

Alveolar Ridge Regeneration with Equine Spongy Bone: A Clinical, Histological, and Immunohistochemical Case Series

Danilo Alessio Di Stefano, DDS;* Luciano Artese, MD;† Giovanna Iezzi, DDS, PhD;‡
Adriano Piattelli, MD, DDS;§ Stefano Pagnutti, BSc;¶ Marcello Piccirilli, BSc;**
Vittoria Perrotti, DDS, PhD††

ABSTRACT

Background: In the case of localized ridge atrophy, a ridge augmentation procedure, with the use of bone substitutes and barrier membranes, may then be necessary.

Purpose: The aim of the present study was a clinical, histological, and immunohistochemical evaluation of an equine spongy bone in alveolar ridge augmentation procedures.

Materials and Methods: Five patients showing horizontal mandibular ridge defects participated in this study. A ridge augmentation was performed through an onlay apposition of equine bone covered by a titanium-reinforced membrane. After 6 months of healing, five bone cores from nonaugmented sites (control) and five from augmented sites (test) were retrieved.

Results: In test sites, no postoperative complications occurred. Horizontal bone width increased from ≤ 4 to ≥ 7 mm. In control sites, the newly formed bone represented 33%, and in test sites, 35% of the total area. The mean value of the microvessel density was 25.6 ± 3.425 per mm^2 in controls, while 33.3 ± 2.5 vessels per mm^2 in the test sites were found ($p < .05$). Both groups showed a high intensity (++) of vascular endothelial growth factor expression in the newly formed bone, while a low intensity (+) was found in the mature bone.

Conclusion: Equine bone appeared to be biocompatible and to be associated with new vessel ingrowth. Within the limits of the small sample size, the present study indicated that equine bone could be used in mandibular ridge augmentations.

KEY WORDS: bone regeneration, equine bone, microvessel density, vascular endothelial growth factor

INTRODUCTION

Dental implants placed in deficient ridges have higher failure rates than those placed in ridges with a normal bone height.¹ In the case of a localized ridge atrophy,

a ridge augmentation procedure, with the use of bone substitutes and barrier membranes, may then be necessary.¹⁻⁵ Onlay grafts have been successfully used either in the presence of wide alveolar defects or when it is necessary to increase the horizontal diameter of the alveolar crest to obtain a good aesthetic result and to insert the implants in a correct way.^{1,6,7} On the other hand, the augmentation of resorbed mandibles with an interposed iliac crest graft can present surgical and prosthetic complications, resulting in a greater implant failure than using short implants.⁸ The gold standard graft material for bone regeneration is autologous bone, because it contains vital bone cells.⁹⁻¹³ The harvesting of autologous bone grafts, however, requires an additional surgical procedure and may be associated with hospitalization, higher costs, longer treatment time, and some

*Private practice, Milan, Italy; †professor of pathology, Dental School, University of Chieti-Pescara, Italy; ‡research fellow, Dental School, University of Chieti-Pescara, Italy; §professor of oral pathology and medicine, Dental School, University of Chieti-Pescara, Italy; ¶Private Practice, Arcugnano, Italy; **research assistant, Dental School, University of Chieti-Pescara, Italy; ††research fellow, Dental School, University of Chieti-Pescara, Italy

Reprint requests: Prof. Adriano Piattelli, MD, DDS, Via F. Sciucchi 63, 66100 Chieti, Italy; e-mail: apiattelli@unich.it

© 2008, Copyright the Authors

Journal Compilation © 2008, Wiley Periodicals, Inc.

DOI 10.1111/j.1708-8208.2008.00104.x

morbidity at the donor site (ie, pain and discomfort).^{14,15} To avoid these complications, the use of other bone substitutes, for example, allografts or xenografts, has been proposed.

Xenografts are bone grafts that have been taken from a donor of another species. These natural materials, thanks to their chemical-physical characteristics similar to those of the human bone, show great osteoconductive properties.^{16,17} Most of the xenogenous materials used in bone regeneration procedures are either of bovine or porcine origin.¹⁸ To our best knowledge, there are no studies on equine-derived bone substitutes, apart from the few articles on equine bone protein extract, which showed its ability to induce osteoblastic differentiation of human bone marrow-derived mesenchymal stem cells¹⁹ or to induce ectopic bone formation in a rat model.²⁰ The material used in the present study was an equine-derived bone substitute material commercially available (Osteoplast Flex®, Bioteck Srl, Arcugnano, Vicenza, Italy), which was deantigenated through a low-temperature (maximum 37°C) enzymatic process that should not alter its mineral component.

In order to have a bone formation and a successful repair at the grafted site, blood supply and a close contact between the implanted material and the vascularized tissue are fundamental requirements.^{21,22} Angiogenesis is the process of vascular induction, and it has an important role in wound healing, inflammatory diseases and tumors, and in endochondral and intramembranous ossification in bone growth.^{23–25} Bone formation is closely linked to blood vessel invasion²⁶, and angiogenesis precedes osteogenesis.^{27–30}

Vascular endothelial growth factor (VEGF) is a multifunctional angiogenic mediator that potently increases microvascular permeability, stimulates endothelial cell proliferation, induces proteolytic enzyme expression,³¹ attracts endothelial cells and osteoclasts, and stimulates osteoblast differentiation.^{22,32–34} It has been involved in peri-implant healing processes,³⁵ in osteoclastogenesis,³⁶ in bone resorption,³⁴ and in wound healing.³⁷ Localized VEGF delivery has been demonstrated to be beneficial for bone regeneration in numerous animal models by promoting neovascularization, bone turnover, osteoblast migration, and mineralization.³⁸ VEGF production seems to be the major mechanism in which angiogenesis and osteogenesis are tightly related during bone repair.^{27,38}

One of the methods of assessing the presence of blood vessels in a tissue is to count the microvessels to evaluate the microvessel density (MVD).³⁹ MVD has already been used in a few studies to evaluate the presence of blood vessels and the angiogenetic properties of bone substitute materials.^{40–42} Moreover, MVD has been extensively studied in tumors and seemed to correlate with VEGF expression.⁴³

The aim of the present study was to conduct a clinical, histological, and immunohistochemical evaluation of a partially demineralized equine spongy bone used in association with a titanium-reinforced membrane for alveolar ridge augmentation procedures.

