Immunoexpression of Cbfa-1/Runx2 and VEGF in sinus lift procedures using bone substitutes in rabbits

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Abstract

Objectives: To analyze and compare the expression of core binding factor-1 (Cbfa-1)/Runx2 and vascular endothelium growth factor (VEGF) in sinus lift procedures using bovine hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP).

Material and Methods: Twenty-four male rabbits that had undergone bilateral sinus lift procedures were divided into three groups, according to the sinus filling material: Group 1: autogenous bone graft; Group 2: bovine HA; and Group 3: β-TCP. All groups were sacrificed after 7, 14, 30 and 60 days, for microscopic, histomorphometry and immunohistochemistry analysis.

Results: Microscopic analysis showed a similar bone repair pattern between the tested groups. New bone formation, soft and medular tissue, remaining material or particulate bone graft area were obtained by histomorphometric analysis. After 14 days, statistically significant differences in new bone formation were found between Group 1 (27.76/C6 7.8) and Groups 2 (14.22/C6 3.2) and 3 (11.1/C6 7.7). After 30 days, statistically significant differences (P<0.05) were detected in bone formation between Groups 1 (31.39/C6 36.5) and 2 (14.13/C6 3.2). The last period showed improved bone formation in Group 2. Also, Group 2 showed higher Cbfa-1/Runx2 immunoexpression when compared with Group 3. No remarkable differences were observed in VEGF immunoexpression among groups.

Conclusion: Taken together, both biomaterials allowed bone tissue growth in a conductive pattern and did not interfere with bone remodeling in the late period, with a slight improvement in bone tissue formation when using HA, confirmed by marked expression of Cbfa-1 at initial periods.

Maxillary sinus lift is an established surgical procedure indicated for the improvement of posterior maxillary bone height when enough bone is not present for the installation of endosseous implants. Although autogenous bone graft is still considered the gold standard graft material for a number of reasons already published elsewhere (Block & Kent 1997; Crespi et al. 2007; Gerressen et al. 2009; Sbordone et al. 2009), natural and synthetic biomaterials are being used for this purpose, with variable results (Hurzeler et al. 1997; Ozyuvaci et al. 2003; Velich et al. 2004; Orsini et al. 2005; Zijderveld et al. 2005). Particulate material is preferred for filling cavities, serving as a scaffold that promotes a tri-dimensional matrix in order to stabilize and maintain the shape of the filled area. It also allows and supports cell
migration and angiogenesis, resulting in new bone formation during repair [Spector 2008; Walsh et al. 2008]. Most of these so-called bone substitutes present osteoconductive capacity, while only a few are osteoinductive. Satisfactory results highlight some biomaterials in the field of dental implantology, such as the bovine hydroxyapatite (HA) [Hurzeler et al. 1997; Valentini & Abensur 1997; Piattelli et al. 1999; Orsini et al. 2005; Scarano et al. 2006] and β-tricalcium phosphate (β-TCP) [Lu et al. 2004; Horch et al. 2006; Somanathan & Simunek 2006].

Although a number of studies have focused on the clinical behavior of bone substitutes, only a few present the molecular mechanisms of bone formation associated with these materials [Zerbo et al. 2005; Knabe et al. 2008], taking into consideration some differences in tissue response. It is known that some molecules are directly involved in osteoblast cells’ differentiation, like the core binding factor-1 (Cbfa-1), a transcription factor necessary for the activation of this process, regulating the genes responsible for the synthesis of bone-specific proteins [Franceschi et al. 2003; Garant 2003; Afzal et al. 2004]. The expression of Cbfa-1 is related to osteoblasts’ transition from the proliferative to the differentiation cell phase [Pratap et al. 2003].

Another important molecule is the vascular endothelium growth factor (VEGF). The production of VEGF is the main mechanism that associates angiogenesis and osteogenesis during bone repair [Street et al. 2002]. Along with the fibroblastic growth factor, it is known to stimulate the production of proteases and plasminogen activators by endothelium cells, degrading the vascular basal membrane and allowing the proliferation and migration of endothelial cells. These events lead to revascularization, which is crucial for bone tissue repair [Carano & Filvaroff 2003; Hsiong & Mooney 2006]. In addition, there are evidences that VEGF exerts a chemotactic effect on osteoblasts [Mayr-Wohlfart et al. 2002].

