Combining Scaffolds and Osteogenic Cells in Regenerative Bone Surgery: A Preliminary Histological Report in Human Maxillary Sinus Augmentation

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ABSTRACT

Purpose: The following case series evaluated the maxillary sinus augmentation responses to tissue-engineered bone graft obtained by a culture of autogenous osteoblasts seeded on polyglycolic–polylactic scaffolds and calcium phosphate.

Materials and Methods: Sinus floor augmentation was performed bilaterally in five patients (mean age 58.4 years) with tissue-engineered bone (test site – Oral Bone®, BioTissue, Freiburg, Germany) or calcium phosphate (control site – Biocoral, Novaxa Spa, Milan, Italy). Biopsies were harvested 6 months after sinus augmentation for histometric evaluation. Volumetric measurements were taken at baseline and 6 months after the surgical procedure.

Results: The mean of vertical bone gain was 6.47 ± 1.39 mm and 9.14 ± 1.19 mm to test and control sites, respectively. The histological sections depicted mature bone with compact and cancellous areas. All biopsies contained varying percentages of newly formed bone and marrow spaces. The mean of bone tissue in the grafted area was 37.32 ± 19.59% and 54.65 ± 21.17% for tissue-engineered bone and calcium phosphate, respectively.

Conclusion: Within the limits of the present report, the histological data in humans confirmed that tissue-engineered bone and calcium phosphate allowed newly formed bone after maxillary sinus augmentation.

KEY WORDS: bone regeneration/bone grafts, bone tissue engineering, maxillary sinus augmentation

INTRODUCTION

Bone regeneration techniques are of paramount importance in modern regenerative surgery. Complete integration of the regenerate bone into vital bone structure is an essential requisite for long-term success. Bone augmentation procedures before dental implant insertion are carried out with autografts, allografts, or composite materials.[1–5] Autogenous bone has been always considered the most successful grafting material, even in difficult contexts.[6] However, the use of autogenous bone has some limitations, such as limited availability and morbidity at donor site,[7–9] which could reduce its application.[10]

The bone tissue engineering is a fusion of the recent discoveries in the field of molecular cell biology that applies the most innovative methods of the reconstructive surgery.[11] Bone tissue engineering aims to construct the ideal bone graft material, characterized by the same

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biological and structural properties of native bone[12,13] manipulating three essential elements, such as biomaterials, growth factors, and osteogenic cells. In particular, the use of biomaterials combined with osteogenic cells represents a fascinating opportunity for bone tissue engineering, with the purpose to obtain bone regeneration, even in difficult contexts, where biomaterials alone have demonstrated poor clinical performance.[14]

Therefore, the aim of this study was to evaluate the maxillary sinus augmentation grafted with an engineered bone tissue that is obtained by a culture of autogenous osteoblasts seeded on polyglycolic–polylactic (PLGA) scaffolds (Oral Bone®, BioTissue, Freiburg, Germany) or calcium phosphate (Biocoral®, Novaxa Spa, Milan, Italy).

MATERIALS AND METHODS

Patients

Five nonsmokers (two males and three females, from 45 to 64 years old, with an average age of 58.4 years), without systemic diseases and with good general health status, have been enrolled in this case series. All selected patients presented in both sides of the posterior maxilla missing teeth in the whole premolar and molar regions, with severe atrophy,[15] which enable sufficient implant anchorage. The patients received information on all proposed treatment, and they provided signed informed consent prior to treatment. Sinus floor augmentation was performed bilaterally using tissue-engineered bone graft (test site – Oral Bone) or calcium phosphate (control site – Biocoral).

