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Original paper

Immunohistochemical evaluation of autogenous mandibular bone grafts integration:

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Abstract Alveolar ridge augmentation with autogenous bone grafts is a frequent procedure used in Implant Dentistry. This paper presents an immunohistochemical assessment of the integration of mandibular autografts, applied in maxillary bone defects. Seven adult dogs were used in the study. The work methodology was established through maxillary and mandibular morphometry. The posterior mandibular body was considered the donor region and the lateral region of the maxilla the recipient area. Bilateral maxillary bone defects were performed on the predetermined receiving sites, which were later augmented with mandibular grafts. Fragments of hard tissue from the grafted sites were harvested 90-100 days after the surgical interventions and immunohistochemically evaluated. The immunohistochemical study proved the existence of bone regeneration in the case of mandibular corticocancellous autografts applied at the maxillary level, being an efficient procedure for assessing their integration.

Keywords Corticocancellous autografts, tissue regeneration, immunohistochemistry, osseointegration

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Background

Oral rehabilitation with implant-supported dental prostheses has become a routine procedure for the treatment of edentulous patients in everyday dental practice. In cases of severely atrophied alveolar ridges, it is necessary to perform augmentation of the available bone volume in order to enable implants placement and to obtain a long-term favourable outcome.

Introduction

Dental implant therapy has gained a lot of popularity in modern dentistry. The success is conditioned by the adequate dimensions of the available bone, which, if inadequate, could be reconstructed through autogenous bone grafts, either before, or at the time of implant placement; this surgical procedure facilitates new bone formation (A. SCARANO & al. [1], A. POLL [2], B.S. MCALLISTER and K. HAGHIGHAT [3]).

Bone healing in autogenous bone grafting depends on the migration of osteogenic cells (osteoblasts) at the level of the bone defect, and the exclusion of cells which inhibit bone formation (for example, epithelial cells and fibroblasts). Positive outcome of the procedure is conditioned by the elimination, at the level of the grafting region, of epithelial and connective tissues, by the stability of the fibrin clot, by the lack of graft mobility, and by the primary closure of the wound (W. BECKER & al. [4], K. SCHENK & al. [5]).

Bone autografts are appreciated because they are non-immunogenic and they have all of the properties required of a bone material (A.R. AMINI & al. [6]). Due to their higher resistance to resorption and horizontal bone atrophy, they can be used to correct larger bone defects (A. POLL [2]). The use of this type of graft is an efficient procedure in order to obtain the adequate bone volume for implant placement, in the case of severely atrophied alveolar ridges (J.A. GREENBERG & al. [7], B. BAS & al. [8]).

Best results have been obtained with corticocancellous bone grafts. Due to the fact that they have the structural characteristics of the cortical bone and of the cancellous bone, they provide better mechanical resistance and allow an adequate revascularisation that enhances the integration of the graft in the nearby structures (R.A. HARDESTY and J.L. MARSH [9]).

The use of mandibular autografts requires knowledge of the mechanism underlying bone integration and understanding of the particular homeostatic mechanism in the human maxilla and mandible, bones which are in a constant process of modelling and remodelling.

In order to evaluate the post-grafting formation and regeneration of the bone, a histological and immunohistochemical analysis is necessary. The grafting material is fundamental in bone formation.

The immunohistochemical techniques had previously been used in dental medicine for evaluations of the pulp-dentin complex or in order to evaluate the epithelial and connective tissues integration to dental implants (M.J. TUCULINA & al. [10], S.A. GASPAR [11]).

The purpose of the present study was to analyse, from an immunohistochemical perspective, the *in vivo* tissue reactions of mandibular corticocancellous bone autografts used in surgically performed maxillary bone defects, on a standard canine experimental model, determined through research, in order to obtain a scientific perspective regarding their healing.

Material and methods

Seven clinically healthy adult dogs, *Canis familiaris,* 15-20 kg of weight, from the bio base of the Bucharest Faculty of Veterinary Medicine, were used in the study. The research was organised and performed in compliance with the legislation and regulations in force.

The research methodology was established after the previous morphometric analysis of maxillary and mandibular bone structures, completed in other studies (A. POLL & al. [12], A. POLL & al. [13]). Thus, the posterior mandibular body, border region between the mandibular body and the ramus was considered the target donor area, while the target receptor area was the lateral maxillary body, corresponding to the alveolar region of the premolars. Bilateral maxillary bone defects were performed on the predetermined receiving sites by drilling at conventional speed. Each bone defect was afterwards augmented with a mandibular corticocancellous graft. Fragments of hard tissue from the grafted sites were collected 90-100 days after completing the surgical interventions. The harvested elements were macroscopically oriented and there were selected tissue fragments containing areas of interest.

