Significance of the Immunohistochemical Expression of Bone Morphogenetic Protein-4 in Bone Maturation after Maxillary Sinus Grafting in Humans

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ABSTRACT

Background: Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGFβ) protein superfamily and are known to be involved in bone and cartilage formation. Within this family, BMP-4 is one of the most studied members. It has been shown to induce osteogenic differentiation of osteoblasts and osteoprogenitor cells in vitro, but the intimate processes in which this protein promotes and regulates osseous repair still remains unclear.

Purpose: To assess whether the native cellular immunohistochemical expression of BMP-4 correlates with the maturation of bone samples obtained at 6 months after maxillary sinus augmentation.

Materials and Methods: Histopathological and histomorphometrical analyses were performed in all the samples, which were obtained from a total of 58 patients. Immunohistochemical expression of BMP-4 was analyzed in 30 core biopsies obtained from maxillary sinuses grafted with a combination of anorganic bovine bone and autogenous cortical bone [1:1] (AB-group), and 18 biopsies from maxillary sinuses grafted solely with a cortico-cancellous particulate allograft (M-group), all of them after a 6-month healing period. Also, 10 biopsies of native pristine bone were obtained and used as control group (C-group).

Results: Mild to moderate immunohistochemical expression of native granular BMP-4 was present in 56.8% (31.0% AB-group, 22.4% M-group, and 3.4% C-group) ($p = 0.000$, chi-square) of the specimens analyzed. BMP-4 expression was primarily located in the cytoplasm of osteoblasts, osteoclasts, and epithelial cells of the schneiderian membrane. Whereas significant differences were observed in the proportion of mineralized tissue and cellularity between sinuses grafted with anorganic bovine bone, allograft, or nongrafted sinuses, there were no statistically significant differences in the cellular expression of BMP-4 among groups.

Conclusion: Our findings suggest that the native expression of BMP-4 appears to be associated with normal bone homeostasis and reparation in grafted and nongrafted maxillary sites.

KEY WORDS: biomaterials, bone grafting, bone morphogenetic protein 4, immunohistochemistry, maxillary sinus

INTRODUCTION

Bone repair is a complex physiologic process that involves a number of tightly regulated molecular and cellular events. Some molecular signals, such as bone

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morphogenetic proteins (BMPs), may act as bone repair inductors via activation and/or recruitment of pluripotential cells homed in the periostium, bone marrow, bone cortex, and surrounding soft tissues.[1] BMPs are members of the transforming growth factor-β (TGFβ) protein superfamily and are known to be key players in essential embryological processes such as organogenesis and skeleton development in mammals.[2,3] BMPs also play a pivotal role in the regulation of bone formation, maintenance, and repair.[4,5] Since then, many researchers have focused their efforts in testing the inductive potential of BMPs for tissue regeneration in order to use it as a substitute for bone grafting procedures (i.e., maxillary sinus augmentation or horizontal/vertical bone regeneration).

Only some BMPs are capable to promote bone formation in ectopic locations: OP-1 (BMP-7), BMP-2, and BMP-4,[6] but also BMP-6 and BMP-9 may represent effective osteogenic factors for bone regeneration.[7] BMP-4 is also known as BMP2B, BMP2B1, ZYME, OFC11, or MCOPS6.[8,9] BMP-4 gene is located in chromosome 14 q22.2, and it is, so far, one of the most studied BMPs.[8,9] BMP-4 is involved in skeletal repair and bone regeneration processes.[10] It has been shown to induce osteogenic differentiation of osteoblasts and osteoprogenitor cells; and also it seems to promote bone formation, thus playing a crucial role in the onset of bone and cartilage development and fracture repair.[4] Moreover, it is highly produced in early stages of bone repair by immature cells,[11] and its expression is locally sustained during the healing process,[12,13] acting as a stimulator of proliferation and chemotaxis for osteoblasts.[14] In a preclinical study, it was observed that the amount and density of bone formed when recombinant BMP-4 was applied as a grafting material in surgically created defects is significantly higher as compared with a control.[15] This cell-induction mechanism seems to be a key component in bone regeneration, but the all the mechanistic details in which BMP-4 interacts with the wound environment to promote and regulate osseous repair still remains unclear.[2]

