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ORIGINAL ARTICLE

VEGF and MVD expression in sinus augmentation with autologous bone and several graft materials

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OBJECTIVE: The aim of this study was to assess vascular endothelial growth factor (VEGF) expression and microvessel density (MVD) in maxillary sinus augmentation with autogenous bone and different graft materials for evaluating their angiogenic potential.

METHODS: Biopsies were harvested 10 months after sinus augmentation with a combination of autogenous bone and different graft materials: hydroxyapatite (HA, *n* = 6 patients), demineralized freeze-dried bone allograft (DFDBA, *n* = 5 patients), calcium phosphate (CP, *n* = 5 patients), *Ricinus communis* polymer (*n* = 5 patients) and control group – autogenous bone only (*n* = 13 patients).

RESULTS: In all the samples, higher intensities of VEGF expression were prevalent in the newly formed bone, while lower intensities of VEGF expression were predominant in the areas of mature bone. The highest intensity of VEGF expression in the newly formed bone was expressed by HA ($P < 0.001$) and CP in relation to control ($P < 0.01$) groups. The lowest intensities of VEGF expression in newly formed bone were shown by DFDBA and polymer groups ($P < 0.05$). When comparing the different grafting materials, higher MVD were found in the newly formed bone around control, HA and CP ($P < 0.001$).

CONCLUSION: Various graft materials could be successfully used for sinus floor augmentation; however, the interactions between bone formation and angiogenesis remain to be fully characterized.

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Keywords: graft materials/guided bone regeneration; maxillary sinus augmentation; microvessel density; vascular endothelial growth factor

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Introduction

Therapy for bone regeneration is one of the most clinically important goals of the research in the mineralized tissue filed (Franceschi, 2005). Maxillary sinus floor augmentation has been used for occlusal rehabilitation with dental implants in the posterior maxilla (Scarano *et al*, 2006). Currently, several regenerative therapies including synthetic bone grafts, allogenic and xenogenic bone matrix and recombinant growth/differentiation factors have been used for maxillary sinus grafting (Merkx *et al*, 2003; Bosetti *et al*, 2007). However, autogenous bone grafts is still considered the 'golden standard' for bone regeneration because it contains all the components needed for regeneration, such as presence of stem cells and growth factors (Degidi *et al*, 2007; Hallman *et al*, 2001).

Maxillary sinus augmentation as well as bone regenerative procedures share similarities and both are coordinated process involving various biological factors (Huang *et al*, 2005). Indeed, many growth factors, such as fibroblast growth factor (FGF), transforming growth factor (TGF), bone morphogenetic proteins (BMP), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF), expressed during skeletal development and induced in response to injury are believed to regulate the repair of bone tissue (Degidi *et al*, 2006; Carano and Filvaroff, 2003; Bayliss *et al*, 2006). Some of these molecules are also involved in angiogenesis (i.e. FGF, TGF, VEGF) (Dai and Rabie, 2007). VEGF is probably the most important player in the vascular formation during angiogenesis (Byun *et al*, 2007). VEGF is an endothelial-specific growth factor that promotes angiogenesis by stimulating endothelial cell differentiation, proliferation, and migration (Mattuella *et al*, 2007); and plays an important role in bone remodeling by attracting endothelial cells and osteoclasts, and by stimulating osteoblast differentiation (Eriksson *et al*, 2004). The involvement of VEGF in bone formation is also suggested by its interaction with humoral factors that regulate bone homeostasis (Peng *et al*, 2002) and by its

role, not only in bone angiogenesis but also in different aspects of bone development, including chondrocyte differentiation, and recruitment of osteoblasts and osteoclasts (Dai and Rabie, 2007). Moreover, osteoblasts and osteoblast-like cells have been shown to be able to produce VEGF (Byun *et al*, 2007). VEGF is perhaps the most critical driver of vascular formation during angiogenesis and vasculogenesis, (Byun *et al*, 2007) and blood vessels are, in turn, an important component of bone formation and maintenance. Bone formation is closely linked to blood vessel invasion and therefore, the angiogenesis plays a pivotal role in all regenerative processes (Byun *et al*, 2007; Folkman, 1995; Carter *et al*, 2000; Lakey *et al*, 2000; Eckardt *et al*, 2003). VEGF may act indirectly or directly to increase recruitment of mesenchymal stem cells through an enhancement of vascular permeability, which may facilitate migration of host mesenchymal stem cells to the bone regeneration site (Keck *et al*, 1989). VEGF activity is essential for normal angiogenesis and appropriate callus formation and mineralization in response to bone injury.

