Original Article

Osteocalcin and Runx2 Expression in Anterior Maxillary Reconstructions Using Bone Xenografts Associated to Bone Marrow Aspirate Concentrate

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Abstract

Background: It is known that a large number of mediators involved in osteogenesis can influence bone development and repair; however, whether these mediators could be used as markers of bone maturity has yet to be determined. **Aim:** To evaluate the expression of osteocalcin (OC) and Runt-related transcription factor 2 (Runx2) in bone biopsies obtained during the reconstruction of atrophic anterior maxillae using particulate bone xenografts with or without association of autogenous bone marrow aspirate concentrate (BMAC). **Materials and Methods:** Ten patients were distributed into two groups (n = 5), according to the type of grafting material used: Control group (CG), particulate bone xenograft alone, and test group (TG), particulate bone xenograft combined with BMAC. A bone specimen was removed from the graft area 4 months after grafting, before implant placement. The specimens were processed and submitted to immunohistochemical analysis for detection of OC and Runx2. Histomorphometry was used to ascertain the percentage of stained areas in both groups. The Wilcoxon Mann–Whitney U-Test was used in the statistical analysis ($P < 0.05$). **Results:** The immunohistochemical analysis revealed a significantly higher OC expression in the TG than in the CG, namely 27.40 ± 1.34% and 11.40 ± 2.70%, respectively ($P < 0.05$), and a significantly higher Runx2 expression in the TG than in the CG, namely 2.80 ± 0.84% and 0.40 ± 0.55%, respectively ($P < 0.05$). **Conclusion:** The OC and Runx2 expression levels were higher when BMAC was associated with the bone xenograft than when it was not.

Keywords: *Bone marrow aspirate concentrate, osteocalcin, Runx2, xenograft*

Introduction

Loss of bone volume can be caused by different pathogenic processes related to tooth loss, periodontitis, dental trauma, or tumors.[1] Bone dimensional changes often lead to unfavorable local conditions for surgical interventions, e.g., implant placement surgery. Therefore, bone grafting is performed to provide sufficient bone volume. This procedure is one of the most widely used therapies in oral and maxillofacial surgery.[2] Although grafting with autologous bone is still considered the gold standard for bone augmentation,[3,4] this technique has been associated with several disadvantages, including donor site morbidity, pain, impaired function, and limitations in the quantity and quality of available bone.[5]

In 2016, Pelegrine *et al*.[6] published the results of a particulate bone xenograft associated with bone marrow aspirate concentrate (BMAC), as an alternative to performing traditional autologous bone grafting to provide cells for anterior maxillary reconstruction. In that study, the authors found that the use of the BMAC obtained through the BMAC system was associated with a trend toward obtaining a higher rate of mineralized tissue (MT) in maxillary reconstructions.[6] In addition, the risk of complications related to bone marrow aspiration has been found to be 10 times lower than that observed with classic iliac crest graft harvesting.[7] Finally, when complications do arise, those resulting from the BMAC system tend to be less severe than those resulting from the traditional approach.[8]

A number of factors can influence bone development, growth, and repair. The mediators involved in osteogenesis include transcription factors, growth factors, cytokines, metabolites, hormones, mechanical loading, and aging.[7] Molecular markers of bone metabolism are novel

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tools that can detect the dynamics of bone remodeling in terms of bone formation and resorption. Moreover, the greater availability of reliable, cost-effective, sensitive, and specific assays for bone turnover markers can be used to complement the measurement of bone mineral density and maturity level.[8,9] Therefore, the aim of this study was to assess the expression of bone protein markers in biopsies obtained during the reconstruction of atrophic anterior maxillae, using particulate bone xenografts with or without the association of autogenous BMAC.

Materials and Methods

This study was conducted at the implant dentistry outpatient clinic, following approval by the Institution's research ethics committee (approval no. 2.384.284/2017). All of the procedures of this study were performed in accordance with the ethical standards of said committee, and with the Helsinki Declaration of 1975, as revised in 2000 (http://www.wma.net/en/30publications/10policies/b3/). Free and informed consent was obtained from all the patients.

The inclusion criteria were patients who needed implants to replace four maxillary incisors, and who had both canines and 3 mm or less of alveolar ridge remaining. In addition, only patients who attended all of the follow-up appointments and maintained adequate oral hygiene were included. Smokers, pregnant or breastfeeding women, patients who had undergone radio-or chemotherapy to treat neoplastic disease, who had systemic diseases or had undergone (or were undergoing) treatments affecting bone homeostasis, who had sinus disease, or who were allergic to any of the components of the materials used in the study were excluded. Ten patients with a mean age of 52.4 ± 2.2 years were selected after applying these criteria.

The thickness of the patients' alveolar ridge was measured using cone-beam computed tomography (CBCT; i-CAT Classic, Imaging Sciences International, Hatfield, PA, USA). The CBCT unit was set to operate at 120 kVp, 5 mA, and a 20 s exposure time, and generated images in Digital Imaging and Communications in Medicine format with a resolution of 96 dpi, 14-bit grayscale, and voxel size of 0.25 mm.