There is a paucity of studies appraising the significance of local BMP-4 expression in bone core biopsies obtained after implant site development procedures, such as maxillary sinus augmentation. The aim of the present case series study was to assess whether the native cellular expression of BMP-4 correlates with osseous tissue maturation after maxillary sinus augmentation.

MATERIAL AND METHODS

Patient Recruitment

Two different cohorts of patients treated at the University of Granada School of Dentistry and at the University of Michigan School of Dentistry were recruited for this case series study. The conduction of this study was approved by both the University of Granada Ethical Committee and the University of Michigan Institutional Review Board (IRB #00017520). Patients were recruited following these inclusion criteria: healthy adults over 18 years of age, who underwent uni- or bilateral lateral approach sinus augmentation and delayed implant placement with remaining alveolar bone height under 6 mm. Exclusion criteria included use of medication and/or systemic diseases known to affect bone metabolism, alcohol or drug abuse, smoking more than 10 cigarettes per day, pregnancy or attempting to get pregnant, sinus pathology that contraindicated sinus augmentation upon ear-nose-and-throat consult, and previous history of cancer that involved chemotherapy or radiation to the head or neck.

Surgical Procedure

Surgeries were performed under local anesthesia. In all cases, sinus augmentation was performed following a lateral approach technique.[14] A group of patients (AB-group) ($n = 30$) received anorganic bovine bone with a particle size ranging from 250 to 1,000 μm (Bio-Oss®, Geistlich Pharma, AG, Wolhusen, Switzerland) and autogenous bone harvested with a bone scraper approximately in a ratio of 1:1.[16] The sinuses of the second group (M-group) ($n = 18$) were grafted with a cortico-cancellous allograft with a particle size ranging from 600 to 1,250 μm (Mineross®, BioHorizons Inc., Birmingham, AL, USA). An absorbable collagen membrane (Bio-Gide, Geistlich Pharma, AG, Wolhusen, Switzerland) and a rapidly absorbable collagen sponge (Collatape®, Zimmer Dental, Carlsbad, CA, USA) was used in BA-group and M-group, respectively, to cover the lateral window. A rapidly absorbable collagen tape (Collatape, Zimmer Dental) trimmed to size was used on the M-group. A third group of patients who did not need sinus augmentation was used as control group (C-group) ($n = 10$).[16]

Implant Placement and Biopsy Harvest

Implant placement surgeries were performed after a 6-month healing period. At this time, biopsies were

taken using a 2.75-mm internal diameter trephine. Cores of biomaterials were retrieved from the trephines, sectioning the trephine, in order to preserve the samples. Microdent® implants, (Microdent Implant System, Barcelona, Spain) and Astra TX Osseospeed® implants (Dentsply Implants, Mölndal, Sweden) were placed in the AB-group. In the M-group, BioHorizons implants (BioHorizons Inc., Birmingham, AL, USA) were placed. All biopsies in this study were obtained from the first molar position.

