. We also observed a reduction of VEGF expression in the 6-month-old specimens. When we differentiated, both in 3- and 6-month-old specimens, MVD and VEGF positivity (of vessels and stromal cells) between the areas neighboring newly formed bone and areas adjacent to more mature, compact bone, we found a higher MVD value and a higher number of VEGF-positive vessels and stromal cells in newly formed bone and vice versa in areas adjacent to more mature, compact bone. Our results are similar to those reported by Ohtsubo *et al* (2003) who found, in a study of rats, that VEGF appeared in the early stages and gradually decreased, and angiogenesis passed gradually into a more mature stage. It has also been reported that, after fracture in rodents, local levels of endogenous VEGF peak at 5 days and decrease toward normal levels after 10 days (Carano and Filvaroff, 2003). Vascularity was found to be extensive after 3 weeks in bone defect healing (Winet, 1996). After mucoperiosteal flap surgery, new vessels formed on the bone and root side from day 7 to day 14 starting from the periodontal ligament vascular plexus (Nobuto *et al*, 2003).

Our results support the hypothesis that VEGF seems to be essential for blood vessel invasion of the injury site, invasion that has a key role in tissue repair (Carano

and Filvaroff, 2003). VEGF production seems to be a major mechanism that links angiogenesis and new bone formation at the bone repair site (Carano and Filvaroff, 2003). Intravital microscopy and angiographic analysis in bone chamber models show that angiogenesis temporally precedes osteogenesis (Carano and Filvaroff, 2003) and that newly formed bone is found always in very close contact with newly formed blood vessels (Eckardt *et al*, 2003). Our data show a difference in angiogenesis, expressed both in the percentage of vessel and stromal cells positive to VEGF and by the MVD values, between the areas where there was newly formed bone compared with areas where bone maturation processes were occurring, and this, according to us, could point to a close spatial relationship between angiogenesis and osteogenesis.

Future studies should evaluate the angiogenetic properties of several different biomaterials.

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