

Use of Bovine Hydroxyapatite With or Without Biomembrane in Sinus Lift in Rabbits: Histopathologic Analysis and Immune Expression of Core Binding Factor 1 and Vascular Endothelium Growth Factor

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Purpose: Considering the clinical discussion on the necessity of using a barrier membrane in the osteotomy area of sinus lift procedures to prevent fibrous tissue formation in this area and as a physical limit, the aim of this study was to analyze and compare the use of bovine hydroxyapatite (HA) with and without a biologic membrane by histopathologic analysis and immune expression of core binding factor 1 and vascular endothelium growth factor in the sinus lift in rabbits.

Materials and Methods: Sixteen male rabbits underwent bilateral sinus lift procedures and were divided into 2 groups according to the sinus filling material: group 1 received bovine HA (Bio-Oss; Geistlich Pharma AG, Wohlhusen, Switzerland) and group 2 received bovine HA and a nonporous polytetrafluorethylene membrane. All groups were sacrificed after 7, 14, 30, and 60 days for microscopic, histomorphometric, and immunohistochemical analyses.

Results: Microscopic analysis showed a similar bone repair pattern between the tested groups. New bone formation, soft tissue, and the remaining material were analyzed by histomorphometric analysis. No statistically significant differences ($P > .05$) were detected between groups for all periods analyzed. In addition, no remarkable differences were noticed in core binding factor 1 or vascular endothelium growth factor immune expression.

Conclusion: Taken together, these results show that using a biologic membrane does not improve bone repair induced by bovine HA, as shown by histopathologic and immunohistochemical analyses.

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The placement of endosseous implants in edentulous areas of the jaw is frequently limited by inadequate bone volume of the residual ridge.¹ Various techniques have been suggested to augment the residual ridge and prepare the site for implant installation.² Maxillary sinus lift is an established surgical procedure indicated to improve posterior maxillary bone height when enough bone is not present for the installation of endosseous implants. Although autogenous bone graft is still considered the criterion standard graft material for several reasons published elsewhere, natural and synthetic biomaterials are being used for this purpose, with variable results.^{3,4} Particulate material is preferred for filling cavities, serving as a scaffold that promotes a tridimensional matrix to stabilize and maintain the shape of the filled area. It also permits and supports cell migration and angiogenesis, resulting in new bone formation during repair.^{5,6} Most of these so-called bone substitutes present osteoconductive capacity, whereas only a few are osteoinductive. Satisfactory results have highlighted some biomaterials in dental implantology, such as bovine hydroxyapatite (HA).⁷⁻⁹

Guided tissue regeneration, a biologic treatment concept, is aimed at ensuring that cells with the capacity to regenerate a particular type of lost or diseased tissue are allowed to populate the defect/wound during healing, for example, by a physical barrier such as a membrane.¹⁰ Growing evidence suggests that the most appropriate method of treating intraosseous defects is combined therapeutic management involving bone augmentation with a biomaterial and its simultaneous covering with a barrier membrane.¹¹ This is because the method is based on the cumulative regenerative potentials of the materials and their mechanical maintenance at the site of the defect. The biomaterial supports the barrier membrane, thereby preventing its collapse, and the barrier membrane stabilizes the material and ensures its protection at the entrance site.¹² Tawil and Mawla,¹³ considering the need of covering the lateral ostomy site in sinus lift procedures, investigated 29 patients with 61 Brånemark system implants installed in sinuses grafted with Bio-Oss uncovered and covered with Bio-Guide membrane and found that the use of the membrane seemed to improve the quality of graft healing and the survival rate of the implants loaded 6 to 9 months after installation.

However, to the best of our knowledge, there are no studies that have addressed if, and to what extent, with the aid of molecular biology, biologic membranes could improve bone repair associated with HA in the sinus lift in rabbits. Therefore, this study is justified, as are others.