Histopathological and Morphometric Analysis

The biopsies from AB- and M-groups, as well as native bone, were fixed in 10% buffered formalin for 24 hours, decalcified with Decalcifier I®, containing formaldehyde (10% w/v), formic acid (8% w/v), and methanol (1% w/v) (Surgipath softener I® Europe Ltd, Peterborough, UK) for 24 hours in oven at 37°C, and embedded in paraffin. Then, 4-μm sections were cut along the major axis of the biopsy core, and were then deparaffinized and hydrated for staining with hematoxylin-eosin, periodic acid-Schiff, and Masson's trichrome. A millimeter scale in the viewfinder of a microscope (BH2, Olympus Optical Company, Ltd., Tokyo, Japan) with a 400× magnification was used to quantify the paratrabecular osteoblasts, multinucleated osteoclasts, intraosseous osteocytes, fibroblast, inflammatory cells, and number of vessels per mm^2. The results were expressed in terms of the number of events/mm^2. A bone histomorphometric semi-automatic analysis was done on the sections stained with Masson's trichrome, evaluating 10 randomized images with a 100× magnification using a microscope equipped with a digital camera (DP70, Olympus) connected to a computer and using a specialized software (ImageJ® v.1.48, NIH, Bethesda, MD, USA – http://imagej.nih.gov/ij/). Separate measurements of vital bone, graft particles, and nonmineralized tissue were taken and expressed as percentages of each compartment.

Immunohistochemical Analysis

Sections decalcified and embedded in paraffin were dewaxed, hydrated, and heat-treated in 1 mM EDTA pH 8 for an antigen retrieval PT module (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 95°C for 20 minutes. Sections were incubated for 30 minutes at room temperature with the prediluted polyclonal antibody against BMP-4 (Master Diagnóstica, Granada, Spain) to identify intracellular expression. For immunohistochemistry, an automatic immunostainer was used (Autostainer480, Thermo Fisher Scientific Inc., Leicestershire, UK) with the display/amplification peroxidase conjugated micropolymer method and revealed with diaminobenzidine (Ultravision Quanto, Master Diagnóstica). Results of the immunohistochemical analyses were calculated in a semi-quantitative manner using a scale of 0–3 (0, absence; 1, mild [<10% positive cells]; 2, moderate [10–25%]; 3, intense [>25%]).

Statistical Analyses

A statistical software package (SPSS 20.0, IBM Inc., Chicago, IL, USA) was used to perform the statistical analyses. The normality of the distribution of variables was examined with the one-dimensional Kolmogorov–Smirnov test. The bivariate tests used are reported in the table footnotes. Nonparametric Kruskal-Wallis test was used to compare groups. A p value of 0.05 was set as the statistical significance threshold.

RESULTS

Study Population and Clinical Outcomes

A total of 58 patients (53.1% men and 46.9% women) with a mean age of 55 ± 11.09 years (ranging from 23 to 69 years), distributed among the three groups, were included in this study. The AB-group consisted of 30 patients, whereas the M-group was composed of 18 patients. In addition, this study also had 10 patients that conformed the C-group (pristine bone biopsies). Not statistically significant was founded in age or sex in three groups. During the observation period, no failure of the grafting technique was observed. Likewise, no implant failed during the observation period.

Histomorphometric Analysis

Histomorphometric analyses showed greater percentage of mineralized vital bone in samples obtained from the AB-group as compared with samples from the M-group (37.87 ± 15.06% vs 27.59 ± 23.25%). The proportion of mineralized vital tissue found in pristine bone samples (C-group) was higher (45.20 ± 19.50%), as reflected in Figure 1 and Table 1. There was a statistically significant difference ($p = 0.006$) in the amount of bone formation between M-group and pristine bone samples (Table 1). The percentage of nonmineralized tissue observed in samples from AB-group and M-group was lower than