The purpose of this study was an immunohistochemical evaluation of VEGF expression and microvessel density (MVD) in maxillary sinus augmentation with a combination of autogenous bone and different graft materials.

Materials and methods

Study design

A total of 34 patients participated in this study. All patients underwent maxillary sinus augmentation prior to implant placement with a mix of autogenous bone and the following graft materials: resorbable, synthetically-derived non-ceramic form of hydroxylapatite (HA; Osteogen Impladent Ltd, Holliswood, NY, USA; $n = 6$ patients), demineralized freeze-dried bone allograft (DFDBA; Dembone, Pacific Coast Tissue Bank, Los Angeles, CA, USA; $n = 5$ patients), calcium phosphate – $CaPO_4$ (CP; Bone Source, Howmedica Leibinger, Inc., Dallas, TX, USA; $n = 5$ patients), natural polymer – *Ricinus communis* polymer (Polymer – Poliquil, Polímeros Químicos LTDA, Araraquara, SP, Brazil; $n = 5$ patients). As control, autogenous bone was used ($n = 13$ patients). Inclusion and exclusion criteria were the same as in the two previously published studies (Boëck-Neto *et al*, 2002, 2005) where the same protocol was used for evaluating clinically and histomorphometrically new bone formation. All subjects signed an informed consent, which was approved by The Local Ethics Committee for Human Research. At the initial visit, the patients received a clinical and occlusal examination, and periapical and panoramic radiographs were performed to evaluate possible intrasinusal pathologies as well as bony wall morphology.

Surgical protocol

The sinus augmentation procedure was performed by using a round burr in a straight hand-piece speed is provided by the manufacturer, under copious irrigation of saline solution for outlining a large buccal window at the maxillary sinus lateral wall. Care was taken not to penetrate the sinus membrane. Once the outline was completed, a delicate dissection using blunt sinus curettes was performed to push the sinus membrane inward and upward.

The sinus membrane was released without any tension to provide an adequate compartment for the bone grafts. Autogenous bone grafts from the symphysis area were obtained via an intraoral incision from canine to canine. Two rectangular bone blocks were harvested using a straight burr under constant saline irrigation, respecting the midline. The corticocancellous bone blocks were stored in saline solution until they were ground into particles with a surgical bone mill.

The graft material was then placed into the medial aspect of the compartment created in the sinus cavity under meticulous condensation. The sinus buccal window was covered with the mucoperiosteal flap, which was then closed over by interrupted sutures.

Amoxicillin (1 g b.i.d.) was prescribed for 1 week and analgesics as required. Sutures were removed 2 weeks after surgery. At the time of implant surgery, after 10 months healing period, bone cores were harvested using a 2.0×10 mm diameter trephine burr under sterile saline solution irrigation. The bone cores were retrieved through a transcrestal route at a minimum distance of 5 mm from the nearest teeth; the dimension of the bone cores was 2×8 mm. Implants were then inserted, and the second stage surgery was carried out after an additional healing period of 4–6 months. A total of 34 bone cores were retrieved.

Specimen processing

The block biopsies were harvested, fixed in buffered formalin, and decalcified in Morse solution (Morse, 1945). Once decalcified, routine histologic processing and paraffin embedding were done and 5 μm thick tissue blocks on the longitudinal plane were obtained. For each biopsy, sections were selected in the central portion of the bone cores. The original bone crest was not evaluated histomorphometrically.