MATERIALS AND METHODS

Clinical Cases

Five patients (two males and three females with a mean age of 45.5 years, range 32–59 years) participated in this study. All patients were medically healthy, with a noncontributory past medical history, and were treated, starting in November 2001 until June 2005, at the private practice of one of the authors (D.S.). The procedure was explained to the patients, and all the patients signed a written informed consent form.

Inclusion criteria were as follows: mandibular defects in a vestibulolingual direction resulting from prior extractions, crestal width of ≤ 4 mm, crestal height of ≥ 10 mm, age >30 years, moderate smoking (less than 10 cigarettes/day), controlled oral hygiene (overall plaque score of $<20\%$), and absence of any lesions in the oral cavity; in addition, the patients had to agree to participate in a postoperative control program.

Exclusion criteria were as follows: a high degree of bruxism; smoking more than 10 cigarettes/day; excessive consumption of alcohol (about 1 L of wine/day); localized radiation therapy of the oral cavity; antineoplastic chemotherapy; blood, liver, and kidney diseases; immunosuppression; corticosteroids and bisphosphonates therapy; pregnancy; inflammatory and autoimmune diseases of the oral cavity; and poor oral hygiene (overall plaque score of $>20\%$).

At the initial visit, all patients underwent a clinical and occlusal examination, and periapical and panoramic radiographs; computed axial tomography (CAT) scans were performed to evaluate the bony wall morphology and to measure the height and width of the residual ridges.

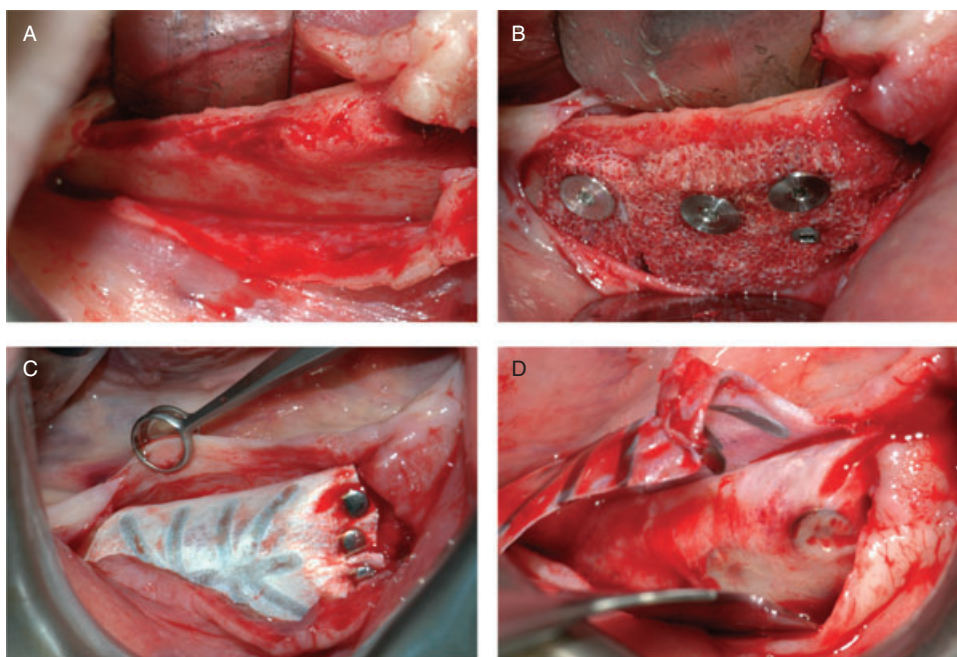


Figure 1 (A) A thin (3 mm) alveolar ridge is present. (B) The equine bone layer has been fixed, with osteosynthesis screws, as an onlay to the alveolar bone. (C) The onlay graft has been covered with a titanium-reinforced expanded polytetrafluoroethylene membrane. (D) The alveolar ridge 6 months after insertion of the implanted material, showing an increase of the ridge width (≥ 7 mm).

Bone Grafting Material Preparation

Bone regeneration was performed through the onlay apposition of an enzyme-deantigenated equine flexible spongy bone layer (Osteoplast Flex, Bioteck, Vicenza, Italy), which ($25 \times 25 \times 3$ mm) had been obtained from an equine femur. This material has been approved for clinical use on patients according to the European standards. The gross residues had been mechanically cleaned from the femurs; the bone had been cut so that the spongy part of the epiphysis or diaphysis was isolated. The spongy fraction had then been cut into pieces, and these underwent enzymatic deantigenation by immersion in a thermostatic bath containing a complex aqueous enzymatic solution at 37°C . The duration of the treatment and the composition of the mixture had been optimized in order to obtain a total deantigenation of the sample. The deantigenated sample had then been partially demineralized by an electrolytic treatment in a slightly acidic solution containing HCl. This treatment rendered the spongy layer flexible when it was rehydrated. The spongy layer was then dehydrated by lyophilization, packaged, and sterilized with 25 kGy beta irradiation.

Surgical and Grafting Procedure

On the same day of the surgery, each patient was draped to guarantee a maximum asepsis, and antibiotic prophylaxis

(Amoxicillin/Clavulanic acid, Augmentin, Glaxo-SmithKline, Verona, Italy) was initiated. One hour before the surgery, the patients were also premedicated with a sedative (Diazepam, Valium 2, Roche, Monza, Milan, Italy) and a common nonsteroidal anti-inflammatory drug (Nimesulide, Aulin, Bayer, Milan, Italy), and then the patients were subjected to mouth rinses with Chlorhexidine 0.2% (Corsodyl, GlaxoSmithKline).