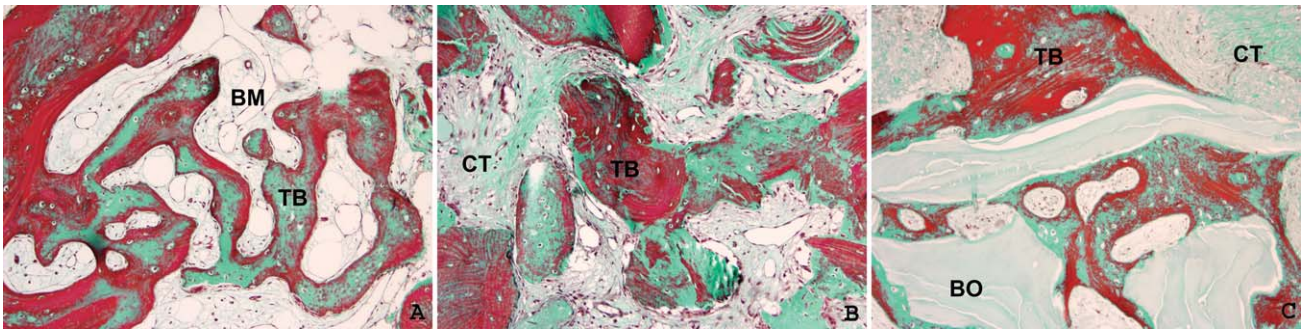

Figure 1 Representative microphotographs of the bone biopsies. (A) Native bone (C-group). (B) Sinus grafted with cortico-cancellous allograft (M-group). (C) Sinus grafted with anorganic bovine bone and autogenous cortical bone (AB-group) (Masson's trichrome, original magnification ×40). BM = bone marrow; BO = anorganic bovine bone particle; CT = connective tissue; TB = trabecular bone.

the percentage found in native bone (C-group), although this difference was not statistically significant (Table 1). Striking statistically significant differences in cellularity composition per mm^2 between the AB- and M-groups were also observed (Table 2). Furthermore, histologic findings indicative of intense bone remodeling were evident in AB-group, namely statistically significant differences in osteoblasts and osteoclasts per mm^2 and number of osteoid lines as compared with the M-group (Table 2).

Immunohistochemical Analyses

Intracellular expression of BMP-4 was observed in a 56.8% of the samples (31.0% AB-group, 22.4% M-group, and 3.4% C-group) ($p = 0.000$, chi-square). However, there were no statistically significant differences in the location and cells in where this protein was expressed. The anorganic bovine bone particles were immunostaining; however, cortico-cancellous particulate allograft and trabecular bone were negative (Table 3 and Figure 2). No correlation could be established between the proportion of mineralized vital tissue and the intracellular expression of BMP-4 (i.e., all Spearman correlation coefficients $p > 0.05$).

DISCUSSION

Maxillary sinus augmentation is considered a highly predictable implant site development option for the atrophic posterior maxilla.[17] The use of many different biomaterials has been proposed in an attempt to replace autologous bone, traditionally considered as the "gold standard," due to concerns associated to availability and second surgical site morbidity. Certainly, maxillary sinus augmentation has been demonstrated to be a predictable procedure after using a variety of grafting materials of different nature, as shown by high long-term survival rates.[18] Additionally, the use of biological agents, such as BMPs, represent a current and interesting line of research. Nonetheless, despite of this fact, the original seeking is still pursuing the ideal biologic response of the graft in order to figure out its behavior, and accordingly, once known accurately the healing events, to provide the proper timeline for implant placement after bone grafting. Actually, it is noteworthy to bear in mind the

TABLE 1 Comparative Morphometric Analysis between the Three Different Study Groups

	AB-Group		M-Group		C-Group		*p* Values
Morphometric Analysis	% $n = 30$ mm^2		% $n = 18$ mm^2		% $n = 10$ mm^2		(K-W Test)*
Mineralized vital bone	37.87 ± 15.06*	0.23 ± 0.10	27.59 ± 23.25	0.18 ± 0.08	45.20 ± 19.50	0.23 ± 0.04	**0.006**†
Remaining biomaterial	21.45 ± 17.09	0.15 ± 0.12	20.58 ± 20.67	0.08 ± 0.09	–	–	0.316‡
Nonmineralized tissue	40.66 ± 18.52	0.25 ± 0.11	52.16 ± 16.29	0.27 ± 0.04	54.45 ± 10.55	0.28 ± 0.04	0.141

Values of percentage (%) and area (mm^2) are expressed as mean ± standard deviation.
*K-W test: Kruskal-Wallis test.
†Statistically significant difference between AB-group (Anorganic Bovine Bone + Cortical autogenous bone) and M-group (Cortico-cancellous allograft).
‡Mann-Whitney *U* test.