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. For unmasking 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 second-

ary biotinylated antibody. After incubation with a chromogen employing 'liquid DAB substrate pack' (BioGenex), the specimens were counterstained with Mayer's hematoxylin and coverslipped. A negative control using the secondary antibody without the primary one was used. 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. These evaluations were performed in randomly selected high power (40×) fields 10 surrounding areas of newly formed bone and 10 surrounding mature bone. Quantitative analysis was performed for VEGF. Two different VEGF staining intensities were assigned: yellow corresponding to low VEGF expression and red corresponding to high VEGF expression. The value was considered low when > 50% of the vessel area was yellow and high when > 50% was red. VEGF was evaluated by using a light microscope (Laborlux S; Leitz, 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 image capturing capacity (Image-Pro Plus 4.5; Media Cybernetics Inc., Immagine & Computer, Milan, Italy).

Immunostaining for CD31 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 3 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-CD31 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 31-related antigen was used to highlight blood microvessels; all morphologic structures with a lumen surrounded by CD 31-positive endothelial cells were considered as blood microvessels. The assessment was carried out at the level of the endothelial cells lining the vessels. Microvessels were counted using an IBAS-AT image analyzer (Kontron, Munich, Germany); for evaluation, a 40× 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 randomly selected and measurements were performed. The values were expressed as number of microvessels per square millimeter (MVD).

Histomorphometry of newly formed bone, residual particles, marrow spaces, was carried out for each case on the whole sample at low magnification (25×). Area occupied by osteoblasts and osteoclasts was measured on 10 randomized fields for each sample at a 40× magnification. These measurements were undertaken by a masked examiner (LA) 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.).

Statistical analysis

The differences between VEGF intensities of expression, MDV values, histomorphometric measurements among the different grafted groups were calculated for each biopsy and then for each group. A non-parametric analysis of variance (Kruskal–Wallis test, $P < 0.05$) and non-paired *t*-test (Mann–Whitney test, $P < 0.05$). All the measurements were expressed as a mean ± standard deviation.

Results

Histologic and histomorphometric results

After 10 months, histologic evaluation revealed the presence of mature bone with compact and cancellous areas in all the five groups examined. Only in some fields, and specifically in the vicinity of graft material particles, newly formed bone with well-organized osteons was observed. The cancellous bone as well as the compact bone exhibited incremental basophilic lines mixed with interposed reversal lines. Where cancellous bone was found, the marrow spaces were ample and frequently filled with a well-vascularized connective tissue with no signs of inflammation or foreign body reaction (Figure 1). In some cases, particles of the implanted materials were seen as irregular vacuolated amorphous masses of basophilic tendency, or as discretely eosinophilic amorphous masses, except for the control group.

The bone formation process was well identified by presence of osteoblasts. In three out of five patients implanted with CP, most of the marrow spaces were empty and only contained fragments of the material (Figure 2). The inflammatory infiltrate was on the average not significant and mononuclear cells were prevalent.

The histomorphometric data are presented in Table 1. All biopsies contained varying percentages of newly formed bone, residual particles and marrow spaces. The sinuses augmented with autogenous bone (control group) and DFDBA presented higher mean values of newly formed bone ($P < 0.05$) (Figure 3). No significant differences in the percentage of newly formed bone were found between control group *vs* DFDBA ($P > 0.05$), and among HA *vs* CP and *vs* polymer ($P > 0.05$) groups. Regarding the percentage of residual particles, HA and polymer groups presented the highest mean values ($P < 0.05$); however, the percentage of marrow spaces were not significant among the groups ($P > 0.05$) (Figure 4).