The enrolled patients were distributed into two groups (n = 5), according to the grafting material to be used: Control group (CG), particulate bone xenograft alone (500–1000 μm granules; Bio-Gen; Bioteck, Vicenza, Italy), and test group (TG), particulate bone xenograft combined with BMAC (Bone Marrow Procedure Pack; Harvest Technologies, Plymouth, MA, USA. A web-based software tool was used for randomization (www.randomization.com). Collagen membranes (Biocollagen; Bioteck, Vicenza, Italy) were placed over the bone grafts in all the maxillary augmentation procedures in both groups, following the principles of guided bone regeneration. All the patients were dentally rehabilitated using osseointegrated implants and fixed prostheses at the end of the study.

Bone marrow aspirate concentrate method

Bone marrow was harvested and processed in the operating room using the BMAC system (Harvest Technologies), following the manufacturer's instructions. In brief, 30 mL of bone marrow aspirate was obtained through a puncture performed in the upper posterior iliac crest, using a bone marrow needle, and heparinized in 30 mL syringes containing 1 mL of heparin at a concentration of 5000 U/mL. This procedure was conducted in an outpatient setting under local anesthesia, using a 2% xylocaine solution without a vasoconstrictor [Figure 1]. The syringe filled with bone marrow was then connected to a filter bag, to which 8 mL of anticoagulant citrate dextrose-A solution was added. After homogenization, a new syringe was attached, and the filtered 30 mL was removed. The bone marrow aspirate was transferred into disposables, which were then placed in a centrifuge (SmartPReP 2; Terumo BCT, Lakewood, CO USA). Two phases were obtained within the tube after 14 min of centrifugation, namely plasma supernatant and precipitated bone marrow cell concentrate [Figure 2]. The plasma was removed using syringes provided in the kit, after which approximately 4 mL of the cell concentrate was aspirated.

Surgical procedure

As previously described,[6] all the patients were treated with local anesthesia (Mepiadre; DFL, São Paulo, SP, Brazil), and a full-thickness flap was raised to provide access to the alveolar ridge. A 21-mm carbide burr (Ar N 701; Jota Rotatory Instruments, Ruthi, Switzerland) was used for decortication, aimed at enhancing vascularization. The particulate bone xenograft was spread over the bone in both groups, in order to cover the exposed bone evenly and to provide adequate graft thickness.

In the TG, the bone graft was mixed with the BMAC before placing it in the atrophic site. The graft was covered with an equine collagen membrane in both groups. The flaps

Figure 1: Aspiration of the bone marrow

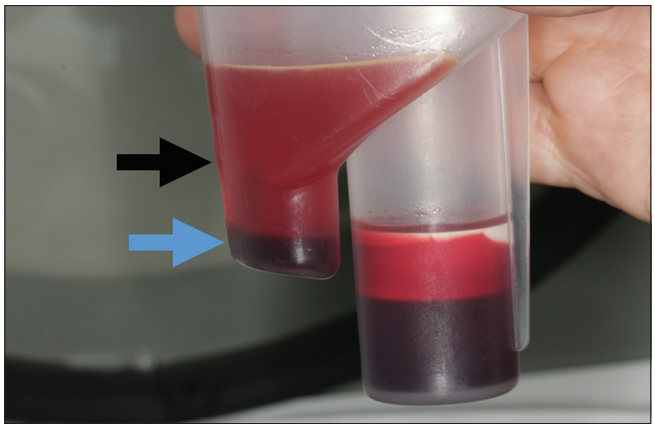

Figure 2: Bone marrow after centrifugation. Note the plasma supernatant (black arrow) and the bone marrow concentrate cells (blue arrow)

were then repositioned and sutured with interrupted single 4-0 nylon sutures. Four months after grafting, a cylindrical biopsy was obtained from the graft site using a trephine bur with an internal diameter of 2 mm [Figure 3a and b], after which the dental implants were installed as planned.

Specimen preparation

The cassettes containing the bone specimens were taken to the pathology laboratory for preparation of the silanized slides. A microtome (Leica RM2245; Buffalo Grove, IL, USA) was used to produce 3-µm-thick fragments; four fragments were placed on each slide. The slides were kept in an incubator at 6°C for 1 h before initiating the immunohistochemical analysis.

Rabbit primary antibodies for Runx2 (monoclonal ab192256, Abcam, Cambridge, MA, USA) and osteocalcin (OC) (polyclonal ab93876, Abcam, Cambridge, MA, USA) were used to investigate the behavior of the biomaterials with respect to bone tissue repair reactions: Osteoblastic activity (Runx2) and bone mineralization and maturation (OC). The specimens were bathed twice in xylol for 10 min, at room temperature, to remove excess paraffin from the histological sections, and then for 3 min in decreasing alcohol gradients, to hydrate the specimens, namely three times in absolute alcohol, one in 95%, and one in 85% alcohol. After washing in running water, the specimens were rinsed three times in distilled water. Retrieval of the Runx2 (citrate with pH 6.0) and OC antigenic sites was performed with Tris ethylenediaminetetraacetic acid (EDTA), pH 9.0, in a pressure cooker for 3 min at 97°C, followed by a new wash in running water, and an additional three rinses in distilled water. Blocking of endogenous tissue peroxidase was done by immersion in a 6% hydrogen peroxide and methanol solution (1:1) for 15 min, followed by washing in running water, rinsing in distilled water, and bathing twice in a 0.05 M TRIS buffer solution with pH 7.4, for 5 min.