TABLE 2 Comparative of Cells Counts per mm² between Samples Obtained from the Two Experimental Groups

	Osteoblasts/mm²	Osteoclasts/mm²	Osteocytes/mm²	Fibroblasts/mm²	Vessels/mm²	Osteoid Lines	Infl. Cells/mm²
AB-group	101.84 ± 54.71*	19.70 ± 16.39	113.71 ± 44.39	121.50 ± 77.99	32.26 ± 15.51	18.20 ± 9.74	45.85 ± 79.66
M-group	49.73 ± 50.04	1.97 ± 1.96	95.52 ± 65.11	28.23 ± 26.32	10.56 ± 11.01	3.40 ± 3.27	69.62 ± 42.75
C-group	23.38 ± 23.57	4.03 ± 6.85	159.67 ± 28.90	17.53 ± 15.75	15.34 ± 4.85	2.30 ± 1.88	20.45 ± 12.69
p Values†	**0.002**	**0.000**	0.140	**0.000**	**0.000**	**0.000**	**0.000**

*Values are expressed as mean ± standard deviation; AB-group: anorganic bovine bone + cortical autogenous bone; M-group: cortico-cancellous allograft; C-group: control group.
†Mann-Whitney *U* test between AB- and M-groups.

maxillary sinus cavity supply an excellent environment in the maxillofacial region that permits studying the healing process in bone grafting.

Findings from the present study showed that BMP-4 displayed a mild granular cytoplasmic expression in osteoblasts, osteoclasts, and/or epithelial cells of the schneiderian membrane in more than 50% of samples obtained. BMP-4 was expressed in a strong granular manner in eight out of the 34 normal tissues studied by Alarmo and colleagues in 2013,[17] which indicates that it is likely to have important in normal tissue function. BMP-4 is expressed by numerous epithelial cells, such as cells of the stratified squamous epithelium of the oral cavity and the stratified squamous epithelium of the esophagus, among others tracts of the alimentary canal. Additionally, it was identified in the transitional epithelium of the bladder and ureter and in the lung cells lining the alveoli. In nonepithelial tissues, granular BMP-4 expression was identified in the red pulp area of the spleen.[17] BMP-4 expression in multiple normal and tumor tissues reveals its importance beyond development. Nonetheless, there is still a dearth of studies reporting cellular bone expression of BMP-4 and its biological implications to draw conclusive statements.[4,17]

Generally speaking on a cellular level, BMPs are able to modulate cell proliferation, differentiation, survival, migration, and even cell fate.[18] Findings from experiments performed in animal models indicate that BMP-4 plays an important role in the initiation and regulation of both endochondral and membranous osseous repair.[4] BMP-4 has been broadly investigated in distraction osteogenesis, mainly in rat model. In these studies,[19–21] the expression of BMP-4 in mesenchymal undifferentiated cells in the fracture callus, particularly in preosteoblasts and osteoblasts, was observed in early and late stages of bone regeneration.[11] Nonetheless, one previous preclinical study in rats showed the presence of this protein in osteoclasts actively participating in matrix remodeling, as well as within periosteal cells along the areas of activity.[2] In the present study, this protein has been observed not only in osteogenic cells and osteoclasts, like in a previous study in humans,[22] but also in cells lining the schneiderian membrane (Figure 2). Within the limits of our knowledge, this has not been described previously in human samples in the literature.