Several proteins and genes are involved in bone formation and repair. Different from osteoinductive

molecules, such as bone morphogenetic proteins from the transforming growth factor β superfamily, involved in the recruitment and differentiation of pluripotential mesenchymal-like cells,¹⁴ core binding factor 1 (Cbfa-1) is a transcription factor necessary for the activation of osteoblast differentiation, regulating the genes responsible for the synthesis of bone-specific proteins.¹⁵ Expression of Cbfa-1 is related to osteoblast transition from the proliferative to the differentiation cell phase.¹⁶

Another important molecule is vascular endothelium growth factor (VEGF). The production of VEGF is the main mechanism that associates angiogenesis and osteogenesis during bone repair.¹⁷ Different cell types can secrete VEGF, including osteoblasts. According to Wang et al,¹⁸ it indirectly induces osteoblast proliferation and differentiation by stimulating endothelial cells to produce osteoanabolic growth factors. Also, with fibroblastic growth factor, it is known to stimulate the production of proteases and plasminogen activators by endothelium cells, degrading the vascular basal membrane, and permitting proliferation and migration of endothelial cells. These events lead to revascularization, which is crucial for bone tissue repair.^{19,20} In addition, there is evidence that VEGF produces a chemotactic effect on osteoblasts.²¹

With this information in mind, the present study investigated the expression of Cbfa-1 and VEGF in the reconstructed maxillary sinus with and without barrier membranes to analyze the biologic relevance of this mechanical barrier on tissue repair when using HA as a bone substitute.

Materials and Methods

Sixteen adult male New Zealand rabbits, with a mean weight of 3.5 kg, underwent sinus lift surgical procedures and were divided into 2 groups according to the graft material: group 1 received bovine HA (Bio-Oss; Geistlich Pharma AG, Wohlhusen, Switzerland) and group 2 received bovine HA (Bio-Oss) and a nonporous polytetrafluorethylene membrane (Gore-Tex; W.L. Gore and Associates, Elkton, MD), as previously established by our group.²²

SURGICAL PROCEDURE AND EXPERIMENTAL GROUPS

All experimental protocols used in this study were approved by the ethical committee for animal research of Sagrado Coração University (Bauru, SP, Brazil). At the beginning of the experimental design, all animals underwent surgical procedures for the performance of bilateral sinus lift procedures. General anesthesia was obtained by intramuscular administration of 1% ketamine (Francotar; Virbac Ltda, São Paulo, Brazil) and a sedative, 2% chloridrate of xy-

lazine (Virbaxyl 2%, Virbac Ltda), in the recommended dose. Local anesthesia was also performed with 2% mepivacaine and adrenaline (1:100,000) to reduce bleeding in the surgical site. The sinus lift surgical procedure was performed according to Xu et al.²³ A trephine bur with an internal diameter of 5 mm was used to delineate the diameter of the bone window for maxillary sinus access. The osteotomy proceeded with a round diamond bur under copious irrigation with saline solution. The sinus membrane was carefully elevated, permitting the insertion and condensation of the graft materials that were mixed with venous blood for better agglutination. Nonporous polytetrafluorethylene membrane (Gore-Tex) was used in group 2 only. Afterward, the tissues were repositioned and sutured.

HISTOLOGIC PROCEDURES

The animals were sacrificed 7, 14, 30, and 60 days after surgery with an overdose of anesthetics, and the sinuses were retrieved en bloc. The specimens were immediately fixed in 10% formalin (Merck, Darmstadt, Germany) for 48 hours, washed in tap water for 24 hours, and immersed in buffered 4% ethylenediamine-tetraacetic acid for demineralization. Longitudinal semiserial histologic slices were obtained from the specimens, so that the entire sinus could be visualized, and stained with hematoxylin and eosin and Masson trichrome.

MORPHOMETRIC ASSESSMENT

Five regions of the maxillary sinus from each sample stained by Masson trichrome were blindly analyzed by 1 expert observer at 10× magnification. The images were digitally captured (Eclipse 80i; Nikon, Tokyo, Japan) and visualized with Image Pro-Plus 5.1 for Windows (Media Cybernetics Inc, Silver Spring, MD). The areas were expressed in square micrometers and the obtained measurements were summed, representing the total area of each sinus.

