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VASCULAR ENDOTHELIAL GROWTH FACTOR AND e-NITRIC OXIDE SYNTHASE-MEDIATED REGENERATIVE RESPONSE OCCURRING UPON AUTOLOGOUS AND HETEROLOGOUS BONE GRAFTS

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Bone regeneration procedures allow oral rehabilitation with dental implants also in edentulous ridges with severe bone atrophy. The integration of grafted materials with the host tissue can initiate regenerative, inflammatory and apoptotic response. Since molecular mechanisms exist at the basis of such response, the aim of this work is to investigate, by immunohistochemical analyses, the expression of proteins involved in the graft integration process, in parallel to clinical and histological modifications, occurring on sites treated with extraoral autologous bone graft deriving from the parietal region of the calvaria (eAB), intraoral autologous bone graft deriving from mandibular ramus (iAB) and heterologous bone graft from swine (hB) in human patients. In our study, the immunohistochemical expression of BSP, VEGF, eNOS in eAB samples was significantly higher ($p < 0.05$) compared to values recorded in iAB and hB samples. The inflammatory response, investigated by iNOS expression, was found lower in all autologous samples (eAB and iAB) compared to hB, at statistically significant values. Moreover, the expression of the pro-apoptotic molecule, Bax, resulted significantly lower ($p < 0.05$) in eAB than in iAB and hB samples. These values, together with the low number of apoptotic cells detected in autologous samples, suggest a good regenerative response when extraoral autologous bone graft is used in comparison to the response from the other grafts, and also suggest the use of calvaria graft as a predictable therapeutic procedure for repairing severe bone defects in oral and maxillofacial surgery, not only by clinical and biomechanical criteria, but also from a biomolecular aspect.

After dental extractions, alveolar bone undergoes remodeling phenomena, leading to both vertical and horizontal bone loss (1). In addition to these phenomena, and to the generally poor quality of the bone in the upper distal regions, maxillary sinus pneumatization process occurs, determining an insufficient residual bone height to ensure primary stability of the implant placement (2). Novel classification of residual bone in the posterior maxilla considers, besides the width and the height of the residual alveolus, also the intermaxillary relationship (3). To correct these severe bone defects and to have a good prosthetic result, the use of onlay bone grafts, in addition to sinus floor lift, represents

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a valid therapeutic solution (4).

Different materials have been proposed and used for bone grafting (5-10); among these, autologous graft, and in particular membranous bone graft, could be considered the elected material for augmentation of craniofacial skeletal atrophy (11-12). A significant amount of cortical and cancellous bone may be harvested from the parietal region, as already described (13), even if for a monocortical bone regeneration, intra-oral donor sites, such as mandibular ramus, may furnish a sufficient bone quantity, with few complications and moderate post-operative discomfort for the patient (14). On the other hand, different xenografts utilized for sinus filling have shown a clinical performance comparable to autologous grafts (11).

Since at the basis of such response molecular mechanisms exist, different molecules may be investigated to evaluate regenerative, inflammatory and apoptotic response in sites treated with extra-oral autologous bone graft (eAB), intra-oral autologous bone graft (iAB) and heterologous bone graft (hB), in parallel to clinical and histological modifications.

Regenerative response was evaluated by immunohistochemical analysis of the expression of three molecules differently involved in bone regeneration and remodeling processes: Procollagen Type I, Bone Sialoprotein (BSP) and Vascular Endothelial Growth Factor (VEGF). Procollagen Type I is shown to be the major extracellular matrix protein of bone and, physiologically, it provides the protein basis for bone architecture (15). BSP is an extracellular matrix protein with important functional roles in the regulation of extracellular matrix deposition and mineralization. BSP has also been shown to promote osteoclastic resorption of mineralized surfaces (16). VEGF is able to induce the growth of new blood vessels and, in addition, it plays an important role in the maintenance and development of endothelial fenestrations (17-20).

Furthermore, two molecules belonging to Nitric Oxide Synthase (NOS) family proteins have been investigated: the endothelial eNOS (NOS-3) and inducible iNOS (NOS-2) (20). eNOS catalyzes the production of Nitric Oxide (NO), a key regulator of blood pressure, vascular remodeling and angiogenesis (21-22). iNOS is a calcium-independent molecule which is found to be expressed in response to endotoxins and to inflammatory cytokines in macrophages and many other cell types (23-25). In parallel to regeneration and inflammatory response, apoptosis occurrence was evaluated. Apoptosis involves a series of biochemical events leading to a variety of morphological changes, including blebbing, loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation, and ends with cell death. Pro-apoptotic Bax and survival Nf-kB molecules have been investigated. Bax, a member of the Bcl2 family, is one of the most important regulators of programmed cell (26-27), while Nf-kB is a transcription factor playing a vital role in many physiological and pathological processes, primarily by controlling the genes regulating cell differentiation, survival and proliferation (28). We checked Nf-kB expression in order to understand whether, together with induction of cell death, repair and survival mechanisms were activated.