Table 1 Mean values and standard deviations of newly formed bone, residual particles, marrow spaces, osteoblasts and osteoclasts present in all graft material groups

Histometric variables	*Control (C,* n = *13)*	*HA (*n = *6)*	*DFDBA (*n = *5)*	*Calcium phospate (*n = *5)*	*Polymer (*n = *5)*
Newly formed bone (%)*	39.04 ± 1.54[b]	30.15 ± 1.6[a]	39.36 ± 1.5[b]	34.2 ± 1.9[b]	25.2 ± 1.7[a]
Residual particles (%)*	19.8 ± 1.9[a]	30.12 ± 3.8[b]	19.8 ± 1.7[a]	16.9 ± 1.3[a]	39.0 ± 1.4[b]
Marrow spaces (%)[ns]	39.6 ± 1.9[a]	39.7 ± 1.4[a]	39.7 ± 1.5[a]	39.3 ± 2.0[a]	39.4 ± 1.5[a]
Osteoblasts*	13.2 ± 1.6[a]	19.4 ± 3.6[c]	8 ± 1.7[a]	13 ± 1.5[b]	9 ± 1.4[a]
Osteoclasts*	4.6 ± 1.8[a]	8.2 ± 1.9[b]	3 ± 0.89[a]	6.2 ± 1.3[b]	3.8 ± 0.7[a]

DFDBA, demineralized freeze-dried bone allograft; HA, hydroxyapatite.
Significance of difference within the group was tested using Mann–Whitney test (*$P < 0.001$). Different letters next to mean ± s.d. indicate statistically significant differences in the distribution of histometric variable results during experimental design. ns, no significant $P > 0.05$.

Immunohistochemical results

Microvessel density

The MVD count was performed in the areas surrounding newly formed bone and mature bone. These vessels were distributed in the peripheral and central portions of marrow spaces (Figure 5). In all the groups examined, greater values of MVD were always found in the peripheral areas of marrow spaces, specifically, in the proximity of newly formed bone. When comparing the different grafting materials, the highest MVD in the newly formed bone were found in the control, HA and CP groups ($P < 0.001$), with statistically significant differences among HA *vs* polymer groups ($P < 0.01$), control *vs* DFDBA ($P < 0.05$) and, control *vs* polymer ($P < 0.01$). Regarding MVD in the areas surrounding mature bone significant differences were found among control *vs* DFDBA and control *vs* polymer ($P > 0.05$).

VEGF

Vascular endothelial growth factor was expressed in all groups with different intensities of expression among the groups. The evaluation was performed at the level of the area surrounding newly formed and mature bone. In all the groups, higher intensities of VEGF expression were prevalent in the newly formed bone, while lower intensities of VEGF expression were predominant in the areas of mature bone, indicating that VEGF expression was enhanced in the front of osteoconduction. The group showing the highest intensities of VEGF expression in the newly formed bone was the HA group, but statistically significant differences were only found between HA *vs* DFDBA and HA *vs* polymer ($P < 0.05$) (Figure 6). Also lower intensities of VEGF were mainly expressed by HA group, however, significant differences were found among control *vs* DFDBA, control *vs* polymer and, HA *vs* polymer ($P < 0.05$) (Table 2).

Discussion

The majority of studies in maxillary sinus floor augmentation have focused on new bone formation around several graft materials (Scarano *et al*, 2006; Hallman *et al*, 2001; Boëck-Neto *et al*, 2002, 2005). In our study, we evaluated the relationship between angiogenesis and bone formation after maxillary sinus augmentation with several graft materials. Angiogenesis is an important process in wound healing. The degree of vascularization seems to be the key of the success of any bone graft procedure. Blood vessels are essential for transport of nutrients and oxygen, as also as an important way to deliver circulating osteogenic factors and stem cells in the bone (Costa *et al*, 2004). Therefore, formation of an appropriate vascular bed is needed to support the bone forming mass and to ensure bone mass maintenance (Degidi *et al*, 2007; Huang *et al*, 2005; Degidi *et al*, 2006).