IMMUNOHISTOCHEMISTRY

Paraffin was removed with xylene from 4- μ m serial sections and the sections were rehydrated in graded ethanol and then pretreated in a microwave with citric acid buffer 0.01 mol/L (pH 6) for 3 cycles of 5 minutes each at 850 W for antigen retrieval. The material was preincubated with 0.3% hydrogen peroxide in phosphate buffered saline (PBS) solution for 5 minutes for inactivation of endogenous peroxidase and then blocked with 5% normal goat serum in PBS solution for 10 minutes. The specimens were then incubated with anti-Runx2 monoclonal primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 1:200 or anti-VEGF2 monoclonal primary antibody (Santa Cruz Biotechnology) at a con-

centration of 1:200. Incubation was carried out overnight at 4°C within a refrigerator. This was followed by 2 washes in PBS for 10 minutes. The sections were then incubated with a biotin-conjugated, secondary antirabbit immunoglobulin G antibody (Vector Laboratories, Burlingame, CA) at a concentration of 1:200 in PBS for 1 hour. The sections were washed twice with PBS followed by the application of a preformed avidin-biotin complex conjugated to peroxidase (Vector Laboratories) for 45 minutes. The bound complexes were visualized by the application of a 0.05% solution of 3-3'-diaminobenzidine solution and counterstained with Harris hematoxylin. For control studies of the antibodies, the serial sections were treated with rabbit immunoglobulin G (Vector Laboratories) at a concentration of 1:200 in place of the primary antibody. In addition, internal positive controls were performed with each staining bath.

STATISTICAL ASSESSMENT

Results obtained from the morphometric analysis were subjected to nonparametric tests using analysis of variance and post hoc Tukey test, with *P* less than .05 considered statistically significant.

Results

MICROSCOPIC ANALYSIS

At 7 days, group 1 showed biomaterial granules inside the maxillary sinus that were surrounded by a highly vascularized granulation tissue, presenting a moderate mononuclear inflammatory infiltrate. Osteogenic activity was more evident in the bone walls (Fig 1). In group 2, biomaterial granules surrounded by granulation tissue were observed inside the sinus.

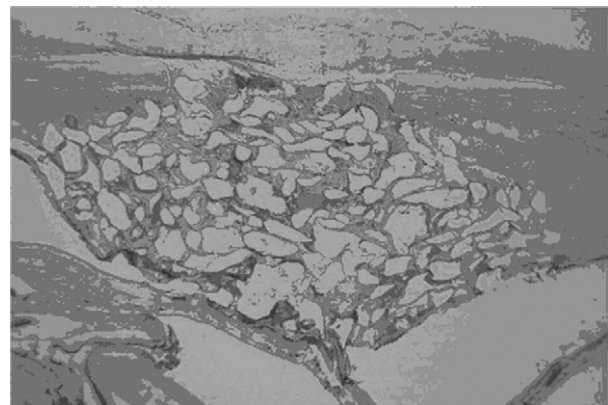


FIGURE 1. Photomicrograph showing bovine hydroxyapatite 7 days after surgery (hematoxylin and eosin stain, ×20 magnification).

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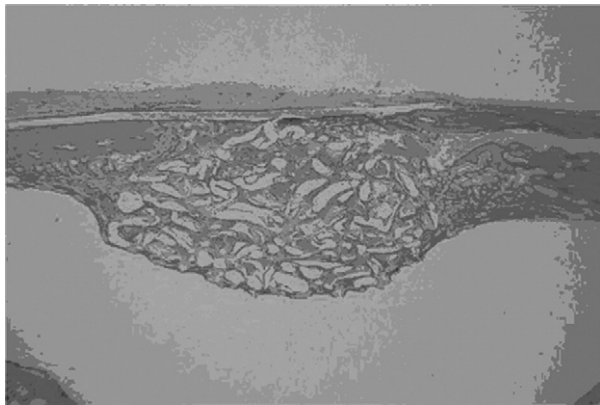


FIGURE 2. Photomicrograph showing bovine hydroxyapatite and biomembrane 14 days after surgery (hematoxylin and eosin stain, ×20 magnification).