Taking this information into consideration, the present study aimed to analyze and compare the expression of Cbfa-1/Runx2 and VEGF in sinus lift procedures using HA and β-TCP, by means of immunohistochemistry.

Material and methods

All experimental protocols involving animals conformed to procedures described in the Guiding Principles for the Use of Laboratory Animals and the study approved by the Animal Committee of University of Sacred Heart, USC.

Twenty-four adult male New Zealand rabbits, mean weight 3.5 kg, were divided into three groups, according to the sinus filling material, as follows: Group 1: Control, particulate autogenous bone graft, Group 2: Bovine HA [Bio-Oss®, Geistlich Pharma AG, Wohllhusen, Switzerland] and Group 3: β-TCP [Cerasorb®, Curasan-AG, Kleinostheim, Germany].

Surgical procedure and experimental groups

All the experimental protocols used in this study were approved by the Ethical Committee for Animal Research, Sagrado Coração University (USC), Bauru, SP, Brazil. At the beginning of the experiment, all animals underwent surgeries for bilateral sinus lift procedures. General anesthesia was induced by an intramuscular administration of 1% ketamine [Francotar, Virbac Ltda, São Paulo, Brazil], along with with a sedative, 2% chloridrate of xylazine [Virbaxyl 2%, Virbac Ltda], in the recommended dose. Local anesthesia was also administered with 2% mepivacaine with adrenalin [1 : 100,000] in order to reduce bleeding in the surgical site. The sinus lift surgical procedure was performed according to Xu et al. [2005]. A trephine bur with a 5 mm internal diameter was used to delineate the diameter of the bone window for the maxillary sinus access. Osteotomy was carried out using a round diamond bur under copious irrigation with saline solution. The sinus membrane was carefully elevated to allow the insertion and condensation of the graft materials, mixed with venous blood for better agglutination. No membrane was used to cover the bone window, and the tissues were repositioned and sutured. The animals were randomly divided into three groups according to the graft material, as follows: Group 1: Control, particulate autogenous bone graft retrieved from the nasal bone, Group 2: Bovine HA (Bio-Oss®, Geistlich Pharma AG) and Group 3: β-TCP (Cerasorb®, Curasan-AG).

Histologic procedures

After 7, 14, 30 and 60 days of the surgery, the animals were sacrificed with an overdose of anesthetics, and both the sinuses of these animals were retrieved en bloc (Fig. 1). The specimens were immediately fixed in 10% formalin [Merck, Darmstadt, Germany] for 48 h and washed in tap water for 24 h, and immersed in buffered 4%
EDTA for demineralization. Longitudinal semi-serial histological slices were obtained from the specimens so that the entire sinus could be visualized, and stained with hematoxylin–eosin and Masson trichrome.

Morphometric assessment
A total of five regions of the maxillary sinus from each sample stained by Masson trichrome were blindly analyzed by one expert observer at × 10 magnification. The images were digitally captured (Nikon – Eclipse 80i, Tokyo, Japan) and sent to the Image Pro-Plus Program – Version 5.1 for Windows (Media Cybernetics Inc., Silver Spring, MD, USA). The areas were expressed in μm² and the measures obtained were summed to represent the total area of each sinus.

Immunohistochemistry
Paraffin was removed with xylene from serial sections of 4μm and the sections were rehydrated in graded ethanol, and then pretreated in a microwave with 0.01 M citric acid buffer (pH 6) for three cycles of 5 min each at 850 W for antigen retrieval. The material was preincubated with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) solution for 5 min for inactivation of endogenous peroxidase and then blocked with 5% normal goat serum in PBS solution for 10 min. The specimens were then incubated with anti-Runx2 monoclonal primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a concentration of 1 : 200 in PBS for 1 h. The sections were washed twice with PBS, followed by the application of a preformed avidin–biotin complex conjugated to peroxidase (Vector Laboratories) for 45 min. The bound complexes were visualized by application of 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 IgG (Vector Laboratories) at a concentration of 1 : 200 in place of the primary antibody. Additionally, internal positive controls were performed with each staining bath.

Statistical assessment
All morphometric data were assessed using the Kruskal–Wallis non-parametric test, followed by Dunn’s test using SigmaStat software (Chicago, IL, USA). The level of statistical significance was set at 5 %.