Under local anaesthesia with 4% articaine and 1:100,000 epinephrine (Citrocartin 100, Molteni, Scandicci, Florence, Italy), a full thickness mucoperiosteal flap was elevated and reflected to expose the underlying ridge (Figure 1A). The vestibular wall of the ridge, including the adjacent control area, was partially decoricalized with a bone collector (Safescraper Twist, Meta, Reggio Emilia, Italy). The flexible spongy bone layer was then rehydrated for about 5 minutes in sterile saline solution at room temperature, and molded by hand in order to adapt to the defect. The spongy layers were then fixed to the vestibular wall of the ridge through transcortical screws using a proper screwdriver (Bioteck Srl, Arcugnano, Vicenza, Italy) (Figure 1B). Finally, the grafting and control sites were covered with a titanium reinforced, expanded polytetrafluoroethylene membrane (W.L. Gore & Associates®, Verona, Italy) (Figure 1C), and the mucoperiosteal flap was sutured.

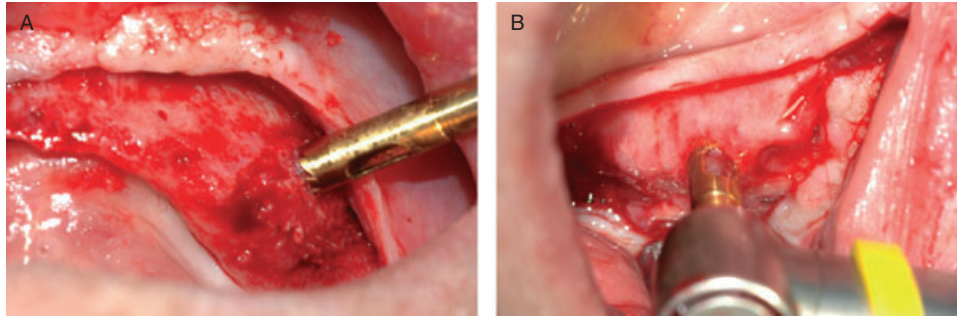


Figure 2 The bone cores were retrieved with a 3.5×10 -mm-diameter trephine in a vestibulolingual direction, (A) in the area immediately behind the graft (control) and (B) in the area where the grafting procedure had been performed (test).

Antibiotic treatment was prescribed for 6 days after the surgery, and analgesics were given as required. Sutures were removed 2 weeks after the surgery. Postsurgical visits were scheduled at monthly intervals to check the course of healing. After a mean of 6 months of healing, the surgical site was reopened (Figure 1D), a second CAT scan was performed, measurements of alveolar ridge width were performed and evaluated by a masked examiner (D.S.), and by means of a surgical template, the implants (XiVE, DENTSPLY-Friadent, Mannheim, Germany) were inserted. A total of 15 implants were inserted, and these included four implants that were 3×11 mm, two that were 4.5×13 mm, two 4.5×11 mm, two 3.8×13 mm, two 3.8×11 mm, two 3×13 mm, and one that was 3.8×8 mm. A 3.5×10 -mm-diameter trephine was used to harvest bone cores, under a cold (4 – 5°C) sterile saline solution irrigation. The bone cores were retrieved in a vestibulolingual direction in the area immediately behind the graft (control) (Figure 2A) and in the area where the grafting procedure had been performed (test) (Figure 2B); the dimension of the bone cores was 3×8 mm. In each patient, two bone cores, a control and a test, were retrieved. A total of 10 bone cores, five from the non-augmented ridges (control) and five from the augmented ridges (test), were retrieved. The second-stage surgery was carried out after an additional healing period of 6 months, and all the implants were restored with a fixed prosthesis. The average follow-up was 40.5 months.

Histologic and Immunohistochemical Procedures

All specimens were fixed in formalin (10% neutral buffered formalin). The specimens were decalcified in a

filtered solution of 37.22 g of ethylenediaminetetracetic acid (sodium salt) in 1 L of distilled water containing 70 mL concentrated hydrochloric acid for 1 to 2 months. The specimens were then dehydrated in ethanol and embedded in paraffin. Three $5\text{-}\mu$ cross-sections were cut for each sample and were mounted on poly-L-lysine-coated slides. Serial sections were cut. One section per sample was stained with hematoxylin-eosin and was used for histologic observations and histomorphometric measurements. Histomorphometry of the newly formed bone and residual implanted material was carried out for each case on the whole sample at low magnification ($\times 25$). The area occupied by osteoblasts and osteoclasts was measured on 10 randomized fields for each sample at a $\times 40$ magnification. These measurements were undertaken by a masked examiner (L.A.) on the hematoxylin-eosin stained sections prior to immunohistochemical staining using a light microscope (Laborlux S, Ernst Leitz GmbH, Wetzlar, Germany) 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 an image capturing capacity (Image-Pro Plus 4.5, Media Cybernetics Inc., Immagine & Computer, Milan, Italy).

One section per sample was then immunostained for CD-31 with the Streptavidine-Biotin-Peroxidase (Strep-ABC) method using a mouse monoclonal antibody (Novocastra Laboratories Ltd, Newcastle, UK). Paraffin sections were dewaxed using xylene, rehydrated, and finally washed in Tris buffer (pH 7.6) for 10 minutes. In order to unmask the antigens, the specimens were placed in a 2.1% aqueous solution of citric acid in a microwave oven. The following steps were optimized by automatic staining (Optimax, BioGenex, San Ramon, CA, USA). The sections were incubated with