Engineered Bone

In all patients, 6 weeks before surgery, a specimen (2×5 mm) of bone marrow has been taken from the posterior area of the mandible, together with 100/150 mL of venous blood sample. The specimen, preserved in a medium containing antibiotics and antimycotic solution, was then transferred to the BioTissue Technologies' laboratories (Biotissue) to be processed in a clean room. In the first 28 hours, cells were enzymatically detached by 0.1% collagenase CLSIII (clostridium hystolyticum, Bio-Chrom, Berlin, Germany) in DMEM/Ham's F12 (Dulbecco's substratum modified by Eagle medium 1:1; DMEM–Invitrogen GmbH, Karlsruhe, Germany). After 3 hours, the cellular suspension was strained and filtered through a 100 mm mesh, washed two times using saline phosphate-buffered solution (PBS; Invitrogen GmbH), and seeded as primary culture in polystyrene culture flasks (Corning, Acton, MA, USA). The medium consisted of DMEM/Ham's F12 (1:1) with 10% of autologous patient serum. During the first two steps, penicillin (10 U/mL) and streptomycin (10 µm/mL) were added prophylactically. Cells were cultured at 37°C with 5% CO_2 and 95% humidified air. Every 3 days, 75% of the culture medium was replaced. Reaching 70% confluence, cells were detached from culture flasks with 0.02% trypsin and 0.02% thylenediaminetetraacetic acid, and then sub-cultivated until a cell number of 16 to 32 million of units was reached in three to four passages. A fraction of these cells was tested separately for osteogenic reproducibility.[16] Cell suspension was subsequently mixed with fibrinogen at a ratio of 3:1 (Tissucol Duo S®, Baxter, Vienna, Austria), until a cellular density of 15 million of cells/mL (±25%) was reached, and soaked into biodegradable scaffolds (Ethisorb Tamponade®, Ethicon, Norderstedt, Germany), with a volume of 100 mL each. The scaffolds were characterized by an unwoven, disk-shaped PLGA structure with a defined size of 8 mm diameter and 2 mm height. Scaffold porosity was very high (>90%). Every single disk was finally capable to carry 1.5 million of autogenous cells. The fibrinogen was polymerized after the addition of thrombin (diluted 1:10 with PBS). After polymerization was completed, cell-seeded constructs were cultured for 1 week in a specific osteogenic medium (Sigma®, Deisenhofen, Germany), made of DMEM/Ham's F12 (1:1) enriched with 5% autogenous serum, ascorbic acid (0.3 mM), dexamethasone (10^{-8} mol/L), and beta-glycerophosphate (10 mM). After 6 to 9 days of three-dimensional culture, the cellular vitality was tested by the measurement of cellular glucose consumption (mg glucose consumption/5 mL of culture medium/48 h). When glucose consumption rates suggested sufficient viability, constructs were stored in a sterile transport medium and transferred to the clinic for the sinus floor elevation procedure, precisely 6 weeks after biopsy was performed.

Maxillary Sinus Augmentation

All patients received antibiotics prior to the surgery. The maxillary sinus augmentation was performed as previously described.[17] Following a horizontal crestal incision and two vertical incisions extending beyond the mucogingival junction, a full-thickness flap was reflected in order to expose the maxillary sinus lateral bone

wall. Under constant irrigation with saline solution, an osseous window of approximately 1×1 cm was demarked and isolated using a round diamond-coated bur. The isolated osseous window was subsequently removed and conserved in saline solution. The schneiderian membrane was exposed and carefully isolated, using specially designed elevators, to avoid undesired perforations. In every patient, engineered bone transplants were used for the augmentation in one of the maxillary sinuses. Six PLGA disks (8 mm diameter $\times$ 2 mm depth, Oral Bone, BioTissue), each carrying 1.5 million of autogenous osteoblasts, were used in every patient, placed and condensed into the depth of the sinus cavity. Control-site cavities were filled with blocks of coral-derived porous hydroxyapatite (Biocoral), properly shaped during the surgical procedure, and granules of the same material. After graft-material placement, the sinus augmentation procedure was completed; the previously isolated osseous window was repositioned to close the sinus lateral wall. Sutures were performed (Supramid®, Novaxa Spa) to ensure complete flap closure.

Volumetric Measurements before and after the Surgical Procedure

Two computed tomography (CT) data sets were acquired for every patient, at baseline and 6 months after maxillary sinus augmentation procedures. The first examinations were performed with specific scannographic templates (90% acrylic resins and 10% barium sulfate). These templates were based on the diagnostic waxing with perforations on the longitudinal axis of the premolar and molar regions, simulating the ideal position where the implants should have been placed. These perforations represented reference points for both quantitative and qualitative bone measurements. The CT data were transferred in the DICOM format to the specific implant navigation software (Simplant®, Materialise, Leuven, Belgium). Simplant allows, through the image-segmentation process, a three-dimensional reconstruction of the maxilla (Figure 1). Moreover, this software enables us, through segmentation tools, to exactly measure bone crest height along transversal sections, corresponding to the longitudinal axis of the scannographic templates, before and after maxillary augmentation. The same navigation software was finally used for the evaluation of the bone density of grafted areas, 6 months after the augmentation procedures. Density measurements were performed to compare the degree of maturation or mineralization of new bone obtained with the grafting material using the scannographic templates as references.

Bone Core Harvesting

At the time of implant surgery, after 6 months healing period, bone cores were harvested using a 2.0×10 mm–diameter trephine bur under sterile saline solution irrigation. The bone cores were retrieved through a transcrestal way at a minimum distance of 5 mm from the nearest teeth; the dimension of the bone cores was almost 2×6 mm. Implants were then inserted, and the second-stage surgery was carried out after an additional healing period of 3 to 5 months. A total of 10 bone cores (two per patient) were retrieved.