Results
Microscopic analysis
7 days
During this period, control group showed irregular fragments of autogenous bone graft, showing irregular surfaces associated with a number of osteoclasts, surrounded by granulation tissue. Eventually, new bone formation was observed on the surface of some bone particles. Specimens from the experimental Groups [1 and 2] exhibited similar histological patterns, with biomaterial granules surrounded by a high vascularized granulation tissue, and with osteogenic activity predominant in the sinus bone walls.

14 days
Remodeling trabecula, marked by basophilic reversal lines, could be seen to be in close contact with the particles of autogenous bone (Fig. 2a). Osteogenic activity could still be observed in the central region of the sinus. Discrete osteogenesis was observed in the highly vascularized granulation tissue surrounding HA particles, as well as woven bone deposition on the biomaterial surface [Fig. 2b]. Round granules of β-TCP were easily identified, allowing a few depositions of woven bone (Fig. 2c).

30 days
Control sinuses were filled by remodeling bone, presenting marked reversal lines. A few particles of the grafted bone were seen, as well as discrete osteogenic areas. Highly vascularized loose connective tissue was predominant in medullar spaces. Well-organized bone trabeculae were seen surrounding the HA granules [Fig. 3a], whereas remodeling bone was observed in contact with β-TCP particles. Eventually, areas of osteogenic activity could be observed [Fig. 3b].

60 days
Sinuses filled by autogenous bone presented thin mature bone trabeculae after 60 days. Mature bone was also observed on the surface of HA biomaterial granules [Fig. 4a], whereas remodeling trabeculae were visualized in the β-TCP group,
marked by basophilic reversal lines. Med-
ular tissue showed intense vasculariza-
tion [Fig. 4b].

Histomorphometrical statistical
assessment

7 days
No statistically significant differences were
found among the groups after 7 days of the
surgery in terms of the areas of new bone
formation, soft tissue and remaining mate-
rial [Table 1].

14 days
During this period, a statistically signifi-
cant difference was observed in Group 1 in
terms of new bone formation in compar-
ison with the experimental groups. How-
ever, Group 2 presented less bone
formation, as in Group 3, but significantly
less soft tissue \(25.22 \pm 9.4\), which was
similar in Groups 1 \(55.51 \pm 7.7\) and 3
\(41.88 \pm 7.3\). Both biomaterials’ granules
were evident after 14 days [Table 2].

30 days
Particulate autogenous bone grafts were
indistinguishable at this period. Significant
bone formation was noted in Group 2
\(14.13 \pm 3.2\) compared with Groups 1
\(31.39 \pm 36.5\) and 3 \(18.05 \pm 1.4\). No
statistically significant differences were de-
tected among the experimental groups in
terms of soft tissue and remaining material
[Table 3].

60 days
In the last period, bone formation was
similar in both experimental groups, as
well as the remaining material; however,
soft tissue was significantly different be-
tween Groups 2 \(10.52 \pm 2.26\) and 3
\(40.62 \pm 8.46\) [Table 4].

Immunohistochemistry
Cbfa-1/Runx2 immunoreactivity could be
seen in medullar tissue for the control
group mainly in the early periods of this
study, i.e. 7 and 14 days after surgery [Fig.
5a]. In the group exposed to bovine HA,
Cbfa-1/Runx2 immunoexpressivity was
seen circumjacent cells to the biomaterial
with a moderate pattern for all periods
evaluated [Figs 5b and 6a, b]. In the groups
exposed to \(\beta\)-TCP, a weak Cbfa-1/Runx2
immunohistochemistry was evidenced fol-
lowing tissue repair [Fig. 5c].

In VEGF immunohistochemistry, this
could be detected in cells involving capil-
lary walls inside the defect in the control
group [Fig. 7a]. Bio-Oss™ and Cerasorb™
displayed VEGF expression following tis-
ue repair indistinctly when compared
with the control group [Fig. 7b].

Discussion
The various available biomaterials indi-
cated for the maxillary sinus lift procedure
show different biological behavior accord-
ing to their origin, shape, size, porosity and
degradation rate. It is already known that
these differences act directly on the rate
and time of bone formation. In order to
elucidate some aspects of this behavior, the present study proposed to observe the process of new bone formation in reconstructed maxillary sinuses using two bioactive materials, a bovine HA and a β-TCP, by means of immunohistochemistry, focusing on vascularization and osteoblasts’ differentiation. Although both materials have shown satisfactory clinical results, being well accepted and used extensively by the dental implantology community, it has been observed that some differences exist in tissue repair.