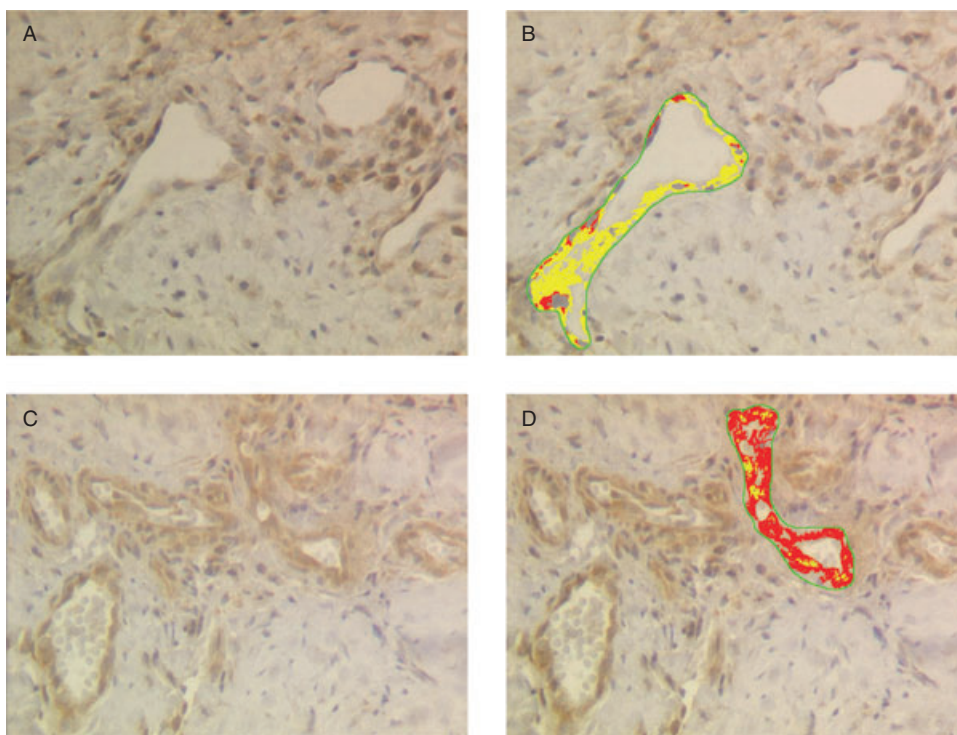


Figure 3 (A) Vascular endothelial growth factor (VEGF) showing a low positive staining of endothelial cells lining the blood vessels. (B) Low intensity (+) of VEGF expression. (C) VEGF high positive staining of endothelial cells lining the blood vessels. (D) High intensity (++) of VEGF expression. VEGF staining (alkaline phosphatase anti-alkaline phosphatase) 30 $\times$.

primary anti-CD-31 monoclonal antibody diluted 1:50 for 30 minutes at room temperature. The slides were rinsed in buffer (Automated IHC Wash, Novocastra Laboratories Ltd), and the immunolabeling reaction was completed according to the Strep-ABC-Peroxidase manufacturer's instructions (Novocastra Laboratories Ltd). After incubation with diaminobenzidine (DAB) chromogen solution, the specimens were counterstained with Mayer's hematoxylin, and coverslips were applied. The antibody against human CD-31-related antigen was used to highlight blood microvessels; all structures with a lumen surrounded by CD-31 positive endothelial cells were accounted as blood vessels. However, only vessels that had a diameter of less than 3 μ , presented a vessel wall thickness of less than 1 μ , and had 1 or more endothelial cells that lined the lumen were considered microvessels and enumerated.³⁹ The microvessel count was performed using an IBAS-AT image analyzer (Kontron, Munich, Germany). For evaluation, a $\times 400$ magnification was used, and the individual microvessel profiles were circled to prevent the duplication or omission of any microvessels. For each case, 10 high-power fields, corresponding to 1.1 mm², were randomly selected, and measurements were performed. The values

were expressed as the number of microvessels per mm² (MVD).

One section per sample was immunostained for VEGF using the Strep-ABC method. Paraffin sections were dewaxed by xylene, rehydrated, and finally washed in Tris buffer (pH 7.6) for 10 minutes. In order to unmask the antigens, a microwave oven and a 2.1% aqueous solution of citric acid related to VEGF monoclonal antibody (Diapath, Martinengo, Bergamo, Italy) were used. Subsequent steps were optimized by automatic staining. Sections were incubated for 30 minutes with an anti-VEGF antibody diluted 1:100 at room temperature. The slides were rinsed in buffer (Automated IHC Wash), and the immunoreaction was completed according to the Strep-ABC-Peroxidase manufacturer's instructions. After incubation with DAB chromogen solution, the specimens were counterstained with Mayer's hematoxylin and were coverslipped.

The assessment of VEGF expression was carried out at the level of endothelial cells lining the vessels using the image analysis software described earlier. Two different VEGF staining intensities were assigned: yellow corresponding to low VEGF expression (Figure 3, A and B), and red corresponding to high VEGF expression

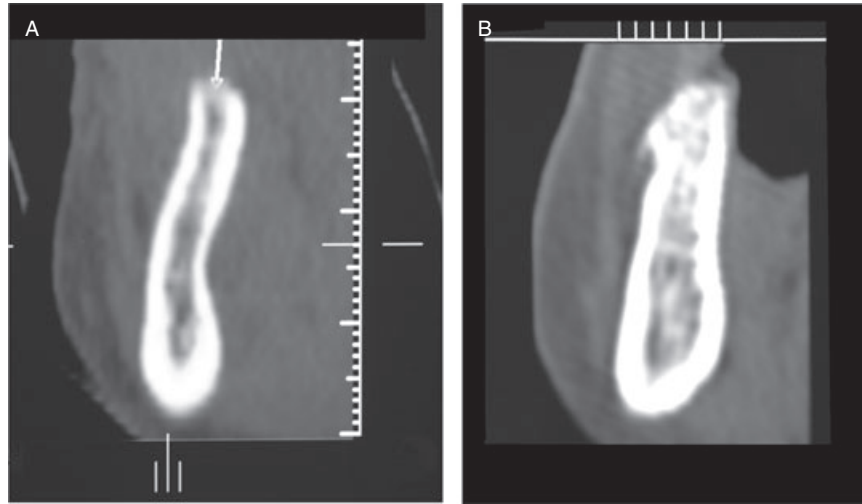


Figure 4 Computed axial tomography (CAT) scans (A) before and (B) after surgery revealing that a 7-mm bone width has been achieved.