The present study shows that angiogenesis and bone formation seem to be related. VEGF and MVD were

Table 2 Mean values and standard deviations of intensity of expression of MVD and vascular endothelial growth factor (VEGF) present in all graft material groups

Immunohistochemical variables	*Control (C,* n = *13)*	*HA (*n = *6)*	*DFDBA (*n = *5)*	*Calcium phosphate (*n = *5)*	*Polymer (*n = *5)*
MVD*					
Area surrounding newly formed bone	39.08 ± 0.5[a]	29.2 ± 3.0[a]	21.8 ± 1.9[b]	31.8 ± 1.9[a]	18.8 ± 2.3[b]
Area surrounding mature bone	21.6 ± 0.3[a]	13.2 ± 2.7[b]	7.4 ± 1.1[b]	10.6 ± 1.1[b]	7.4 ± 1.1[b]
VEGF*					
Area surrounding newly formed bone					
High intensity	32.8 ± 1.0[a]	38.2 ± 2.3[a]	13 ± 1.1[b]	31 ± 1.5[a]	14.2 ± 1.3[b]
Low intesity	13 ± 0.0[a]	14.8 ± 1.9[a]	9.9 ± 2.0[b]	11.6 ± 1.1[a]	9.3 ± 1.3[b]
Area surrounding mature bone					
High intensity	14.8 ± 0.0[a]	11 ± 1.5[a]	7.2 ± 1.3[b]	9.6 ± 1.9[b]	7.8 ± 0.8[b]
Low intesity	31.50 ± 2.1[a]	22.8 ± 2.3[b]	14.4 ± 1.9[c]	20.2 ± 1.9[b]	13.4 ± 1.8[c]

DFDBA, demineralized freeze-dried bone allograft; HA, hydroxyapatite.
Significance of difference within the group was Tested using Mann–Whitney Test (*$P < 0.001$). Different letters next to mean ± s.d. indicate statistically significant differences in the distribution of histometric variable results during experimental design. ns, no significant $P > 0.05$. Two different VEGF staining intensities were assigned: low (yellow) and high (red).

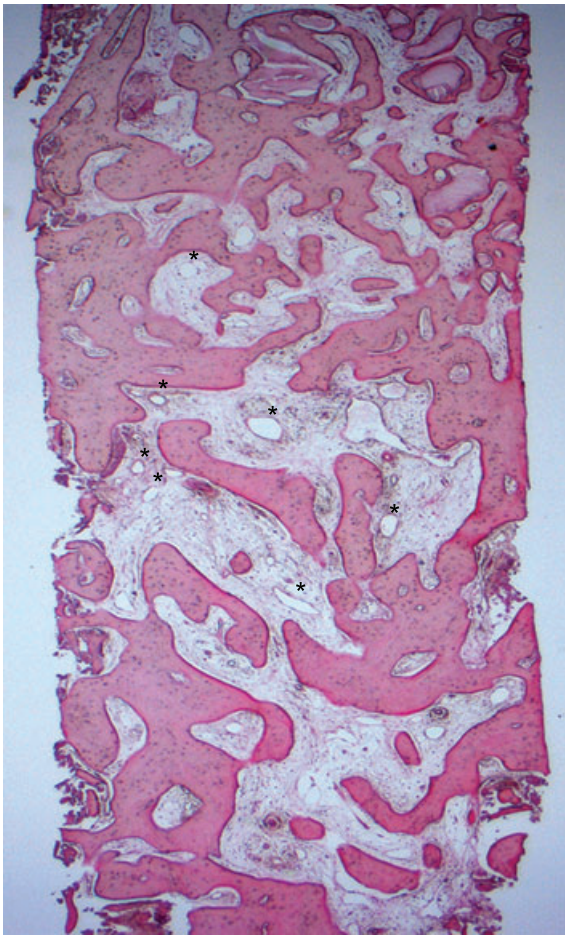

Figure 1 Regularly distributed vascular structures (*) located in marrow spaces are observed. No inflammatory cells are detected. H&E 25×