Thus, the aim of this work is to investigate the molecular mechanisms involved in the graft integration process, in parallel to clinical and histological modifications occurring on eAB, iAB and hB in human patients.

MATERIALS AND METHODS

Patients

Ten patients (8 male and 2 female) with ages ranging from 45 to 65 years, with inadequate bone volume in the posterior maxilla, who were scheduled for bone augmentation procedures followed by implant placement to rehabilitate the masticatory function, were enrolled in this study.

All patients gave written informed consent in accordance with the Local Ethics Committee, in compliance with Italian legislation and with the code of Ethical Principles for Medical Research involving Human Subjects of the World Medical Association (Declaration of Helsinki).

Before surgical procedures, the patients underwent complete anamnesis, and clinical and radiographic examinations. They all had healthy systemic conditions, including the absence of any disease that would contraindicate surgery. The exclusion criteria were: uncontrolled periodontal disease, severe illness, unstable diabetes, drug abuse, a history of head and neck irradiation, chemotherapy. Pre-operative radiographic

evaluation included orthopantomograms and computed tomography.

Patients were divided into three groups, according to the severity and morphology of the bone defect, mirroring Classes C, E and F of the Chiapasco's Classification of the Posterior Maxilla (2004) (3). Patients in Class C received only maxillary sinus lifting by deproteinized swine heterologous bone (Group hB, including 4 patients); patients in Class E received vertical onlay grafts of intraoral autologous bone (Group iAB, 3 patients); while extraoral autologous bone graft was preferred in patients with vertical and horizontal defects, in Class F (Group eAB, 3 patients). However, in all the patients included in Groups iAB and eAB, maxillary sinus lifting was necessary.

Surgical procedures

Extraoral autologous bone blocks were withdrawn from the parietal region of calvaria, utilizing the split-in-situ calvarial graft, under general anesthesia. After skin incision and reflection of flaps, the outline of the strips were drawn with a piezoelectric instrument and the fragments were detached with differently-angled scalpels.

Intraoral autologous bone blocks were harvested under local anaesthesia from mandibular ramus. The donor site was exposed through a full-thickness incision and reflection of mucoperiosteal flaps. After incision, bone blocks (dimensions about 10×3×4 mm) were obtained from the anterior edge of the ramus with a piezoelectric surgical device.

The bone blocks were shaped according to the dimension of the defects. The cortical bone of the recipient site was perforated with a 1 mm diameter round bur to increase the blood supply from endosseous vessels and the bone blocks were fixed with a lag-screw to rebuild the alveolar ridge. All gaps between the bone blocks and the recipient sites were packed with autologous bone chips.

Heterologous bone was commercially available as swine corticocancellous bone particles (Osteobiol; Tecnoss, Coazze, Italy) width about 600 µm. Sinus floor lifting and bone graft apposition were performed simultaneously. After the first surgery, patients had monthly check-ups with clinical and radiological examination with periapical X-rays in the grafted area.

Post-operative healing was uneventful for all the patients, therefore, after about 6 months they all underwent a second surgery for implant placement. Bone specimens were collected by trephine bur from the reconstructed sites during implant placement, in order to have significant specimens of bone regenerated with extraoral, intraoral autologous bone graft and with deproteinized swine heterologous bone.

Light microscopy and immunohistochemistry

Bone samples, fixed in phosphate-buffered formalin solution, were decalcified in EDTA solution according to data sheet (MIELODEC kit, Bio-Optica, Milano, Italy), dehydrated by ascending alcohols and xylene, and then paraffin embedded. The samples were then de-waxed (progressively lower concentrations of xylene and alcohol) and processed for hematoxylin-eosin staining and for immunohistochemical analyses

In order to detect Procollagen Type I, BSP, VEGF, eNOS, iNOS, Bax, Nf-kB proteins, immunohistochemistry was performed on five tissue sections for each experimental point, by means of an immunoperoxidase Histostain-Plus kit (Zymed Laboratories Invitrogen, Carlsbud, CA). Slides were incubated in the presence of goat anti-Procollagen Type I polyclonal antibody (Santa Cruz Biotechnology, CA, USA), mouse anti-BSP monoclonal antibody (Calbiochem Merck, Cambridge, MA, USA), rabbit anti-VEGF, anti-iNOS, anti-Nf-Kb polyclonal antibodies (Santa Cruz Biotechnology, CA, USA), mouse anti-eNOS monoclonal antibody (BD Transduction Laboratories, Lexington KY), and mouse anti-Bax monoclonal antibody (Santa Cruz Biotechnology, CA, USA). Sections were incubated in the presence of specific HRP-conjugated secondary antibodies. Peroxidase was developed using diaminobenzidin chromogen (DAB) and nuclei were hematoxylin counterstained. Negative controls were performed by omitting the primary antibody.