Milani and colleagues have recently reported immunohistochemical expression of BMP2 and BMP7, not

TABLE 3 Comparative Immunohistochemical Expression of BMP-4 between the Three Different Study Groups

	AB-Group	M-Group	C-Group	*p* Values (Kruskal-Wallis test)
Osteocytes	0.03 ± 0.18	0.05 ± 0.22	0.40 ± 0.55	**0.012**
Osteoblasts	0.55 ± 0.72	0.81 ± 0.81	0.20 ± 0.45	0.217
Osteoclasts	0.50 ± 0.78	0.71 ± 0.90	0.00 ± 0.00	0.186
Inflammatory infiltrate	0.17 ± 0.38	0.38 ± 0.50	0.00 ± 0.00	0.093
Remaining graft particle	0.37 ± 0.47	0.00 ± 0.00	0.00 ± 0.00	**0.002***

Values are expressed as mean ± standard deviation. *Statistically significant difference between AB-group (Anorganic Bovine Bone + Cortical autogenous bone) and M-group (Cortico-cancellous allograft) (Mann-Whitney *U* test).

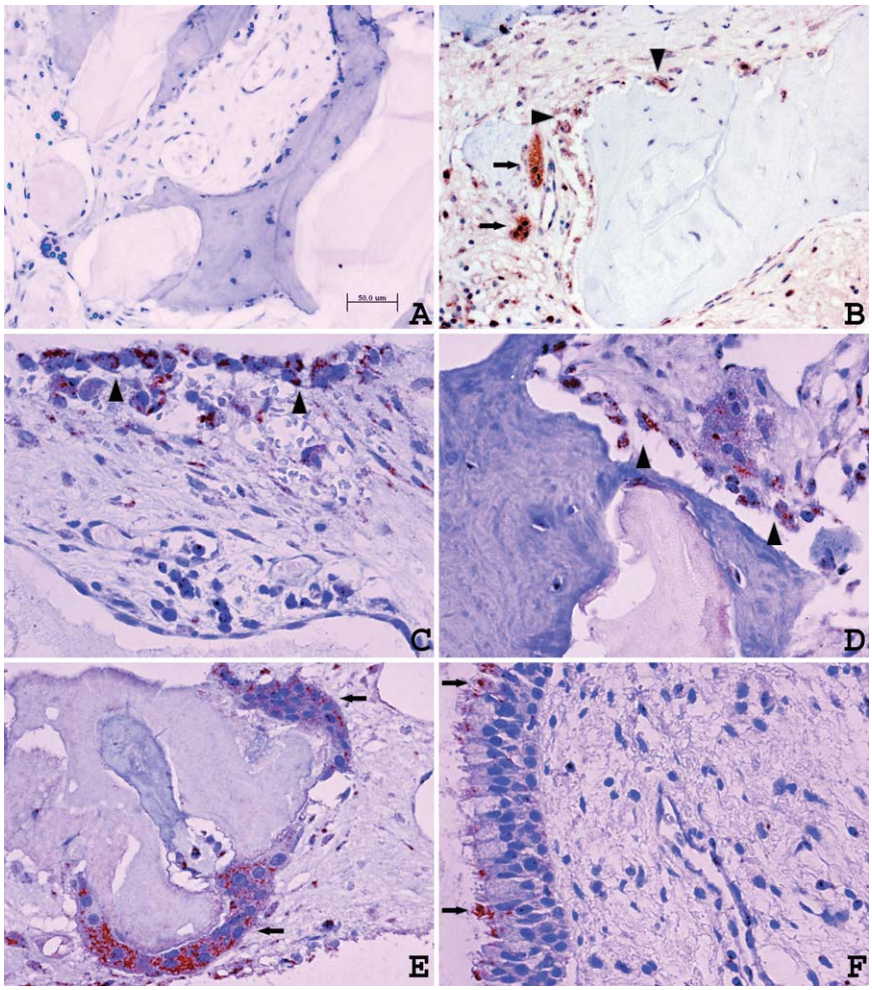

Figure 2 Immunohistochemical expression of BMP-4 in bone samples. (A) Isotype control. Bar 50 micrometers. (B) Osteoblast (arrow head) and osteoclast (arrow) BMP-4 expression in native bone (C-group). (C) Moderate cytoplasmic expression of BMP-4 in osteoblasts (arrow head) in sinus grafted with cortico-cancellous allograft (M-group). (D) Moderate cytoplasmic expression of BMP-4 in osteoblasts (arrow head) in sinus grafted with anorganic bovine bone and autogenous cortical bone (AB-group). (E) Moderate granular cytoplasmic expression of BMP-4 in osteoclasts (arrow) in sinus grafted with anorganic bovine bone and autogenous cortical bone (AB-group). (F) Expression in apical border of epithelial cells from the schneiderian membrane (arrow). (Peroxidase conjugated micropolymer method, original magnification ×200).