Specimen Processing and Histometric Analyses

The biopsies were processed (Precise 1 Automated System®, Assing, Rome, Italy) to obtain thin ground sections as previously described.[18] The specimens were dehydrated in an ascending series of alcohol rinses and embedded in glycol methacrylate resin (Technovit® 7200 VLC, Heraeus Kulzer GmbH & Co., Wehrheim, Germany). After polymerization, the specimens were sectioned lengthwise along the larger axis of the specimens, using a high-precision diamond disk, to about 150 µm and ground down to about 30 µm. Two slides were obtained from each specimen. The slides were stained with basic fuchsin and toluidine blue. Histomorphometry of newly formed bone and marrow spaces were carried out for each case on the whole sample at a low magnification (25×). These measurements were undertaken using a light microscope (Laborlux S, Ernst Leitz GmbH, Wetzlar, Germany) connected to a high-resolution video camera (3CCD, JVC KY-F55B, JVC, Yokohama, Japan) and interfaced to a monitor and personal computer (Intel Pentium III 1200 MMX, Intel Corporation, 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 Version 4.5, Media Cybernetics Inc., Silver Spring, MD, USA).

RESULTS

Clinical and Radiographic Assessment

None of the patients presented complications following sinus augmentation. Maxillary sinuses filled with

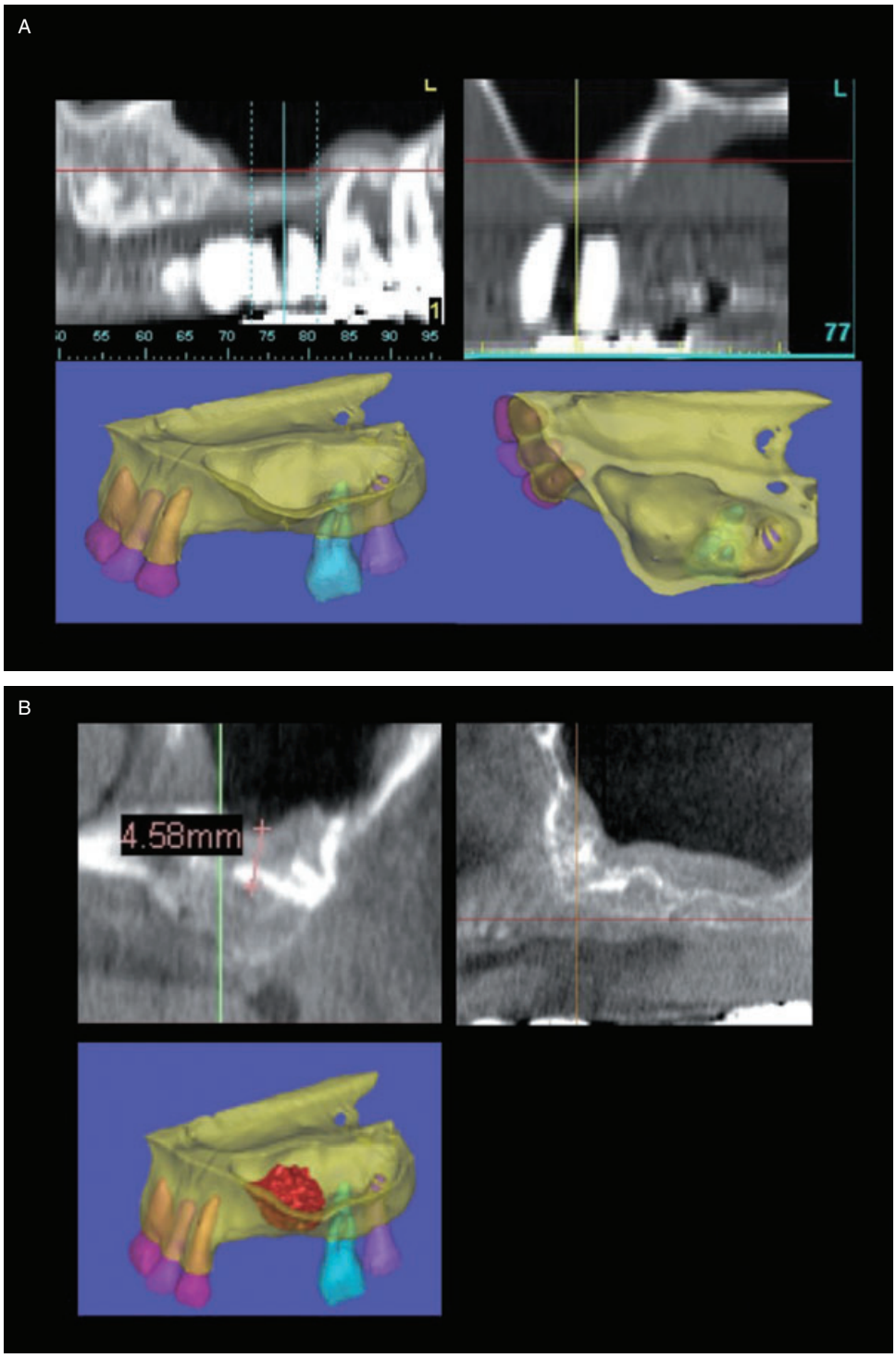

Figure 1 *A*, Preoperative computed tomography and three-dimensional reconstruction showed atrophy of the posterior maxilla; *B*, Computed tomography and three-dimensional reconstruction 6 months postsurgery showed a significant average vertical bone gain with the bone tissue-engineered graft.