Slides were then observed by means of light microscopy (Leica, Heidelberg, Germany) equipped with a Coolsnap video camera for computerized images (RS Photometrics, Tucson, AZ).

Computerized morphometry measurements and image analysis

After digitizing the images, a MetaMorph Software System (Universal Imaging Corporation, Molecular Device Corporation, PA, USA) (Crysel Instruments, Rome, Italy) was used to evaluate Procollagen Type I, BSP, VEGF, eNOS, iNOS, Bax and Nf-kB expression. Image analysis of protein expression was performed on ten fields for each section, through the quantification of the threshold area for immunohistochemical brown per field of light microscope observation. MetaMorph assessments were logged to Microsoft Excel and processed for Standard Deviations and Histograms. Statistical analysis was performed using the analysis of variance (ANOVA). Probability of null hypothesis of 0.1% ($p < 0.05$) was considered statistically significant.

TUNEL staining

Terminal-deoxinucleotidyl-transferase-mediated dUTP nick end-labeling (TUNEL) is a method of choice

Table I. *Comparison of the expression levels of the investigated proteins determined by direct visual counting in eAB (extraoral autologous bone sample) and iAB (intraoral autologous bone sample).*

Molecular Expression	eAB samples (%)	iAB samples (%)	T TEST	P
Pro Collagen type 1	17.9	22.9	P = 0.054	NS
Bone Sialoprotein	27.02	19.48	P = 0.034	X
VEGF	15.27	11	P = 0.018	X
e-Nos	17.15	9.8	P = 0.021	X
i-Nos	8.96	9.36	P = 0.07	NS
Bax	3.01	1.29	P = 0.043	X
Nf-Kb	1.92	6.97	P = 0.031	X

X: $p<0.05$; XX: $p<0.01$; XXX: $p<0.001$; NS: not significant

Table II. *Comparison of the expression levels of the investigated proteins determined by direct visual counting in samples from eAB (extraoral autologous bone sample) and hB (heterologous bone sample).*

Molecular expression	eAB samples (%)	hB samples (%)	T TEST	P
Pro Collagen type 1	17.9	27.04	P = 0.073	NS
Bone Sialoprotein	27.02	23.3	P = 0.031	X
VEGF	15.27	12.8	P = 0.043	X
e-Nos	17.15	16.5	P = 0.039	X
i-Nos	8.96	12.12	P = 0.035	X
Bax	3.01	7.10	P = 0.04	X
Nf-Kb	1.92	6.48	P = 0.007	XX

X: $p<0.05$; XX: $p<0.01$; XXX: $p<0.001$; NS: not significant

Table III. *Comparison of the expression levels of the investigated proteins determined by direct visual counting in samples from iAB (intraoral autologous bone sample) and hB (heterologous bone sample).*

Molecular expression	iAB samples (%)	hB samples (%)	T TEST	P
Pro Collagen type 1	22.9	27.04	P = 0.068	NS
Bone Sialoprotein	19.48	23.3	P = 0.041	X
VEGF	11	12.8	P = 0.12	NS
e-Nos	9.8	16.5	P = 0.0065	XX
i-Nos	9.36	12.12	P = 0.019	X
Bax	1.29	7.10	P = 0.011	X
Nf-Kb	6.97	6.48	P = 0.071	NS

X: $p<0.05$; XX: $p<0.01$; XXX: $p<0.001$; NS: not significant

Table IV. *TUNEL analysis.*

SAMPLES	Number of apoptotic cells for field
eAb	12.0 ± 1.0
iAB	1.4 ± 0.2
hB	16.3 ± 1.5

The presence of DNA fragmentation was quantified by direct visual counting of fluorescent labeled nuclei at 20x magnification ± SD. Green fluorescence labels positive nuclei. Ten fields for each experimental point were examined and values were obtained by computed average of positive cells.

for a rapid identification and quantification of apoptotic cells. DNA strand breaks, yielded during apoptosis, can be identified by labeling free 3'-OH termini with modified nucleotides in an enzymatic reaction. Paraffin embedded tissue sections were heated in an oven at 60°C for 30 minutes, de-waxed, rehydrated and pre-incubated with 0.1%Triton and 0.1% sodium citrate PBS 1x solution. After the permeabilization step, sections were exposed to TUNEL mixture, according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). After two rinses in PBS, slides were mounted by using glycerol solution (10 µg mL−1) and analyzed under fluorescent microscope (Leica Cambridge Ltd, Cambridge, UK). Five slides from each sample were assessed, and apoptotic cell count was performed on five fields per slide. Negative control was performed by omitting the incubation in the presence of the enzymatic mixture.