(Figure 3, C and D). A semiquantitative analysis was performed choosing five random fields for each specimen ($\times 30$ magnification). The value was considered low (+) when $>50\%$ of the vessel area was yellow, and high (++) when $>50\%$ was red. All the immunohistochemical measurements were carried out by a masked examiner (L.A.).

Statistical Analysis

Differences between CAT scan measurements before and after the alveolar ridge regeneration with equine spongy bone layer were evaluated using a paired *t*-test; $p < .05$ was considered significant after testing the normal distribution using the method of Kolmogorov and Smirnov. The same analysis was used for testing differences between the control and test MVD values. All the measurements were expressed as mean $\pm$ standard deviation.

RESULTS

Clinical Findings

The implanted material used in this study proved to be elastic and flexible; it was easily handled and could be cut to the necessary length and width in order to obtain the desired shape. The material was not friable, and it was easily fixed to the alveolar bone with osteosynthesis screws.

Regenerated ridges healed uneventfully. No post-operative complications were present after the ridge

augmentation procedures and at the time of the implant surgery. No evidence of serious adverse local (ie, foreign body reaction, pain, dysesthesia, inflammation, membrane exposition, or dehiscences) was observed in any patient throughout the study. No implanted material was identified in the regenerated sites. The newly formed bone was macroscopically similar to the surrounding bone. By comparing CAT scans 6 months before and after the surgery, it was found that the crestal bone was adequate for implant placement in all patients. Horizontal bone width increased from an initial average value of ≤ 4 mm (Figure 4A) to a final value of ≥ 7 mm (Figure 4B) with statistically significant differences ($p < .0001$) (Table 1). All the inserted implants were osseointegrated, and no failures were reported at the last follow-up.

Control Sites

The bone cores obtained from nonregenerated sites showed a mature compact bone with regularly distributed vascular structures of large and medium dimensions located in marrow spaces and in Haversian canals (Figure 5A). The newly formed bone represented 33% of the total area, and of this, 85% was lamellar and 15% was woven. In some areas, it was possible to observe a few osteoblasts depositing osteoid matrix. In the lingual portion of the core, osteoblasts represented 7% and osteoclasts represented 5% of the total, while in the vestibular portion of the core, osteoblasts represented 14%, and osteoclasts 2%. Low intensities (+yellow) of VEGF

TABLE 1 Computed Axial Tomography Scan Measurements before and after the Alveolar Ridge Regeneration with Equine Spongy Bone Layer

Subject	Ridge Width Measurements before Regeneration	Ridge Width Measurements after Regeneration	<i>p</i> Value
1	3.8	7.1	$p < .0001$
2	4.0	7.3	
3	3.7	7.0	
4	3.8	7.0	
5	4.0	7.3	
Mean $\pm$ standard deviation	3.86 ± 0.13	7.14 ± 0.15	

Paired *t*-test.

expression were prevalent in the proximity of more mature bone tissue, while high intensities (red ++) were found around the newly formed bone (Figure 6A). The mean value of MVD in the control bone was 25.6 ± 3.425 per mm^2 .

Regenerated Sites

In all specimens, vital, lamellar bone with some woven bone, residual implanted material, and cell-rich and well-vascularized connective tissue was observed. The newly formed bone represented 35% of the total bone tissue, and of this bone, 80% was lamellar and 20% was woven. The residual implanted material particles represented 30% of the total bone tissue and presented marked staining differences from the host bone because of a lower affinity for the staining. Many trabeculae, which were being actively remodeled, were identified by the presence of abundant osteoblasts, a thick layer of

osteoid and resorption lacunae. In the lingual portion of the core, osteoblasts represented about 8% of the surface area, while the osteoclasts represented 7%. In the vestibular portion of the core, osteoblasts represented 10 to 15%, while osteoclasts represented 4%. No inflammatory cell infiltrate was present around the material or at the interface with bone. Small- and middle-sized vessels (VEGF ++) were found in contact to the implanted material, while the larger vessels, found near the mature, lamellar bone, expressed a lower VEGF activity (+). The mean value of the MVD was 33.2 ± 2.5 per mm^2 . Most of the vessels were of small size (Figure 5B), but none of them were present between the bone substitute and the newly formed bone.

The statistical analysis showed that the differences between the test and the control sites were statistically significant with respect to the MVD values ($p < .0170$) (Table 2).

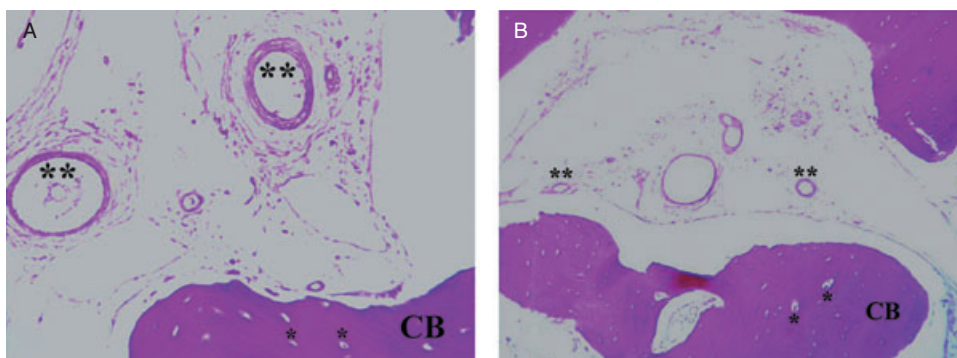


Figure 5 (A) Control sample showing the presence of compact bone (CB) with many osteocyte lacunae (*), and regularly distributed vascular structures of large and medium dimensions (**) located in marrow spaces and in Haversian canals. (B) Test sample showing the presence of CB with many osteocyte lacunae (*) and marrow spaces. Small vessels (**) are mainly located in the proximity of the newly formed bone. H&E 30 $\times$.