only intracellular, but also around xenograft particles in a socket preservation model in humans.[23] This phenomenon has been related in the literature regarding other proteins expression, as osteopontin or TRAP-1.[24] In the present study, regarding the expression of BMP-4 in function of the different grafting biomaterials studied (i.e., xenograft and allograft), it was observed that the remaining particles of anorganic bovine xenograft got impregnated with this protein, which might be related to the superior rate of bone formation promoted by this biomaterial as compared with the allograft alone. Furthermore, it was found that the samples obtained from sinuses grafted with xenograft in combination with autologous bone presented a similar proportion of mineralized vital tissue to the native bone biopsies. Interestingly, this finding has been previously reported in a previous study.[22] Moreover, when we compared both biomaterials with pristine bone, no significant differences were found in the location or intensity of BMP-4 expression. However, statistical differences were observed in the proportion of samples expressing BMP-4 relative to the biomaterial used. Expression of BMP-4 in pristine bone occurred only in the 3.4% of the cases. This control osseous tissue exhibits signs of moderate remodeling, because of the less marked cellularity as compared with the bone graft samples, which may justify the low of expression of BMP-4. In contrast, higher cellularity, vascularity, and number of osteoid

lines were observed in both the AB-group and M-group (Table 2). These results are in concordance with previous studies conducted in humans that demonstrated higher remodeling rates in grafted areas versus pristine bone. In spite of the histomorphometric components were similar in both tissues, the cellular composition, vascularization, and number of osteoid lines were rather superior in grafted areas versus quiescence and mature bone.[22] Furthermore, in the present study, a significantly higher amount of mineralized tissue formation, in correlation with a higher, and also statistically significant, expression of BMP-4 in AB-group in comparison with M-group and C-Group (Table 1) was noticed. A feasible explanation for these findings could be the biological activity associated to the grafting biomaterials overtime.[25] Cortico-cancellous allograft particles showed a higher expression of BMP-4 than pristine bone, indicating a more marked biological activity at 6 months, in terms of bone formation.[26] M-group samples appear to exhibit a faster particle remodeling rate than AB-group, but its biological activity is not as robust as in AB-group, according to the observed cellularity, vascularity, degree of mineralization and BMP-4 expression. Hence, it seems plausible to infer that a higher expression of BMP-4 could indicate a tendency of the AB-group to show a more sustained biological activity than the other group (i.e., allograft) during the healing process that follows maxillary sinus augmentation.

The use of BMPs as sole grafting materials or in combination with other scaffolding bone biomaterials has been broadly studied in dentistry and other medical fields. Clinical trials have shown significant differences in bone formation[26] when this protein is used, being faster and higher.[27,28] In the present study, the relation between native expression of BMP-4 and a superior bone maturation in maxillary sinus augmentation was associated to the biomaterial used. However, this paper shows some limitations, as including to use only one immunohistochemical marker (BMP-4) to have analyze only one time point per sinus, due to ethical reasons, or the high variability in the estimates (high SD). Consequently, results may be interpreted with caution, and further randomized clinical trials with larger number of samples are required to support these results.

CONCLUSIONS

Our findings suggest that the native expression of BMP-4 appears to be associated with normal bone homeostasis and reparation in grafted and nongrafted maxillary sites.

DISCLOSURE

The authors do not have any financial interests, either direct or indirect, in the products or information listed in this manuscript.

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