engineered bone transplants revealed a significant average vertical bone gain of 6.47 ± 1.39 mm while the calcium phosphate presented 9.14 ± 1.19 mm (Tables 1 and 2). The average bone density in the areas of the bone tissue-engineered transplants was 192.76 HU (Hounsfield unit) 6 months after surgery. The bone density relative to the hydroxyapatite graft was 820.65 HU (Figures 1 and 2).

TABLE 1 Average (mm) of Vertical Bone Gain, Obtained in the Computed Tomography Scans at Baseline and after 6-Month Healing of Maxillary Sinus Graft Augmentation Using Engineered Bone Tissue

Patient	1			2			3			4			5		
Virtual position of the implant	24	25	26	24	25	26	15	16	17	25	26	27	14	15	16
Residual bone crest height (mm)	9.2	4.8	3.3	8.7	5.2	2.9	8.0	4.0	2.5	9.1	5.2	3.1	8.9	4.9	3.5
Bone crest height after sinus augmentation (mm)	14.6	14.7	9.3	13.2	12.2	8.7	14.7	10.8	7.2	14.8	13.3	8.1	16.0	13.1	10.7
Vertical height gain (mm)	5.4	9.9	6.0	4.5	7.0	5.8	6.7	6.8	4.7	5.7	8.1	5.0	7.1	8.2	7.2

Histological Findings

After 6 months, histological evaluation revealed the presence of mature bone with compact and cancellous areas in varied degree in both groups. The cancellous bone exhibits as the compact areas, incremental basophilic lines mixed with interposed reversion lines. The medullary spaces were ample and almost always filled with a well-vascularized connective tissue with no signs of inflammation or foreign body reaction (Figure 3). The spaces were filled with fatty marrow, interposing areas of fibrosis that were sometimes dense. In some cases, particles of the implanted material, seen as irregular vacuolated amorphous masses of basophilic tendency or as discretely eosinophilic amorphous masses, could be found mainly in the calcium-phosphate group. Giant cells, characteristic of the resorption process, involved some of these hydroxyapatite fragments. Sometimes the particles of Biocoral were partially or totally involved by fibrous tissue or integrated in the bony mass.

The bone formation process was well identified by the presence of osteoblasts, and the harvesian system was well preserved. The inflammatory infiltrated is on the average nonsignificant with prevalence of mononuclear cells. In some situations the calcium-phosphate blocks were present, close to the bony wall with the absence of osteogenic activity (Figure 4). Blocks of hydroxyapatite were also seen in superficial areas, surrounded by a fibrous capsule and, in some instances, by inflammatory giant cells.

The biopsy cores retrieved from the engineered bone contained a mean of $37.32 \pm 19.59\%$ and $62.67 \pm 27.71\%$ of bone and medullar spaces, respectively. The sinuses grafted with calcium phosphate presented a mean of $54.65 \pm 21.17\%$, $17.56 \pm 5.03\%$, and $27.78 \pm 16.31\%$ of newly formed bone, medullar spaces, and remaining particles, respectively.

DISCUSSION

The present case series presented a newly formed bone after maxillary sinus augmentation with engineered bone and calcium phosphate. Modern bone tissue engineering techniques have the aim to obtain a bone substitute with ideal properties from the structural and biological point of view. Biologically, this material should reproduce the same features of autogenous bone, such as osteogenic, osteoconductive, and even osteoinductive properties.[19,20]

The recent progresses of the molecular biology allowed harvesting and culturing osteogenic cells to seed on biomaterials, to differentiate into functional osteoblasts, and to finally graft material into bone defects. At the same time, it must be structurally and mechanically

TABLE 2 Average (mm) of Vertical Bone Gain, Obtained in the Computed Tomography Scans at Baseline and after 6-Month Healing of Maxillary Sinus Graft Augmentation Using Calcium Phosphate

Patient	1			2			3			4			5		
Virtual position of the implant	14	15	16	14	15	16	25	26	27	15	16	17	24	25	26
Residual bone crest height (mm)	8.4	5.3	2.8	7.5	6.0	3.2	8.9	4.2	3.4	10.2	5.1	3.0	7.7	5.3	2.2
Bone crest height after sinus augmentation (mm)	16.5	15.7	13.0	14.2	15.8	14.2	16.8	12.8	12.2	18.3	14.8	12.9	16.0	15.7	11.4
Vertical height gain (mm)	8.1	10.4	10.2	6.7	9.8	11.0	7.9	8.6	8.8	8.1	9.7	9.9	8.3	10.4	9.2