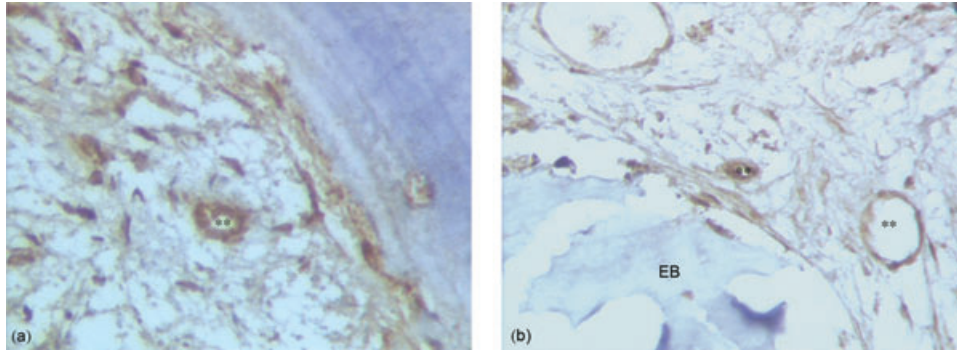


Figure 6 (A) Control sites: high intensities (++) of vascular endothelial growth factor (VEGF) expression were found in a small vessel (**) around the newly formed bone. (B) Regenerated sites: small- and middle-sized vessels (**) (VEGF ++) were found in contact to the implanted material (equine bone). VEGF staining (alkaline phosphatase anti-alkaline phosphatase) 40 $\times$.

DISCUSSION

The interactions between bone formation and angiogenesis still remain to be fully elucidated as physiological angiogenesis during bone remodeling is undefined.⁴⁴ It is well known that angiogenesis is essential for the replacement of cartilage by bone during skeletal growth and regeneration.⁴⁵ A strong relationship between revascularization and osteogenesis in and around autogenous bone grafts has been observed, and the presence of vascular sprouts from the recipient bed has been reported to be intimately related to the development of a new bone.²¹ Furthermore, a significant relationship has been reported between vessel number and bone formation.⁴⁶ In a previous study from our laboratory in sites regenerated with Bio-Oss® (Geistlich Söhne AG, Wolhusen, Switzerland) at 3 and 6 months, it was found that in the areas surrounding the newly formed bone, MVD values were higher than in the areas of more mature, compact bone.⁴¹ A similar trend was shown by VEGF, which

stained 65% of all vessels around the newly formed bone and only 30% in the areas of mature, compact bone.⁴¹ These findings could indicate a close spatial relationship between angiogenesis and osteogenesis. In the present study, it was found that smaller vessels were in close contact with the newly formed bone, while larger vessels were located at a distance. The smaller vessels presented a high intensity of VEGF expression, while the larger, more distant vessels showed a lower VEGF expression. This is also in agreement with a previous study by Cetinkaya and colleagues³¹ where blood vessels with large dimensions were seen in the destruction stage of periodontal disease, while in the healing stage, large areas were occupied by numerous blood vessels with smaller dimensions.

Regenerating tissues have higher metabolic needs and thus require a dense capillary network during repair.²⁴ In the present specimens, it was found that in the sites regenerated with equine bone at 6 months, there was a statistically significant higher quantity of

TABLE 2 Microvessel Density (MVD) Values of the Test and Control Samples

Sample	Control MVD values	Test MVD values	<i>p</i> Value
1	21.4	31.9	<i>p</i> < .0170
2	23.3	32.7	
3	25.7	36.3	
4	29.9	30.1	
5	27.9	35.4	
Mean $\pm$ standard deviation	25.64 $\pm$ 3.41	33.28 $\pm$ 2.54	

Paired *t*-test.

vessels than in the control site. These findings are similar to those reported in a previous study from our laboratory, where 3 months after a sinus augmentation with Bio-Oss®, the mean values of MVD were similar to those of control bone, while at a later period (6 months), there was a significantly higher MVD count in the regenerated sites when compared with the control sites. In a rabbit study, where calcium sulfate was used to fill 6-mm-wide defects, at 4 weeks, a higher MVD value was found in the sites regenerated with calcium sulfate when compared with the control sites.⁴⁰ Taken together, these findings supported the link between regeneration and revascularization of differentiating tissues. Therefore, in the present specimens regenerated with equine bone, the presence of the newly formed bone in a slightly greater amount than in the control could be explained by the capacity of this material to support a new vessel formation.

In regions where there is a poor vascularity, the undifferentiated pluripotential cells are shunted into a chondrogenic rather than an osteogenic pathway.⁴⁷ In a study in rabbits, using an antiangiogenic substance, Mair and colleagues⁴⁸ found that in areas of extensive bone formation, the inhibition of blood vessel formation negatively affected the osseointegration process.

VEGF production seems to be a major mechanism that links angiogenesis and new bone formation at the bone repair sites,²⁷ and newly formed bone was always found in very close contact with the newly formed blood vessels.²⁶ In a study on the expression of VEGF in a rat model at destructive and healing stages of periodontal disease, a statistically significant difference was found in the number of blood vessels in the healing group and in the diameters of blood vessels in the destructive group when compared with the control.³¹ In the present study, no differences in the VEGF expression between the control and test samples were found; the same pattern of VEGF expression, that is, high intensities close to the newly formed bone and low intensities next to the mature bone, was present. Moreover, in the regenerated sites where the highest mean MVD values were found, there was also a slightly higher amount of newly formed bone.

In all the specimens in the present study, the newly formed bone was present. Most of the implanted material was surrounded by bone. The bone was observed in tight contact with the implanted material, and no gaps or fibrous tissues were observed at the interface. Further-

more, no inflammatory cell infiltrate or foreign body giant cells were observed, and these findings support the good biocompatibility of this material. The percentage of newly formed bone was similar to the values reported using other xenografts of different origin.^{41,42}

In conclusion, the results of the present study show that the equine graft material used was biocompatible, and its usage was associated with new blood vessels ingrowth during healing, which has been found to be extremely important in bone formation. Within the limits of the small sample size, these findings show that the equine spongy material used in the present study can be safely and successfully used to perform mandibular ridge augmentations. Further studies on well-controlled animal models need to be carried out for a systematic evaluation of this new bone substitute material.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Rachel Sammons (University of Birmingham, School of Dentistry, Birmingham, UK) for the English review and revision of the paper.