RESULTS

Grafts for alveolar ridge augmentation induce a reaction in the human organism, as it recognizes the biomaterial as a non-self element. The organism reacts by initiating several processes, including inflammatory and apoptotic responses, which can be investigated by morphological and molecular approach.

Morphological analysis

Morphological analysis was assessed by light microscope after hematoxylin-eosin staining. This staining highlights a new bone formation in all the specimens. eAB samples and iAB samples show a great number of osteoblasts, recognizable by their polyhedral form (Fig. 1A - B). Osteoblasts are arranged in lines and localized at the section periphery. Large areas of extracellular matrix can be also recognized, uniformly stained and organized in concentric plates surrounding Haversian canals. In the extracellular matrix, disc-shaped cavities could also be distinguished, showing depressions in the face of the disc, the bone gaps, in which osteocytes were entrapped. Moreover, in eAB samples, bone spicoles, quite completely fused, can be observed (Fig. 1A).

In hB samples, the grafted biomaterial particles are still clearly distinguishable from native bone tissue, for scarce viable cells. The sizes of the bone particles remain unchanged and no evidence of resorption can be detected. Moreover, a smaller number of cell nuclei can be observed, differently from eAB and iAB samples, which show a large number of cavities in which osteocytes are entrapped. In addition, areas with non-self biomaterial appear more stained than areas of autologous bone (Fig. 1C).

The study of the molecular modifications occurring after the different bone graft insertions and the evaluation of the clinical applicability and the ability of integration of these grafts was then performed (Tables I, II, III).

Immunohistochemical analysis reveals an increased expression of Procollagen Type I in hB samples in comparison to the eAB and iAB samples. However, no significant differences in its expression are detected between the samples of different origin (Fig. 2). BSP expression results significantly higher in the eAB sample than those in iAB and hB (Fig. 3).

Densitometric values obtained from immunohistochemical analysis reveal an increased expression of VEGF in eAB samples. VEGF expression shows a statistically significant value in eAB samples in respect to iAB and hB samples (Fig. 4).

Analysis of the inflammatory state

Immunohistochemical analysis reveals high expression of eNOS in eAB samples, and lower in iAB samples. In parallel, in hB samples, iNOS was significantly more expressed than in autologous bone grafts samples. Otherwise, eAB and iAB samples do

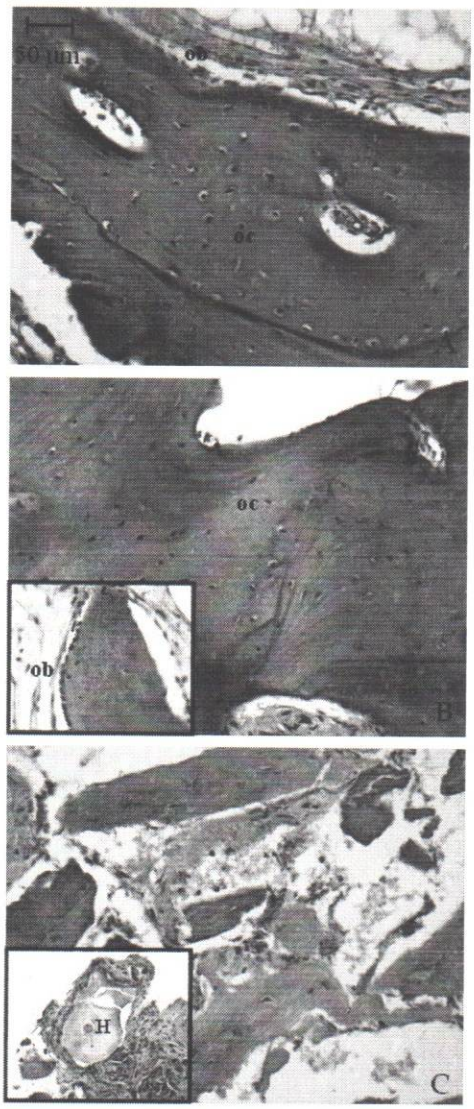


Fig. 1. *Hematoxylin-eosin staining of human alveolar bone in different experimental conditions. Magnification 20x. Insets magnification 40x.* ***A)*** *extraoral autologous bone sample;* ***B)*** *intraoral autologous bone sample, the inset shows a large number of polyhedral osteoblasts which supply new bone;* ***C)*** *heterologous bone sample, the inset shows concentric sheets which edge Haver's canal. ob: osteoblasts, oc: osteocytes; H: Haver's canal*

not show statistical significant differences in iNOS levels of expression between them (Fig. 5). The value of eNOS/iNOS ratio was also evaluated as mean of eNOS and iNOS expression in samples from each site. The eNOS/iNOS ratio resulted elevated in eAB samples compared to iAB and hB samples.