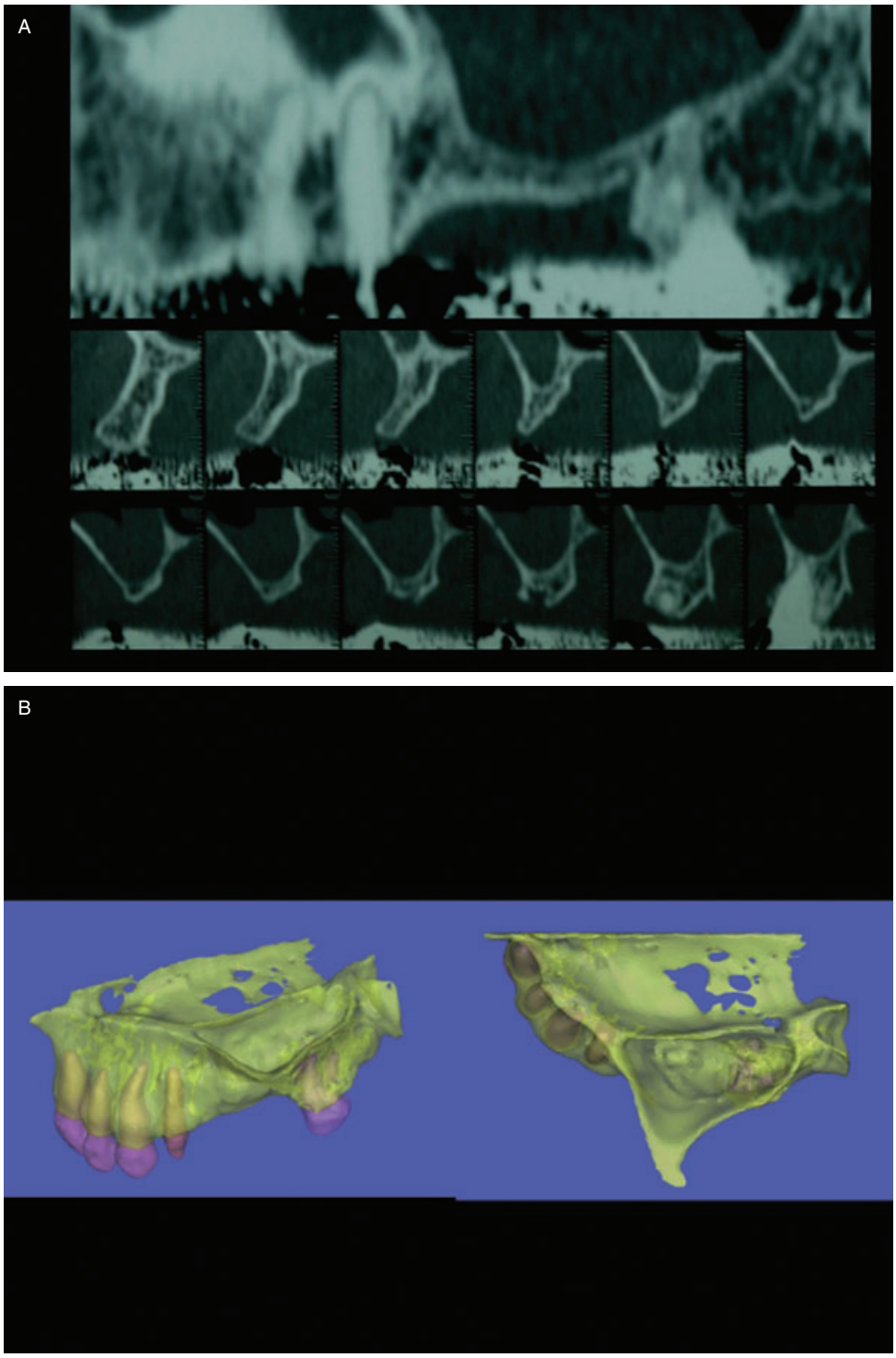

Figure 2 *A*, Preoperative computed tomography. *B*, Three-dimensional reconstruction depicted severe atrophy of the posterior maxilla.

able to sustain cell activity with the advantage of unlimited availability.[21] However, an ideal delivery scaffold to sustain cellular activity in the bone graft site is currently under research.[22]

Even if the technique presented in this case series has been previously described with interesting results in previous studies on maxillary sinus augmentation in humans,[23] this strategy for engineered bone-transplant creation does not seem to guarantee sufficient clinical success. In fact, the PLGA synthetic polymeric scaffolds are characterized by a fast resorption rate, representing an unfavorable factor for bone regeneration. These data