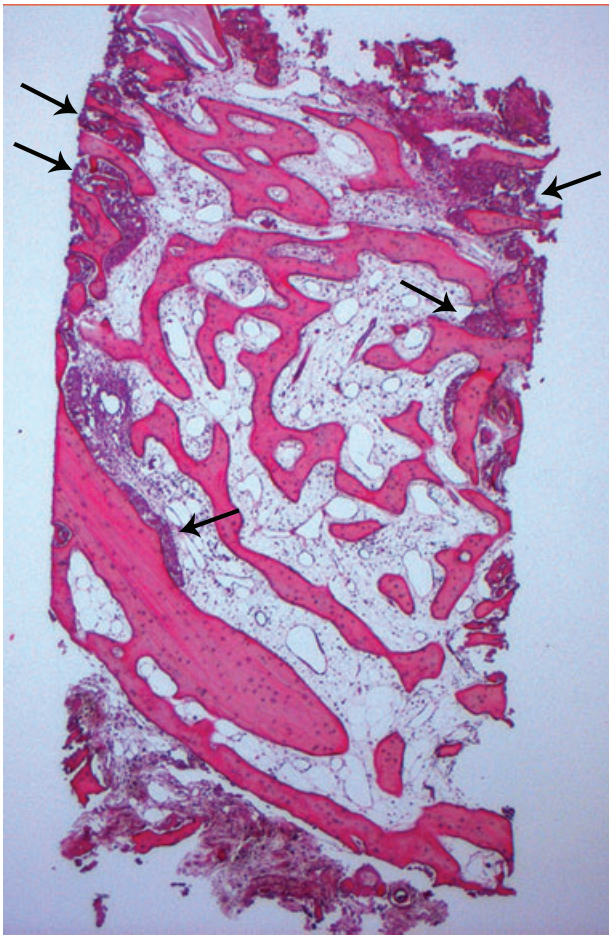

Figure 2 Calcium phosphate. It is possible to observe newly formed bone, while residual particles (arrows) in contact to bone tissue are very scarse. H&E 25×

expressed in all biopsies with different intensities, but higher values were mainly found in the newly formed bone around biomaterial particles. In general, the sinuses augmented with HA and calcium phosphate presented very similar results for the histomorphometric and immunohistochemical variables, when compared

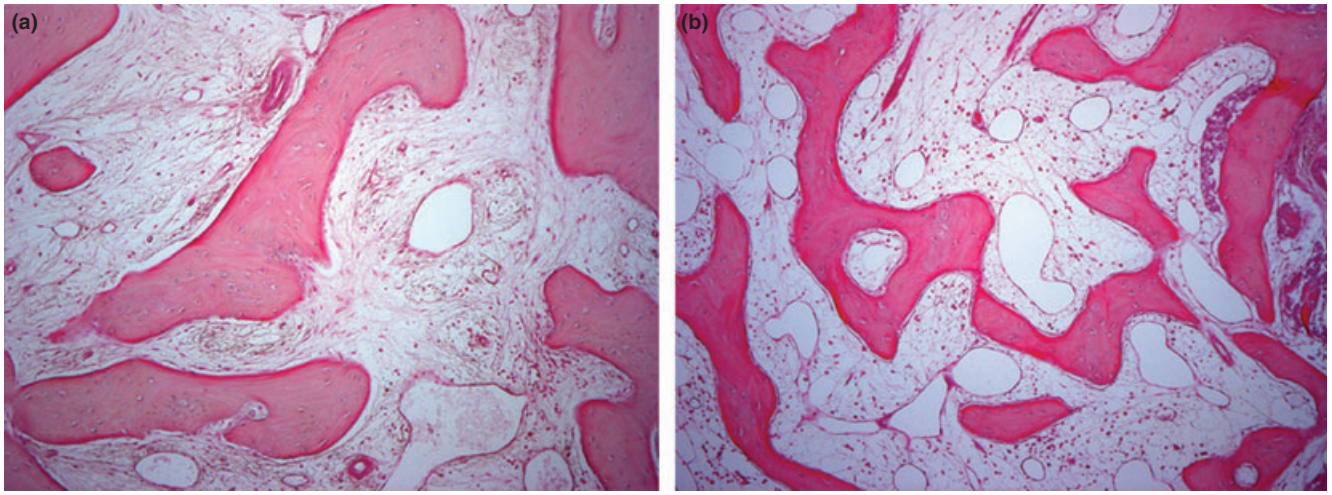

Figure 3 The sinuses augmented with autogenous bone (**a**) and demineralized freeze-dried bone allograft (**b**) presented high means of newly formed bone. H&E 100×