Detection of apoptotic and repair response

TUNEL analysis highlights a greater apoptotic response in hB samples. Sites regenerated with autologous bone graft, in particular in iAB, disclose a lower number of apoptotic cells (Table IV).

In parallel, iAB samples show the lowest Bax level. On the other hand, the level of the pro-apoptotic molecule Bax is significantly higher in hB samples than in eAB and iAB ones (Fig. 6).

Finally, the expression of Nf-kB was investigated. hB and iAb samples do not disclose any significant difference concerning Nf-kB expression, while in eAB samples the expression of this molecule results significantly lower than in other specimens (Fig.7).

DISCUSSION

Correction of large bone defects in the posterior maxilla requires a delicate approach in order to obtain a functional implant restoration and to prevent construction of non-axial prosthetic solutions. Current surgical reconstructive protocols have to also consider the horizontal and vertical relationship of the maxilla and mandible regions and not only the residual bone volumes due to bone resorption and sinus hyper-pneumatization (3-5). In addition, different biomaterials have been used and a great deal of research carried out to study the interactions occurring between the grafts and the host tissues. Most of these studies focused on the clinical differences and benefits in choosing one or another biomaterial. In this study, different molecules were investigated in order to assess, from a biological point of view, the bone tissue response to autologous graft and to heterologous bone graft. As donor sites for autologous grafts, calvaria and mandibular ramus were preferred, as extra-oral and intra-oral donor sites, respectively. Both sites belong to intramembranous bone, since its superiority to endochondral grafts in craniofacial defect repairs had already been demonstrated. Moreover, many authors agree that, independently of the embryologic origin, the number of the corticals and the macroscopic architecture of the tissue harvested may affect clinical success in bone grafting (12, 29). In fact, despite the

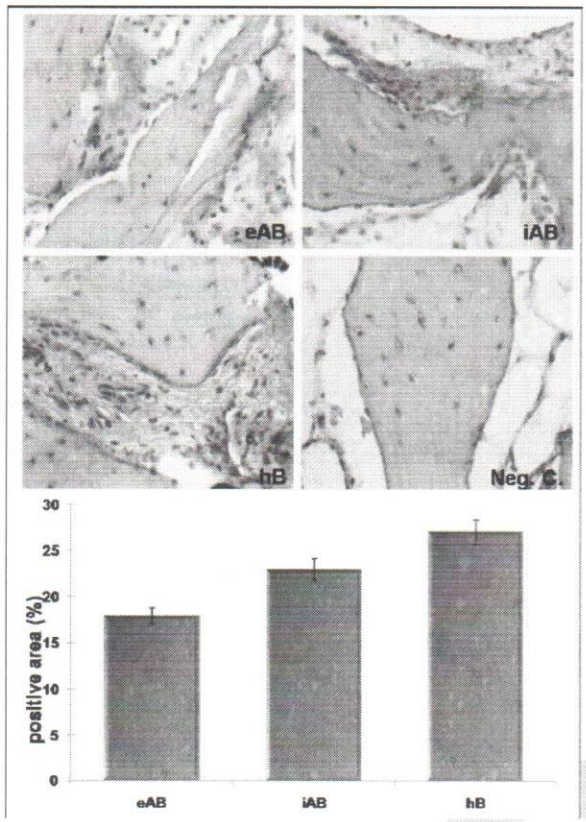


Fig. 2. *a) Immunohistochemical detection of Procollagen Type I in human alveolar bone in different experimental conditions. Magnification 20x. eAB extraoral autologous bone sample; iAB: intraoral autologous bone sample; hB: heterologous bone sample; Neg. C. negative control;* ***b)*** *Densitometric analysis of Procollagen Type I positive area ± SD determined by direct visual counting of ten fields (mean values) for each of five slides per sample at 20x magnification.*