Bio-Oss® is a low-resorbable deproteinized bovine xenograft, chemically and physically identical to human bone, in the form of cortical granules, presenting 75–80% porosity and a large-mesh interconnecting macro- and micropore system that facilitates angiogenesis and osteoblast migration [Orsini et al. 2005], while Cerasorb® consists of 1–2-mm-diameter spherical granules of >99% of pure β tricalcium phosphate with 5–20 μm interconnecting micropores and 40–50% porosity, being absorbed by a combination of hydrolytic and cellular degradation processes [Wiltfang et al. 2002]. According to Merten et al. [2001], it is important that the interconnecting pores are not <5 μm, for faster degradation and micro-osseointegration.

One of the advantages of β-TCP over HA pointed out by Walsh et al. [2008] is its faster resorption. In fact, our results showed a higher degradation rate of β-TCP, meanwhile, lower rates of bone formation were maintained for all experimental periods, when compared with the HA group, which may not be negatively interpreted. Zerbo et al. [2003] observed positive immunostaining for osteopontin and bone sialoprotein in [pre] osteoblast, young osteocytes, and in some, the connective tissue cells around or in the particles of β-TCP in human sinus lift specimens, suggesting that these cells were following the osteogenic differentiation pathway. On the other hand, Artzi et al. [2004] reported that the low-lasting presence of Bio-Oss® incorporated into bone creates a dense cancellous network, by strengthening of bone tissue mass, and improves the ability to withstand loading forces transmitted by dental implants. Orsini et al. [2005], using light microscopy, and scanning and transmission electron microscopy, described in detail the contact between Bio-Oss® and bone tissue in the healing process of human sinuses 6 months after the procedure, highlighting tightly adherent bone 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. Our results demonstrated higher Cbfa-1/Runx-2 expression in the group exposed to bovine HA when compared with specimens exposed to β-TCP. Taken as a whole, it seems that Bio-Oss® and Cerasorb® were able to adequately allow osteoblast differentiation following bone repair, especially in the early phases of the process, with bovine HA exerting a more intense effect.

A higher cell migration in bone defects filled by Bio-Oss® was also demonstrated, when compared with non-filled defects [Tapety et al. 2004]. In the present study, in the latest periods of the healing process, 30 and 60 days, a higher statistically significant quantity of bone formation was confirmed in the HA group in comparison with the control group using an autogenous bone graft. These evidences may explain the stronger immunostaining of Cbfa-1/Runx-2 in the HA specimens than in the β-TCP specimens during all periods of our study, possibly maintained by the prolonged stimulus caused by the presence of HA granules.

Inert implants, like some metals, make bone formation possible, allowing mechanical adhesion to the material, called biological fixation. However, the bioactive materials considered, such as the tested biomaterials, induce a biological response in the interface area, leading to a chemical adhesion with bone, named bioactive fixation, due to 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 (Hench & Best 2004). Also, data have shown that a local increase in calcium and phosphate ions stimulates osteoblastic differentiation in vitro (Sugimoto et al. 1993).

However, cell migration and differentiation occur in healing tissue only if sufficient vascularization is present. Especially in bone tissue, an intimate relation exists between blood vessels and bone cells (Yeh & Lee 1999; Peng et al. 2002). VEGF is involved in angiogenesis, i.e., stimulating the proliferation of new capillaries from already existing blood vessels (Hsiong & Mooney 2006), a common situation under repair conditions. Herein, we were able to evaluate the VEGF expression in this setting. Our results revealed that bovine HA or β-TCP was also able to promote angiogenesis in a similar pattern, i.e., a huge number of capillary vessels were found. Therefore, we believed that the immunoeexpression of VEGF found in these groups could also be helpful for bone repair.

Conclusion

Although both materials yielded a satisfactory biological response, the results obtained from the present study reflect the influence of the differences in the nature and morphology of the tested biomaterials on the expression of Cbfa-1/Runx-2 and VEGF during maxillary sinus repair. Bio-Oss® allowed osteoblastic differentiation more intensely when compared with Cerasorb®, although angioblastic cells' expression was similar for both materials, leading to satisfactory bone repair. However, further studies are necessary to elucidate the molecular mechanisms involved in tissue repair in association with these biomaterials.

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