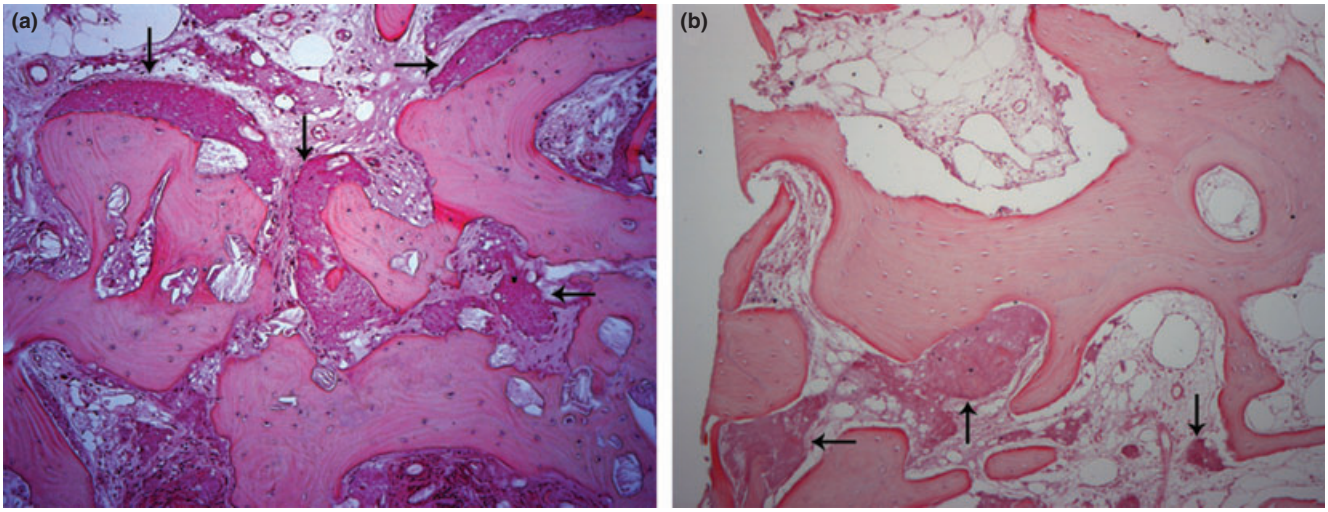

Figure 4 High percentage of residual particles (arrows) were shown by hydroxyapatite (**a**) and polymer groups (**b**). H&E 100×

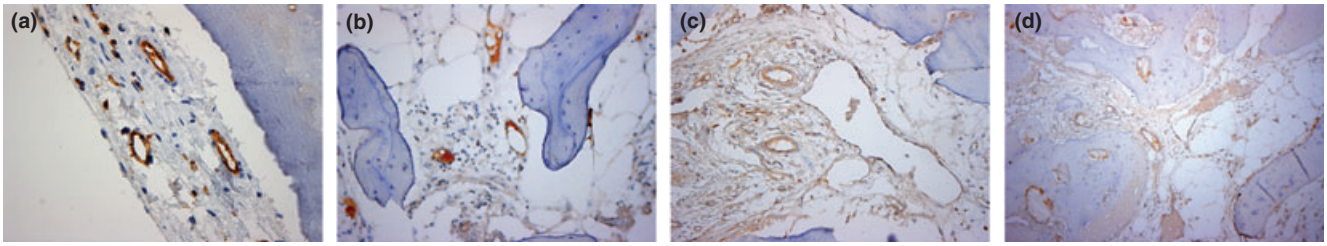

Figure 5 (**a**) Control group showing the highest intensity of vascular endothelial growth factor expression in the newly formed bone followed by hydroxyapatite, (**b**) demineralized freeze-dried bone allograft (**c**) and polymer. (**d**) Vascular endothelial growth factor staining (alkaline phosphatase–anti-alkaline phosphatase method) 200×

with the control group (autogenous bone only). Both the MVD count and the VEGF expression were higher in HA, calcium phosphate and control group. These results support the hypothesis that VEGF seems to be essential for blood vessel invasion of the injury surgical site (Degidi *et al*, 2006; Carano and Filvaroff, 2003).