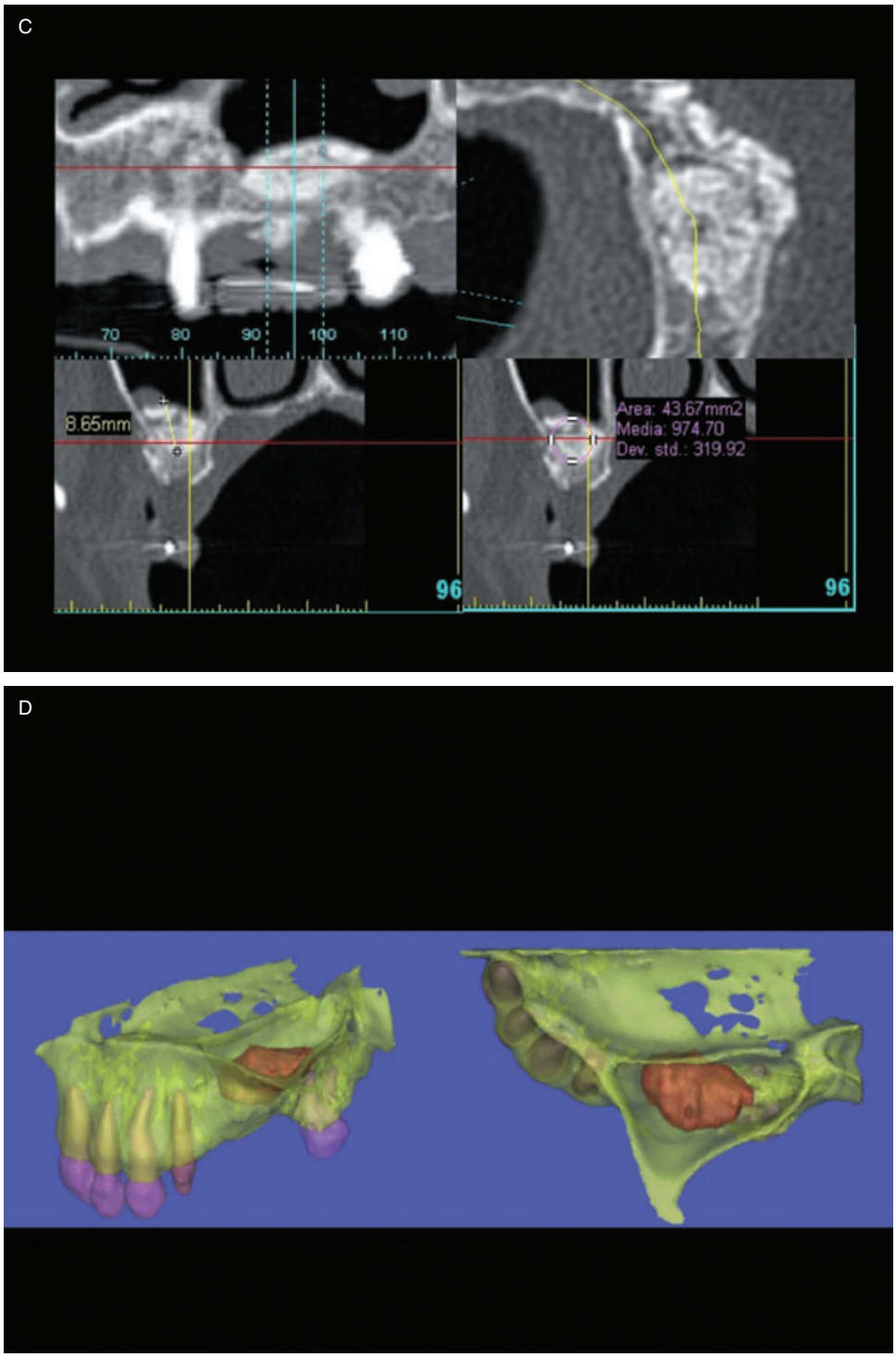

Figure 2 (*continued*) *C*, computed tomography. *D*, Three-dimensional reconstruction 6 months after maxillary sinus augmentation with calcium phosphate.

have been confirmed by another study[24] in a more recent clinical evaluation on maxillary sinus augmentation in 20 patients. In that study, the authors showed the fast resorption of the synthetic polymeric scaffolds at the first weeks postoperative could finally jeopardize bone regeneration, making impossible to guarantee adequate mechanical stability to osteoblasts delivered in the graft site. In fact, osteoblasts must adhere to a stable structure to produce a new bone matrix. The new bone matrix subsequently has to undergo mineralization and