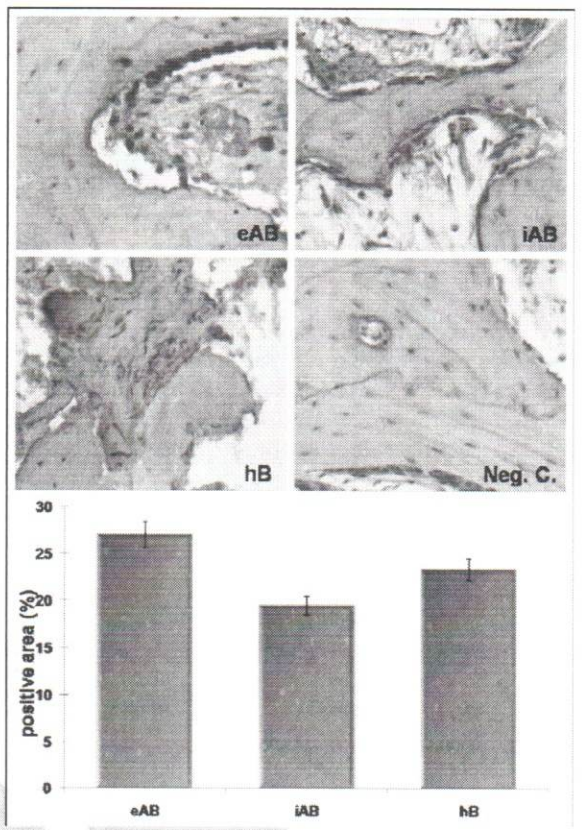


Fig. 3. *a) Immunohistochemical detection of Bone Sialoprotein in human alveolar bone in different experimental conditions. Magnification 20x. Eab: extraoral autologous bone sample; iAB: intraoral autologous bone sample; hB: heterologous bone sample; Neg. C. negative control;* ***b)*** *Densitometric analysis of Bone Sialoprotein positive area ± SD determined by direct visual counting of ten fields (mean values) for each of five slides per sample at 20x magnification.*

same embryologic origin, extraoral bone grafts has been demonstrated to show a lower resorption level during the healing process compared to grafts taken from intraoral donor sites (30). Moreover, extraoral bone graft use is associated with a smaller risk of inflammatory complications, which is probably due to a lower bacterial contamination of the graft during withdrawal (31).

Heterologous bone grafts of different origins are successfully used as filling material for maxillary sinus floor elevation. Even though autologous bone is described as elected grafting material for this surgical procedure (11), the comparable results obtained with heterologous bone grafts lead clinicians to consider the latter biomaterials valuable for sinus lifting (9, 32). In particular, deproteinized heterologous swine bone is successfully used to obtain osteogenesis in guided bone regeneration techniques (33). However, unlike autologous bone grafts, heterologous bone particles work only as osteoconductive biomaterial and their process of biological decomposition is described as slow resorption, since residual

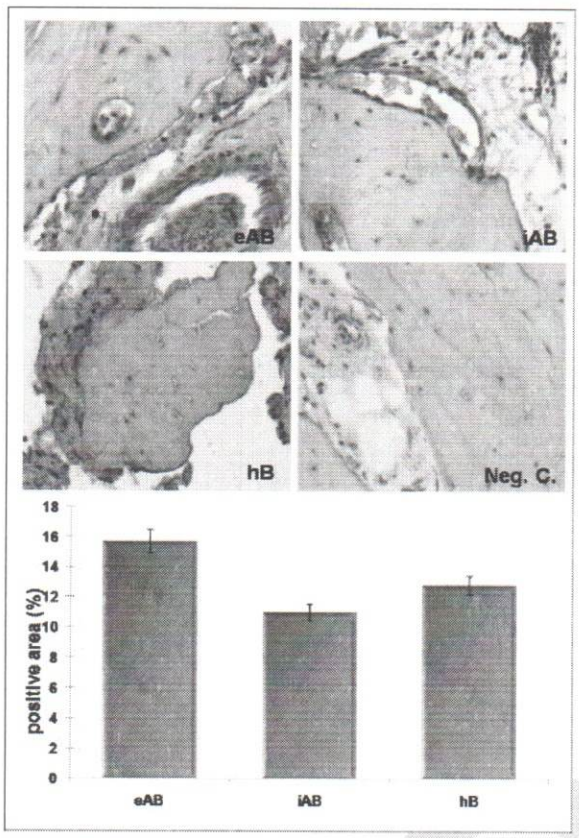
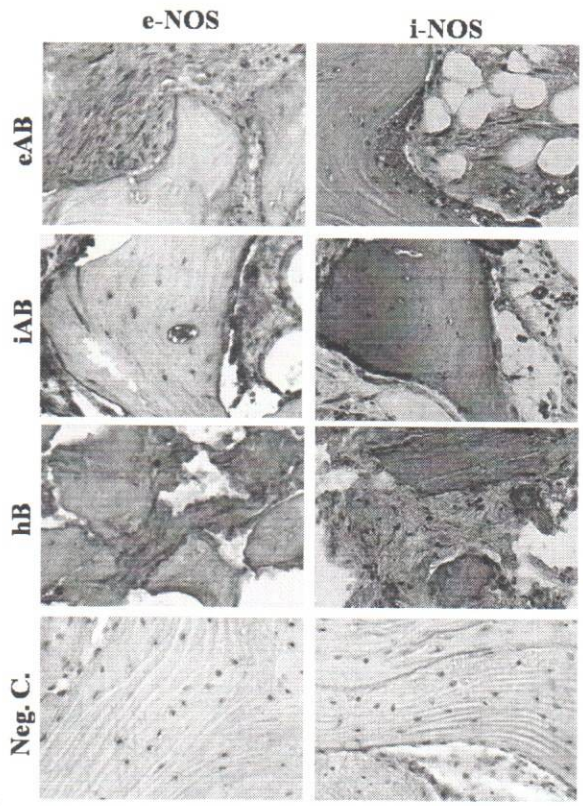