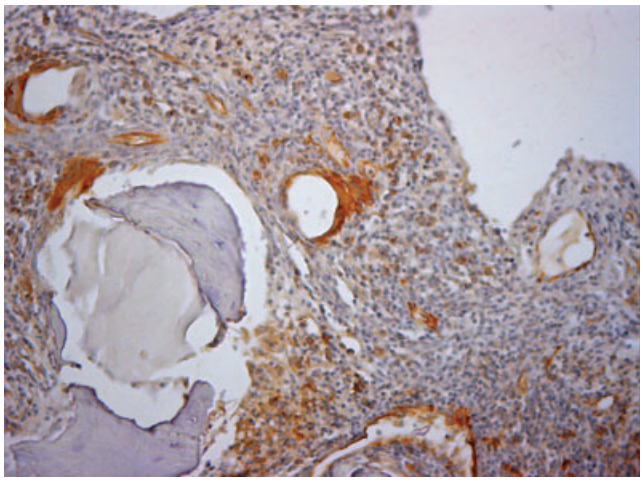

Figure 6 Marrow spaces show CD31+ microvessels (*), located predominantly in the connective tissue. CD31 staining (alkaline phosphatase–anti-alkaline phosphatase method) 200×

In addition, VEGF expression seems to be a major mechanism that links angiogenesis and new bone formation. In this study, newly formed bone was always in close contact with the newly formed blood vessels, in agreement with previous reports (Degidi *et al*, 2006; Eckardt *et al*, 2003) where a close spatial relationship between angiogenesis and osteogenesis was detected. De Marco *et al* (2005) pointed out the importance of blood supply to promote bone growth and graft substitution and they found that the presence of vascular sprouts from the recipient bed was intimately related to the development of new bone. The present 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 pre existing bone and the newly formed bone. In all the groups examined, lower intensities of VEGF expression and lower MVD values were prevalent in the areas surrounding mature bone when compared with newly formed bone. These findings indicate a spatial correlation, between angiogenesis and new bone formation; the expression area of VEGF moved in accordance with the front edge of bone formation.

It has been shown that osteoblasts and osteoblast-like cells can produce VEGF (Byun *et al*, 2007) and, in turn, VEGF could couple angiogenesis and osteogenesis by manipulating the angiogenic response to osteoblast

activity (Dai and Rabie, 2007). In a study by Amir *et al* (2006) it was found that formation of new bone during vertical distraction osteogenesis of the human mandible was related to the presence of blood vessels. A positive correlation was found between the density of blood vessels and the formation of bone in six patients.

This study point out that the success of any bone grafting procedure is related to the degree of vascularization and that the angiogenic potential of these materials is strongly correlated to their potential to promote bone formation. Indeed, our results clearly show that the materials that were more associated with new vessels ingrowths were also the ones showing a greater new bone formation. Moreover, these findings confirm that various bone graft materials could be successfully used for maxillary sinus floor augmentation. The interactions between bone formation and angiogenesis remain to be fully characterized and, in the future, more studies will certainly be necessary to evaluate and elucidate this problem.

Author contributions

Rodolfo Jorge Boëck-Neto: Surgery and study design, Luciano Artese: immunohistochemical analysis, Adriano Piattelli: histological and histomorphometrical analysis, Jamil Awad Shibli: Surgery, Vittoria Perrotti: data analysis, statistical analysis and paper writing, Marcello Piccirilli: specimen processing for histology and immunohistochemistry, Elcio Marcantonio Jr: Surgery and critical revision of the paper.

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