The immunohistochemical analysis was performed by primary antibodies and for visualisation secondary antibodies and streaptavidine complex, the Novolink (Leica) kit were used, as well as liquid DAB chromogen (Leica), as it can be seen in the table below.

Table 1. Work protocol for the immunohistochemical evaluation:

	Primary Antibody	Clone	Source	Manufacturer	Pre-treatment*	Dilution
	SMA	ASM1	Mouse	Leica	-	1:200
	Bcl-2	Bcl2/100/D5	Mouse	Leica	Citrate	1.5:240
	EGFR	EGFR 113	Mouse	Leica	Citrate	1:60
	VEGF	Polyclonal	Rabbit	Thermo Fischer	EDTA	1:100

*The pre-treatment was completed by boiling in EDTA pH 9 buffer or pH 6 citrate, where appropriate, for 15 minutes (3 stages, 5 minutes each), and after every 5 minutes the container was checked and the buffer solution topped off.

The immunohistochemical colorations were completed manually or with the help of the automated Leica Bond III stainer. The protocols used for immunohistochemical staining respect the medical practice guidelines for Anatomical Pathology (S.A. GASPAR [11]).

Results

The favourable evolution and integration of the inserted grafts during the periodic (twice a month) clinical and radiographic controls were observed, with the exception of a single graft which did not present the fixing screw on the 30th day radiographic check-up. This graft was recorded as a failure. Any other post operative complications were not noticed.

The immunohistochemical analysis demonstrated the presence of areas of osteoid material with endothelial cells, lymphocytes and osteoblasts, at the trabecular level, while at the intertrabecular level, blood vessels bordered by pericytes, inflammatory cells and myofibroblasts, which are positive structures for the antibodies used in this study, were found. Tissue regeneration, which is a complex biological phenomenon, implies aside from the stem cells' intervention, participation of certain structural elements from the extracellular environment.

Following, the most suggestive images obtained in this immunohistochemical study are presented, Figures 1-8, with specific details.

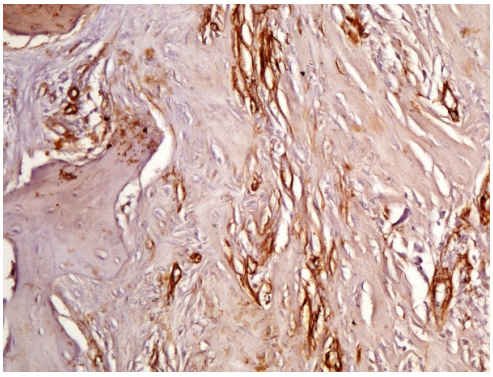

Fig. 1. Negative SMA hypertrophic osteoblasts were identified in irregular masses of osteoid near mature bone trabeculae. SMA x 200.

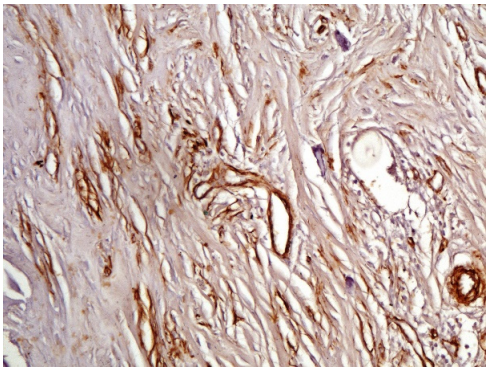

Fig. 2. Intertrabecular blood vessels bordered by pericytes and rare positive myofibroblasts for SMA. SMA x 200.

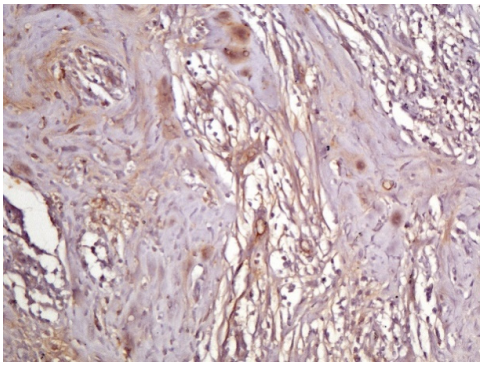

Fig. 3. Deposit area for osteoid material. Positive endothelial cells for Bcl-2, negative osteoblasts for Bcl-2. Bcl-2 x 200.

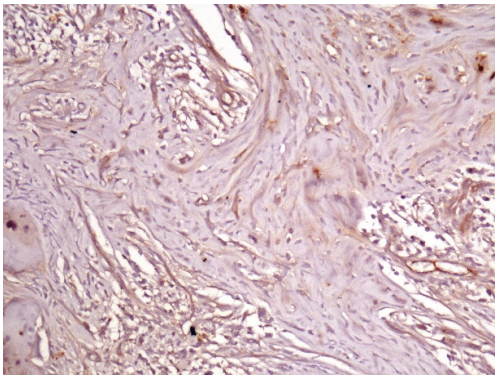

Fig. 4. Osteoid trabeculae with hypertrophic osteoblasts, negative for EGFR. EGFR x 200.