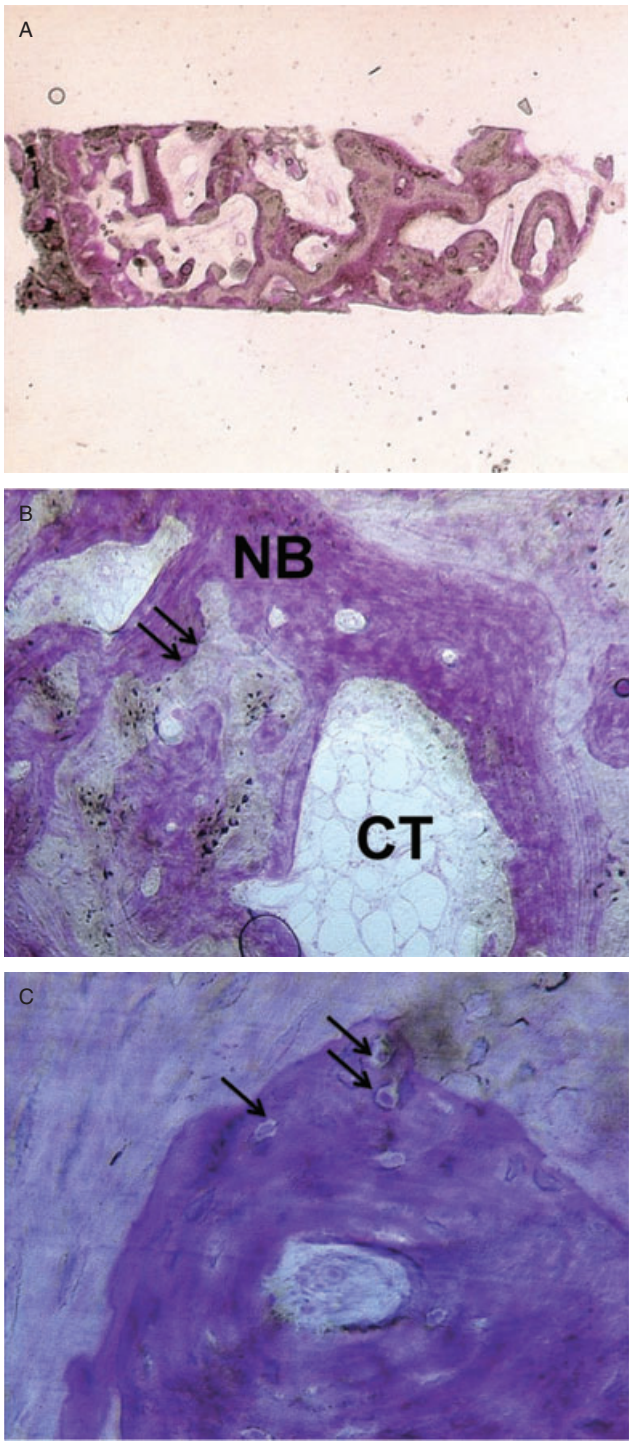

Figure 3 *A*, Panoramic view depicting varied newly formed trabeculae (acid fuchsin and toluidine blue, ×16). *B*, Presence of spaces filled with fatty marrow and connective tissues (CTs). The newly formed bone (NB) is present as the compact areas with incremental basophilic lines mixed with interposed reversion lines (*arrows*) (acid fuchsin and toluidine blue, original magnification ×100). *C*, In areas where the NB bordered cortical bone, compact bone consisting of osteon (haversian canal system) and several osteocytes (*arrows*) is in their lacunae (acid fuchsin and toluidine blue, ×200).

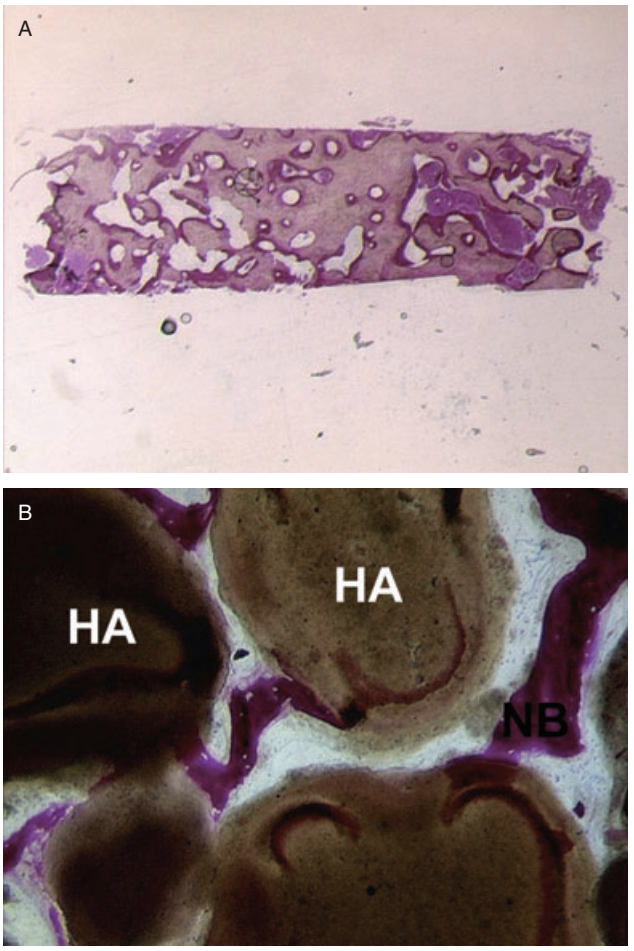

Figure 4 *A*, Lower view of the bone core retrieved from the maxillary sinus grafted with calcium phosphate notes the complete resorption of the graft material (acid fuchsin and toluidine blue, ×16). *B*, Power light view of other ground sections depicting hydroxyapatite (HA) fragments involved by newly formed bone (NB) (acid fuchsin and toluidine blue, original magnification ×140).