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Discrete areas of osteogenesis were observed in the periphery of the sinus.

At 14 days, in group 1, particles of biomaterial surrounded by a highly vascularized connective tissue were seen, presenting slight osteogenic activity, with primary bone deposition especially in the biomaterial surfaces, coming from the sinus walls. In group 2, round granules of biomaterial surrounded by granulation tissue were observed, with some deposition of primary bone (Fig 2).

At 30 days, group 1 showed well organized bone trabeculas surrounding the biomaterial granules. In group 2, close to the material granules, remodeled bone was observed. Eventually, areas of osteogenic activity could be observed.

At 60 days, group 1 showed mature bone on the surface of the biomaterial granules. In group 2, remodeled trabeculas were visualized, marked by basophilic reversal lines. Medullar tissue showed intense vascularization.

MORPHOMETRIC DATA

Regarding newly formed bone, the data revealed no statistically significant differences ($P < .05$) between groups for all periods analyzed. The same was true for

Table 1. STATISTICAL RESULTS FROM HISTOMORPHOMETRIC EVALUATION AFTER 7 DAYS

	New Bone (%)	Soft Tissue (%)
Group 1	5.2 ± 4.8 ^a	48.85 ± 20.04 ^a
Group 2	3.4 ± 3.6 ^a	44.45 ± 31.8 ^a

Different superscript letters indicate significant statistical differences ($P < .05$).

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Table 2. STATISTICAL RESULTS FROM HISTOMORPHOMETRIC EVALUATION AFTER 14 DAYS

	New Bone (%)	Soft Tissue (%)
Group 1	14.22 ± 3.2 ^a	25.22 ± 9.4 ^a
Group 2	10.10 ± 10.6 ^a	21.88 ± 5.3 ^a

Different superscript letters indicate significant statistical differences ($P < .05$). Remaining material in group 1 represents autogenous bone graft.

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soft tissue, ie, no significant differences were noted for all periods established in this study. These findings are presented in Tables 1 to 4.

IMMUNOHISTOCHEMISTRY

In the group exposed to bovine HA, Cbfa-1 immune expression was seen surrounding cells adjacent to the biomaterial with a strong pattern at the initial periods evaluated. The same pattern was found for the group treated with bovine HA and the membrane.

Regarding VEGF immunohistochemistry, Bio-Oss with or without the membrane indistinctly displayed VEGF expression after tissue repair compared with the control group.

Discussion

The various available biomaterials indicated for the maxillary sinus lift procedure present different biologic behaviors according to their origin, shape, size, porosity, and degradation rate. These differences act directly on the rate and timing of bone formation. To elucidate some aspects of this behavior, the present study observed the process of new bone formation in reconstructed maxillary sinuses using a known bioactive material, bovine HA, with or without a biological membrane, by histopathologic analysis and immunohistochemistry, focusing on vascularization and osteoblast differentiation. Although the biomaterial presented satisfactory clinical results, being

Table 3. STATISTICAL RESULTS FROM HISTOMORPHOMETRIC EVALUATION AFTER 30 DAYS

	New Bone (%)	Soft Tissue (%)
Group 1	14.13 ± 3.2 ^a	22.30 ± 5.4 ^a
Group 2	16.05 ± 2.5 ^a	27.12 ± 5.4 ^a

Different superscript letters indicate significant statistical differences ($P < .05$).

Nunes et al. Bovine Hydroxyapatite and Membrane. J Oral Maxillofac Surg 2010.