Fig. 4. *a) Immunohistochemical detection of VEGF in human alveolar bone in different experimental conditions. Magnification 20x. eAB: extraoral autologous bone sample; iAB: intraoral autologous bone sample; hB: heterologous bone sample; Neg. C. negative control; b) Densitometric analysis of VEGF positive area ± SD determined by direct visual counting of ten fields (mean values) for each of five slides per sample at 20x magnification.*



NITRIC OXIDE SYNTHASE	eAB	iAB	hB
eNos	17.15	9.8	16.5
iNos	8.96	9.36	12.12
eNOS/iNOS ratio	1.91	1.05	1.36

Fig. 5. *a) Immunohistochemical detection of eNOS (left panel) and iNOS (right panel) in human alveolar bone. Magnification 20x. eAB extraoral autologous bone sample; iAB: intraoral autologous bone sample; hB: heterologous bone sample; Neg. C. negative control; b) Densitometric analysis of eNOS and iNOS positive area ± SD determined by direct visual counting of ten fields (mean values) for each of five slides per sample at 20x magnification.*

biomaterial particles can be detected even after years (34-35).

Heterologous bone grafts for alveolar ridge augmentation induce a reaction in the human organism, as they are recognized as non-self elements (36-39). The organism reacts by switching on several processes, which can be analyzed and studied by means of morphological and molecular approach.

Regenerative response was investigated by immunohistochemical expression of Procollagen Type I, BSP and VEGF. Procollagen type I is a constitutive molecule, precursor of collagen, abundantly represented in the connective tissue and involved in cellular adhesion mechanisms and tissue architecture maintenance (15). Even if not statistically significant, we found that Procollagen Type I levels resulted higher in hB samples than in eAB and iAB samples. However, a previous study

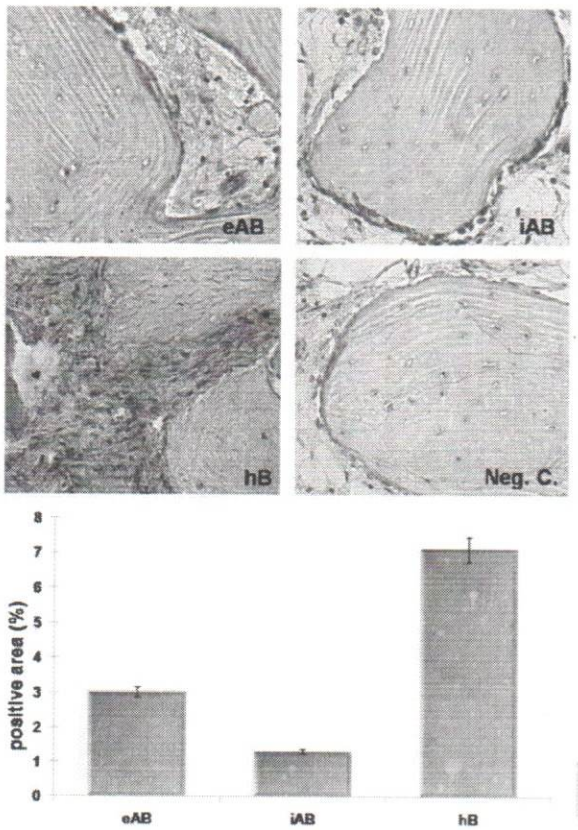


Fig. 6. *a) Immunohistochemical detection of Bax in human alveolar bone in different experimental conditions. Magnification 20x. eAB extraoral autologous bone sample; iAB: intraoral autologous bone sample; hB: heterologous bone sample; Neg. C. negative control; b) Densitometric analysis of Bax positive area ± SD determined by direct visual counting of ten fields (mean values) for each of five slides per sample at 20x magnification.*