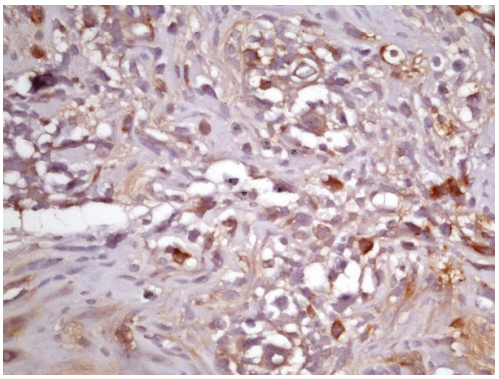

Fig. 5. Detail of Figure 4: positive osteoblasts for EGFR. EGFR x 400.

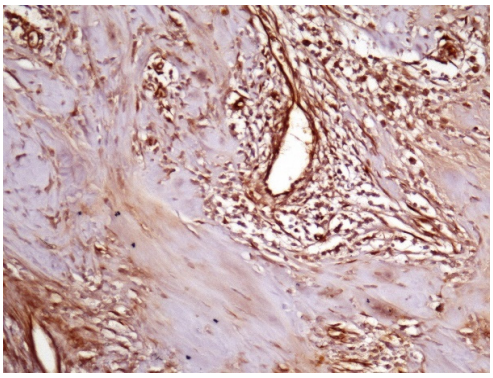

Fig. 7. Osteoid trabeculae with hypertrophic osteoblasts, positive for VEGF. The endothelia of intertrabecular vessels and inflammatory cells positive for VEGF. VEGF x 200.

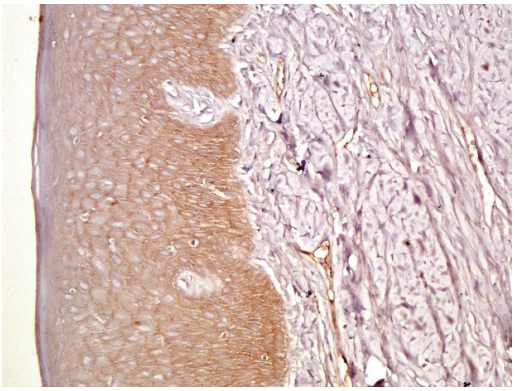

Fig. 6. Internal sample – epithelial and endothelial positivity for EGFR. EGFR x 200.

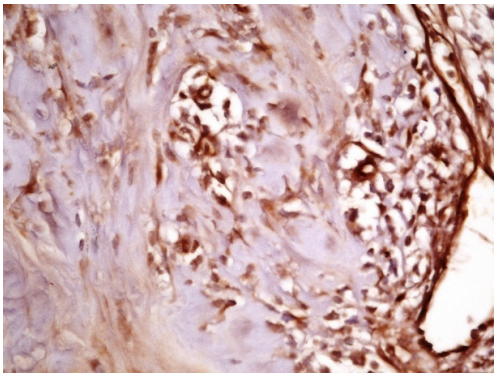

Fig. 8. Detail of Figure 7: positive osteoblasts for VEGF. VEGF x 400.

Discussion

The immunohistochemical analysis revealed positivity for SMA, Bcl-2, EGFR and VEGF in the grafted sites. These findings prove the existence of bone regeneration and the integration of mandibular autogenous grafts. The primary antibody SMA (Smooth Muscle Antibodies) is part of the vascular cells and can be considered a revascularisation and vascular maturation marker. The anti-apoptotic protein Bcl-2 codifies a protein involved in cell death (apoptosis) and plays a part in the cell cycle regulation. EGFR (Epidermal Growth Factor Receptor), a transmembrane glycoprotein, is the receptor for Epidermal Growth Factor (EGF), which plays a part in cellular differentiation and in tissue regeneration. EGF also accelerates the healing process. VEGF (Vascular Endothelial Growth Factor) is a cellular glycoprotein which takes part in angiogenesis through the stimulation of mitosis and of migration of endothelial cells and through the growth of vascular permeability (S.A. GASPAR [11]).

Based on an extensive literature search, carried out using the PubMed database, Yang YQ and collaborators pointed out that VEGF acts as an 'essential mediator' of angiogenesis, process which is closely correlated to osteogenesis. In addition, the same authors showed that VEGF not only functions in bone angiogenesis but also in various aspects of bone development (Y.Q. YANG & al. [14]).

In another recent publication, Grosso A and the co-workers also emphasized the coupling of angiogenesis and osteogenesis for bone regeneration and described the roles VEGF has throughout these processes (A. GROSSO & al. [15]).