Table 4. STATISTICAL RESULTS FROM HISTOMORPHOMETRIC EVALUATION AFTER 60 DAYS

	New Bone (%)	Soft Tissue (%)
Group 1	47.0 ± 12.13 ^a	10.52 ± 2.26 ^a
Group 2	53.2 ± 8.4 ^a	16.7 ± 8.46 ^a

Different superscript letters indicate significant statistical differences ($P < .05$).

Nunes et al. *Bovine Hydroxyapatite and Membrane. J Oral Maxillofac Surg* 2010.

well accepted and largely used by the dental implantology community, our hypothesis is that some differences may exist in tissue repair when using biologic membranes.

Bio-Oss is a low-resorbable deproteinized bovine xenograft, chemically and physically identical to human bone, in the form of cortical granules, presenting 75% to 80% porosity and a large mesh interconnecting macro- and micropore system that facilitates angiogenesis and osteoblast migration.²⁴ Some investigators have stated that the brief presence of Bio-Oss incorporated to bone creates a dense cancellous network by strengthening the bone tissue mass and improving its ability to withstand the loading forces transmitted by dental implants.²⁵ Orsini et al,²⁴ using light microscopy and scanning and transmission electron microscopies, described in detail the contact between Bio-Oss and bone tissue in the healing of human sinuses 6 months after the procedure, highlighting that the bone adhered tightly to the surface of the biomaterial and the presence of an electron-dense layer similar to cement lines or lamina limitans in the particles of the biomaterial. The present results clearly show similar findings between bovine HA with and without a biologic nonporous polytetrafluorethylene membrane after bone repair in rabbits. Our results are in line with other findings describing that exposure to Bio-Oss collagen and Bio Gide Perio membrane in the femurs of rabbits was able to improve bone repair compared with nonfilled bone defects.¹¹ Adeyemo et al¹ also argued that autogenous bone graft covered with Bio-Oss particles resulted in a remarkable increase in the augmented lateral surface of the mandible. Moreover, Stavropoulos et al²⁶ concluded that the membrane material per se does not seem to be a critical factor for the outcome of guided tissue regeneration treatment of intrabony defects with bioresorbable membranes. Taken together, it seems that Bio-Oss improves bone repair independently when using biomembranes in rabbits.

To further elucidate the biologic behavior of these biomaterials on the cellular system, we evaluated the expression of Cbfa-1 and VEGF. Our results showed

higher Cbfa-1/Runx2 expression in the group exposed to bovine HA with or without the presence of a biomembrane. A greater cell migration in bone defects filled by Bio-Oss was also shown.²⁷ These evidences can explain the stronger immunostaining of Cbfa-1 in HA specimens for all periods of the present study, possibly maintained by the prolonged stimulus caused by the presence of HA granules. Taken as a whole, it seems that Bio-Oss with or without a biomembrane can adequately permit osteoblast differentiation after bone repair, especially at early phases in the process.

Inert implants, like some metals, make bone formation possible by permitting a mechanical adhesion to the material, called *biologic fixation*. However, the considered bioactive materials, such as the tested biomaterials, induce a biologic response in the interface area, leading to a chemical adhesion with bone, named *bioactive fixation*, because of the presence of calcium and phosphate. The surface of these materials induces a biologically active carbonated layer of HA that creates an adhesive interface with the tissues. Also, data have shown that local increases in calcium and phosphate ions stimulate osteoblastic differentiation in vitro.²⁸

However, cell migration and differentiation in healing tissue only occur if sufficient vascularization is present. Especially in bone tissue, an intimate relation exists between blood vessels and bone cells.²⁹ VEGF is involved in angiogenesis, ie, stimulating the proliferation of new capillaries from existing blood vessels,²⁰ a common situation in repair conditions. Our results revealed that bovine HA with or without a membrane was also able to promote angiogenesis in a similar pattern, ie, many capillary vessels were found. Therefore, we believe that the immune expression of VEGF found in these groups could be helpful to bone repair.

In conclusion, the present results show that the use of a biologic membrane does not improve bone repair induced by bovine HA, as shown by histopathologic and immunohistochemical analyses. However, further studies are welcomed to elucidate this issue.

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