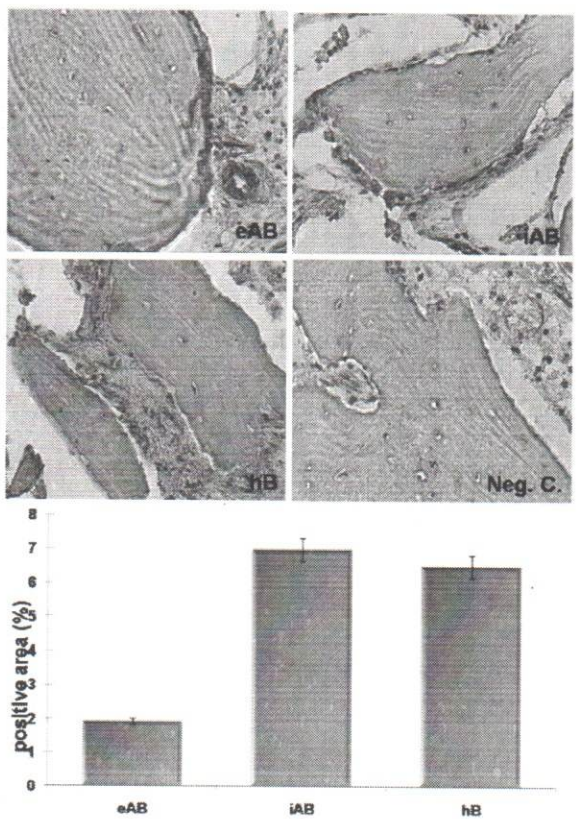


Fig. 7. *a) Immunohistochemical detection of Nf-kB in human alveolar bone in different experimental conditions. Magnification 20x. eAB extraoral autologous bone sample; iAB: intraoral autologous bone sample; hB: heterologous bone sample; Neg. C. negative control; b) Densitometric analysis of Nf-kb positive area ± SD determined by direct visual counting of ten fields (mean values) for each of five slides per sample at 20x magnification.*

demonstrated differences in Procollagen Type I expression between autologous and heterologous bone grafts at the beginning of their incorporation process, while minor differences were noticed thereafter (40-41). This could be due to an increased need of host tissue to restore a scaffold of collagen fibers around heterologous bone particles, in order to allow graft support and integration during the first healing phases and to promote mesenchymal cell migration to the centre of the defects.

BSP is a matrix glycoprotein considered as an index of bone formation and growth; its expression is related to the mature osteoblastic phenotype and up-regulated by factors that induce osteoblast differentiation (16). In our study, the expression of BSP resulted higher in eAB sample, demonstrating new bone formation and stronger remodeling than in sites treated with intraoral autologous bone or heterologous bone particles. The positive staining of BSP suggests that host cells surrounding and invading the graft follow the osteogenic differentiation pathway (42-44). The results support the hypothesis that bone regeneration proceeds up to six months after sinus lifting, thus further increasing bone quality and volume. In our samples the VEGF and eNOS expression was investigated to evaluate the angiogenesis, endothelial maintenance and vascular remodeling processes (17, 21) of

autologous and heterologous bone grafts. In our study, VEGF resulted significantly more expressed in eAB samples than in iAB and hB ones. The higher expression of this protein may be linked to new angiogenic processes occurring predominantly in sites where autologous bone of intramembranous origin was grafted, as elsewhere reported (45-47).

Each grafting material, regardless of its origin, when placed in the host tissue, may induce different inflammatory responses (36-39). For these reason, iNOS expression was checked. When iNOS was lesser expressed along with high eNOS level, the biomaterial resulted better integrated with the host tissue. In our study, minimal inflammation and good vascularization were detected in eAB samples, while iNOS resulted highly expressed in hB samples. These data indicate extensive inflammation and a great amount of new blood vessel formation in eAB samples (Figs. 3 and 5).

We investigated the apoptotic events disclosed by each experimental point, by evaluating the number of apoptotic cells and the expression of markers typically associated to programmed cell death and survival. As the number of apoptotic cells together with Bax pro-apoptotic molecule expression is higher in hB samples, it can be argued that corticocancellous swine heterologous bone may determine apoptosis occurrence among grafted area cells.

Finally, the possibility of repair mechanism activation by such bone grafts led us to check the expression of Nf-kB. It has already been shown that Nf-kB is normally bound to an inhibitor in the citosol; in presence of stress stimuli it is released and within the nucleus it initiates the transcription of many genes belonging to the anti-apoptotic family of Bcl-2 (48-50). From our data, Nf-kB was significantly less expressed in eAB samples than in iAB and hB samples, and not significantly different between the latter, indicating that when apoptosis occurs, Nf-kB-related repair mechanisms are activated at the same time.

Thus, the low number of apoptotic cells, the low expression of iNOS and Bax, together with the high expression of BSP, VEGF and eNOS, could suggest extraoral bone graft, and in particular calvaria graft is an effective and predictable therapeutic procedure to repair severe bone defects, not only according to its clinical and biomechanical long-term behaviour, but also considering it from a biomolecular aspect.

Since tissue integration represents a problem derived from the biocompatibility of biomaterials in terms of inflammation, apoptotis, neo-vascularization and cell survival response, the knowledge of molecular and morphological modifications occurring upon graft placements could help the clinicians to choose the best biomaterial, not only on the basis of easy handling and high sealant capacity, but mainly on the basis of tissue integration.

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