Growth factors, such as Epidermal Growth Factor (EGF) and Vascular Endothelial Growth Factor (VEGF) promote cell proliferation and migration. They are also known to play important roles in fracture repair (V. DEVESCOVI & al. [16]).

The integration of bone autografts depends heavily on their adequate revascularisation, which is independent from the vascular support of the receiving area (M.E. ELSALANTY and D.G. GENECOV [17]).

Growth factors intervene in the revascularisation process through the stimulation of angiogenesis, induction of proliferation and differentiation of osteoblasts and their precursors, enhancing the healing. Graft revascularisation will generate bone neoformation. Also, growth factors lead to an increase in collagen and proteolytic activity, determining a growth in the local number of mesenchymal stem cells (G. ZIMMERMANN and A. MOGHADDAM [18], M.A. SERVIN-TRUJILLO & al. [19]).

The angiogenesis which was highlighted in this experiment in the grafting areas, alongside local areas of inflammation, was also noticed by other authors, who stated that the angiogenesis becomes more evident with the rise of the inflammatory infiltrate (A. BUNGET & al. [20]). Neoangiogenesis involves growth factors and endothelial cell migration and proliferation (D. HINGANU & al. [21]).

An adequate vascular support at the level of the receiving area is also needed for integration of bone grafts. Insufficient blood flow at the bone level or in the surrounding soft tissues, as well as variations in local vascularisation can negatively influence healing, resulting in delayed fusions or non-fusions between the donor and recipient bone elements (A. ORYAN & al. [22]).

Healing of these grafts requires the presence of viable bone cells that produce osteoid and bioactive substances, which modulate this process.

Cellular viability, in the case of bone autografts, is fundamental for the success of the graft, because viable cells differentiate to bone-forming osteoblasts (G. CHIRIAC & al. [23]). Cellular viability, proved by the immunohistochemical study at the level of the maxillary target areas, demonstrates the existence of bone regeneration and the integration of mandibular autogenous grafts.

Lack of, or reduced immunogenicity of grafts used in bone augmentation in oral implantology considerably increase chances regarding the incorporation of grafts in the receiving areas (A. POLL [2]). In this respect, it has been proven that corticocancellous mandibular bone block autografts are biocompatible and osteoconductive and allow new bone formation.

However, this study has certain limitations due to the lack of a control group. Despite all this, it proved great clinical and immunohistochemical results, and the integration of bone autografts took place without dehiscence at the level of the soft tissue. This last aspect was also mentioned by other authors (T. BERGLUNDH and W.V. GIANNOBILE [24], A. ORTIZ-VIGÓN & al. [25]).

Other research groups suggested that the integration of this type of graft is even more difficult due to the slow process of remodelling (A. ACOCELLA & al. [26]).

At the end of this study, it can be stated that the immunohistochemical investigation of the integration of mandibular autografts applied in maxillary bone defects, represents a valuable technique for the evaluation of initial and early phases of healing, statement similar to the conclusions of other authors (F. SCHWARZ & al. [27]). Also, as shown in another study, the immunohistochemical evaluation revealed the presence of specific immuno-competent cells (V. NIMIGEAN & al. [28]).

Due to the fact that many research groups support the implementation and development of alternative methods for the reduction of *in vivo* tests, future studies should precisely determine and clarify the purpose of cells in bone tissue engineering, while maintaining a constant concern for the determination of the bone substitutes which would minimise the need for autogenous bone grafts (W. LILIENBLUM & al. [29]), J. MA & al. [30], Z. SHAFIEI-SARVESTANI & al. [31], V. NIMIGEAN & al. [32]).

In addition, future research should develop prospective studies and should take into account the bone formation direction and the fact that tissue regeneration needs survival and proliferation of bone cells. Furthermore, the experimental research should be done on models that can mimic certain clinical situations.

Conclusions

According to our *in vivo* study on experimental animal model, the use of corticocancellous bone block autografts is a superior alternative, proving to be a predictable procedure for extensive bone augmentation of severely atrophied alveolar bone ridges, with a high success rate.

All the available alternatives for bone augmentation of severely atrophied alveolar ridges present different advantages and limitations. The selection of a certain type of graft depends on the clinician's experience and preference.

Conflict of interests

The authors declare that they have no conflict of interests.

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The stipulations in the European Council's Directive 86/609/EEC, the Directive 2010/63/EU, for the protection of animals used for scientific purposes, were complied with. Also, the study was approved by the Ethics Committee of the Faculty of Veterinary Medicine in Bucharest and the study was in accordance with local laws and regulations.

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Author contribution

Author Victor Nimigean, autor Alexandru Poll, autor Simona Andreea Moraru and autor Maria Justina Roxana Vîrlan have equal contributions to this paper and thus they are all main authors.

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