This work was partially supported by the National Research Council (CNR), Rome, Italy, and by the Ministry of the Education, University, Research (MIUR), Rome, Italy.

REFERENCES

1. Cranin AN, Demirdjan E, DiGregorio R. A comparison of allogeneic and autogenous iliac monocortical grafts to augment the deficient alveolar ridge in a canine model. I. Clinical study. *J Oral Implantol* 2003; 29:124–131.
2. Brugnami F, Then PR, Moroi H, Kabani S, Leone CW. GBR in human extraction sockets and ridge defects prior to implant placement: clinical results and histologic evidence of osteoblastic and osteoclastic activities in DFDBA. *Int J Periodontics Restorative Dent* 1999; 19:259–267.
3. Von Arx T, Wallkamm B, Hardt N. Localized ridge augmentation using a titanium micromesh: a report on 27 implants followed from 1 to 3 years after functional loading. *Clin Oral Impl Res* 1998; 9:123–130.
4. Parodi R, Carusi G, Santarelli G, Nanni F. Implant placement in large edentulous ridges expanded by GBR using a bioresorbable collagen membrane. *Int J Periodontics Restorative Dent* 1998; 18:267–275.
5. Doblin JM, Salkin LM, Mellado JR, Reedman AL, Stein MD. A histologic evaluation of localized ridge augmentation utilizing DFDBA in combination with e-PTFE membranes and stainless steel bone pins in humans. *Int J Periodontics Restorative Dent* 1996; 16:121–129.

6. McCarthy C, Patel RR, Wragg PF, Brook IM. Dental implants and onlay bone grafts in the anterior maxilla: analysis of clinical outcome. *Int J Oral Maxillofac Implants* 2003; 18:238–241.
7. Fukuda M, Takahashi T, Yamaguchi T. Bone grafting technique to increase interdental alveolar bone height for placement of an implant. *Br J Oral Maxillofac Surg* 2000; 38:16–18.
8. Esposito M, Grusovin MG, Coulthard P, Worthington HV. The efficacy of various bone augmentation procedures for dental implants: a Cochrane systematic review of randomized controlled clinical trials. *Int J Oral Maxillofac Implants* 2006; 21:699–710.
9. Schliephake H, Dard M, Planck H, Hierlemann H, Stern U. Alveolar ridge repair using resorbable membranes and autogenous bone particles with simultaneous placement of implants: an experimental study in dogs. *Int J Oral Maxillofac Implants* 2000; 15:364–373.
10. Kohal RJ, Mellas P, Hurzeler MB, Trejo PM, Morrison E, Caffesse RG. The effects of guided bone regeneration and grafting on implants placed into immediate extraction sockets. An experimental study in dogs. *J Periodontol* 1998; 69:927–937.
11. Garg AK. Grafting materials in repair and restoration. In: Lynch SE, Genco RJ, Marx EX, eds. *Tissue engineering. Applications in maxillofacial surgery and periodontics*. Chicago, IL: Quintessence Publishing; 1999: 83–101.
12. Levin L, Barber D, Betts N, MacAfee K, Feinberg S, Fonseca R. Bone induction and the biology of grafting. In: Fonseca R, Davis W, eds. *Reconstructive preprosthetic oral and maxillofacial surgery*. 2nd ed. Philadelphia, PA: W.B. Saunders, 1995; 41–72.
13. Burchardt H. Biology of bone transplantation. *Orthop Clin North Am* 1987; 18:187–196.
14. Cricchio G, Lundgren S. Donor site morbidity in two different approaches to anterior iliac crest bone harvesting. *Clin Implant Dent Relat Res* 2003; 5:161–169.
15. Clavero J, Lundgren S. Ramus or chin grafts for maxillary sinus inlay and local onlay augmentation: comparison of donor site morbidity and complications. *Clin Implant Dent Relat Res* 2003; 5:154–160.
16. Yildirim M, Spiekermann H, Biesterfeld S, Edelhoff D. Maxillary sinus augmentation using xenogenic bone substitute material Bio-Oss in combination with venous blood. A histologic and histomorphometric study in humans. *Clin Oral Impl Res* 2000; 11:217–229.
17. Traini T, Valentini P, Iezzi G, Piattelli A. A histologic and histomorphometric evaluation of anorganic bovine bone retrieved 9 years after a sinus augmentation procedure. *J Periodontol* 2007; 78:955–961.
18. Orsini G, Scarano A, Piattelli M, Piccirilli M, Caputi S, Piattelli A. Histologic and ultrastructural analysis of regenerated bone in maxillary sinus augmentation using a porcine bone-derived biomaterial. *J Periodontol* 2006; 77:1984–1990.
19. El-Sabban ME, El-Khoury H, Hamdan-Khalil R, Sindet-Pedersen S, Bazarbachi A. Xenogenic bone matrix extracts induce osteoblastic differentiation of human bone marrow-derived mesenchymal stem cells. *Regen Med* 2007; 2:383–390.
20. Li H, Springer M, Zou X, Briest A, Bunger C. Ectopic bone induction by equine bone protein extract. *Adv Exp Med Biol* 2006; 585:393–402.
21. De Marco AC, Jardini MA, Lima LP. Revascularization of autogenous block grafts with or without an e-PTFE membrane. *Int J Oral Maxillofac Implants* 2005; 20:867–874.
22. Johnstone S, Logan M. The role of vascular endothelial growth factor (VEGF) in oral dysplasia and oral squamous cell carcinoma. *Oral Oncology* 2006; 42:337–342.
23. Lakey LA, Akella R, Ranieri JP. Angiogenesis: implications for tissue repair. In: Davies JE, ed. *Bone engineering*. Toronto, Canada: Em Squared Incorporated; 2000: 137–142.
24. Said S, Parke W, Neufeld DA. Vascular supplies differ in regenerating and non regenerating amputated rodent digits. *Anat Rec A Discov Mol Cell Evol Biol* 2004; 278A:443–449.
25. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other diseases. *Nature Med* 1995; 1:27–31.
26. Eckardt H, Bundgaard KG, Christensen KS, Lind M, Hansen ES, Hvid I. Effects of locally applied vascular endothelial growth factor (VEGF) and VEGF-inhibitor to the rabbit tibia during distraction osteogenesis. *J Orthop Res* 2003; 21:335–340.
27. Carano RA, Filvaroff EH. Angiogenesis and bone repair. *Drug Discov Today* 2003; 8:980–989.
28. Sojo K, Sawaki Y, Hattori H, Mizutani H, Ueda M. Immunohistochemical study of Vascular Endothelial Growth Factor (VEGF) and Bone Morphogenetic Protein-2,-4 (BMP-2,4) on lengthened rat femurs. *J Craniomaxillofac Surg* 2005; 33:238–245.
29. Villars F, Guillotin B, Amedee T, et al. Effect of HUVEC on human osteoprogenitor cell differentiation needs heterotypic gap junction communication. *Am J Physiol Cell Physiol* 2002; 282:C775–C785.
30. Bouletreau PJ, Warren SM, Spector JA, et al. Hypoxia and VEGF up-regulate BMP-2 mRNA and protein expression in microvascular endothelial cells: implications for fracture healing. *Plast Reconstr Surg* 2002; 109:2384–2397.
31. Cetinkaya BO, Keles GC, Ayas B, Sakalliglu EE, Acikgoz G. The expression of vascular endothelial growth factor in a rat model at destruction and healing stages of periodontal disease. *J Periodontol* 2007; 78:1129–1135.
32. Yee G, Yu Y, Walsh WR, Lindeman R, Poole MD. The immunolocalisation of VEGF in the articular cartilage of sheep mandibular condyles. *J Craniomaxillofac Surg* 2003; 31:244–251.