maturation process. In this way, a fast and extended degradation of the supporting scaffold determines the inevitable failure of bone regeneration because of the collapse of newly formed, immature bone matrix.

The features an ideal delivery system scaffold should possess have been recently elucidated.[25] On the one hand, mechanical support to cells has to be guaranteed until an adequate three-dimensional, mineralized bone matrix has been produced. On the other hand, the scaffold should support the cell from a chemical and biochemical point of view, allowing, by a sufficient vascular contribution, an adequate amount of nutrients, oxygen, molecular signals, and growth factors. Notoriously, the vascular support represents a key factor to obtain new bone formation.[26] For this

reason, the three-dimensional geometry of the scaffold must be studied in order to support adequate neoangiogenesis, creating a strong interconnected new vascular support.[27]

The volume and density of the new regenerated bone observed by means of CT examinations revealed that the osteoblasts obtained from a small autogenous bone core of the patient could be a feasible technique, at least after 6 months follow-up. However, the density obtained after sinus floor augmentation with porous hydroxyapatite was four times higher when compared with engineered bone tissue. The calcium-phosphate coral-derived material (*Genus porites*) evaluated in the present case series presents a chemical composition very similar to bone.[28] Biocoral is constituted by more than 98% of calcium carbonate in crystal form (aragonite) and other elements (F and Sr 0.7–1%, Mg 0.05–0.2%, Na < 1%, K < 0.03%, P < 0.05%, water < 0.5%, and amino acids < 0.026%). Among all these elements, the presence of strontium is fundamental, as it can effectively promote mineralization processes.[29] Biocompatible and osteoconductive, Biocoral possesses an average porosity of 50%, and it is similar to cancellous bone, with an architecture composed by strongly interconnected pores of variable diameter (250–500 μm). Therefore, this composition may explain the higher radiographic density observed for those sinuses.

Calcium-phosphate ceramic materials with defined and interconnected porosity (with pore size ranging from 300 to 400 μm) possess a network of channels and cavities capable to support both vascular invasion and angiogenesis.[25] The geometry of the scaffold seems to be able to influence and even "drive" cellular activity.[13,25] Several authors, using calcium-phosphate ceramic materials, have shown how the first bone apposition preferentially occurs in the concavities of scaffolds.[13,30,31] The osteoblasts, in fact, together with growth factors and specific cytokines, that is, molecular signals that govern cellular activity, tend to preferentially concentrate into cavities.[32] Moreover, the concavities seem to be able to directly stimulate the cells to give complete expression of the osteogenic phenotype. This is a fine and well-regulated mechanism, as the interactions of cells with concavities provide, through the activation of specific molecular bonds mediated by integrins and mechanoreceptors, signals to govern cellular activity and to determine cell fate.[33] The activation of specific mechanoreceptors, as well as molecular bonds related to integrins, determines structural modifications and re-arrangements at the subcellular level, affecting the cell cytoskeleton; structural arrangements at the cytoskeletal level can finally generate chemical signals able to induce cellular functions, in this case the osteogenic phenotype expressions, resulting in new bone apposition.[33]

The Oral Bone material, consisting in PLGA scaffolds, showed scarce efficacy in promoting cellular activity and bone regeneration; for this reason, it is of paramount importance for bone tissue engineering to find other alternates to act as scaffold for osteogenic cell activity. Calcium-phosphate materials, with specific chemical, structural, and geometrical features, could represent in the next future the ideal scaffold to support the biological activity of osteogenic cells, representing a possible alternate to the synthetic polymeric disks used in this work. In fact, calcium-phosphate materials can be technologically modified, chemically and structurally designed with specific architecture, forms, and geometry.[34] All of these properties could drive cell functions, forcing cells to express the desired osteogenic phenotype.[35]

Within the limits of this histological case series, the data confirmed that engineered bone tissue and calcium-phosphate material allowed newly formed bone after maxillary sinus augmentation. However, these results should be considered with caution, and further long-term investigations must be conducted.

CONFLICTS OF INTEREST STATEMENT

The authors have declared no conflicts of interest. [Correction added after online publication 23 October 2009: Conflicts of Interest Statement added.]

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