33. Eriksson C, Nygren H, Ohlson K. Implantation of hydrophilic and hydrophobic titanium discs in rat tibia: cellular reactions on the surfaces during the first 3 weeks in bone. *Biomaterials* 2004; 25:4759–4766.
34. Spanogle JP, Miyanishi K, Ma T, Epstein NJ, Smith RL, Goodman SB. Comparison of VEGF-producing cells in periprosthetic osteolysis. *Biomaterials* 2006; 27:3882–3887.
35. Schultze-Mosgau S, Wehran F, Wichmann M, Schlegel KA, Thorwarth M. Expression of interleukin-1 beta, transforming growth factor beta-1 and vascular endothelial growth factor in soft tissue over the implant before uncovering. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2006; 101:565–571.
36. Wise GE, Yao S. Expression of vascular endothelial growth factor in the dental follicle. *Crit Rev Eukariot Gene Expr* 2003; 13:173–180.
37. Szpadarska AM, Walsh CG, Steiberg MJ, DiPietro LD. Distinct patterns of angiogenesis in oral and skin wounds. *J Dent Res* 2005; 84:309–314.
38. Kent Leach J, Kaigler D, Wang Z, Krebsbach PH, Mooney DJ. Coating of VEGF-releasing scaffolds with bioactive glass for angiogenesis and bone regeneration. *Biomaterials* 2006; 27:3249–3255.
39. Duda DG, Cohen KS, Scadden DT, Jain RK. A protocol for phenotypic detection and enumeration of circulating endothelial cells and circulating progenitor cells in human blood. *Nat Protoc* 2007; 2:805–810.
40. Strocchi R, Orsini G, Iezzi G, et al. Bone regeneration with calcium sulfate: evidence for increased angiogenesis in rabbits. *J Oral Implantol* 2002; 28:273–278.
41. Degidi M, Artese L, Rubini C, Perrotti V, Iezzi G, Piattelli A. Microvessel density and vascular endothelial growth factor expression in sinus augmentation using Bio-Oss. *Oral Diseases* 2006; 12:469–475.
42. Degidi M, Artese L, Rubini C, Perrotti V, Iezzi G, Piattelli A. Microvessel density in sinus augmentation procedures using anorganic bovine bone and autologous bone: 3 months results. *Implant Dent* 2007; 16:317–325.
43. Artese L, Rubini C, Ferrero G, Fioroni M, Santinelli A, Piattelli A. Microvessel density (MVD) and vascular endothelial growth factor expression (VEGF) in human oral squamous cell carcinoma. *Anticancer Res* 2001; 21:689–695.
44. Yao Z, Lafage-Proust MH, Plouet J, Bloomfield S, Alexandre C, Vico L. Increase of both angiogenesis and bone mass in response to exercise depends on VEGF. *J Bone Miner Res* 2004; 19:1471–1480.
45. Aoyama J, Tanaka E, Miyauchi M, et al. Immunolocalization of vascular endothelial growth factor in rat condylar cartilage during postnatal development. *Histochem Cell Biol* 2004; 122:35–40.
46. Barou O, Mekraldi S, Vico L, Boivin G, Alexandre C, Lafage-Proust MH. Relationships between trabecular bone remodeling and bone vascularization: a quantitative study. *Bone* 2002; 30:604–612.
47. Carter DR, Loba P, Polefka EG, Beaupré GS. Mechanical influences on skeletal regeneration and bone resorption. In: Davies JE, ed. *Bone engineering*. Toronto, Canada: Em Squared Incorporated; 2000:358–368.
48. Mair B, Fuerst G, Kubitzky P, et al. The antiangiogenic substance TNP-470 impairs peri-implant bone formation: a pilot study in the rabbit metaphysis model. *Clin Oral Impl Res* 2007; 18:370–375.

Copyright of Clinical Implant Dentistry & Related Research is the property of Blackwell Publishing Limited and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.