The primary antibodies were diluted in 1% bovine serum albumin, titrating 1:1000 for Runx2 and 1:400 for OC,

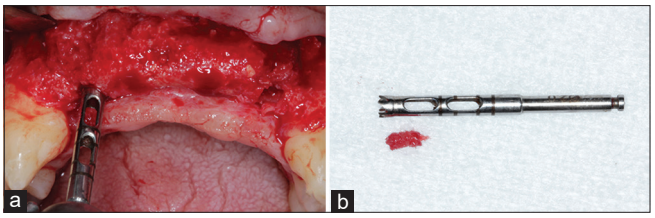

Figure 3: (a) Bone biopsy being harvested with a trephine bur. (b) bone biopsy immediately after removal

and then incubated overnight in a dark humid chamber at 4°C. The secondary antibody to Runx2, Advanced™HPR Link (K4068; DakoCytomation; Carpinteria, CA, USA) was incubated for 30 min and then bathed twice in a 0.05 M TRIS buffer solution with pH 7.4, for 5 min. Subsequently, the HPR-Advanced Enzyme (DakoCytomation; Carpinteria, CA, USA) was incubated for 30 min and then bathed twice in a 0.05 M TRIS buffer solution with pH 7.4 in a dark humid chamber at room temperature, for 5 min.

The secondary antibody to OC, LSAB Biotinylated Link Universal (K0690; DakoCytomation; Carpinteria, CA, USA) was incubated for 30 min and then bathed twice in a 0.05 M TRIS buffer solution with pH 7.4, for 5 min. Subsequently, the LSAB Streptavidin-HPR (DakoCytomation; Carpinteria, CA, USA) was incubated for 30 min. The slides were then bathed twice again in a 0.05 M TRIS buffer solution with pH 7.4, for 5 min, and then incubated in a dark humid chamber at room temperature.

The reaction was developed with diaminobenzidine (DAB K3468; DakoCytomation; Carpinteria, CA, USA) for 10 min in a dark humid chamber. The slides were then quickly rinsed in a 0.05 M TRIS buffer solution with pH 7.4. After washing in running water and bathing in distilled water, the specimens were counterstained by immersion in Mayer's hematoxylin (CI.75290; Exodo Científica, Sumaré, SP, Brazil), for 6 min, washed again in running water, for 6 min, and rinsed again in distilled water.

The specimens were dehydrated in increasingly concentrated ethanol solutions (80%, 95%, and 100%) and cleared in xylol. The slides were mounted in Permount medium (Fisher Chemical Permount Mounting Medium; Abcam, Cambridge, MA, USA) and then analyzed under a light microscope (Eclipse Ci-S; Nikon, Tokyo, Japan).

Digital images of the histological slides were captured with a digital camera (Infinity 1; Teledyne Lumenera, Ottawa, ON, Canada) coupled to a microscope (Nikon) under 40× magnification, and displayed on the screen of a personal computer using Infinity Capture software (Teledyne Lumenera, Ottawa, ON, Canada). All the images were then analyzed by a single calibrated evaluator; Image J software (Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA) was used to quantify the percentage of area stained for OC and Runx2.

Statistical analysis

The Wilcoxon Mann–Whitney U-test was used to compare the results of the study groups. A significance level of 0.05 was adopted.

Results

Figure 4 shows representative histological sections of specimens from both groups (CG and TG) immunostained for Runx2 and OC. OC expression was significantly higher in the TG than in the CG, and Runx2 expression was insignificant in the TG and null in the CG. The mean percentages of the areas immunostained for OC and Runx2 are shown in Tables 1 and 2.

Discussion

In 2016,[6] Pelegrine *et al*. presented the tomographic outcomes of and a complete methodological discussion on the use of particulate bone xenograft associated to BMAC. In that study, the relative quantities (%) of MT and non-MT (NMT) were used to assess the mineralization pattern histomorphometrically. The MT and NMT values reported were 52.3% ±16.78%, and 47.70% ±5.55%, respectively, for the CG, and 65.04% ±20.98% and 34.96% ±10.38%, respectively, for the TG, albeit with no statistically significant difference between them. These results seemed to indicate a trend for higher MT and lower NMT levels in the TG compared with the CG, which could be attributed to the presence of osteogenic cells and osteoinductive growth factors within the bone marrow, as also reported by Pelegrine *et al*.[10] and Aloise *et al*.[11] Therefore, based on the findings of these previous studies, we decided to investigate whether using protein markers of bone maturity could enhance our understanding of particulate bone xenograft associated with BMAC.

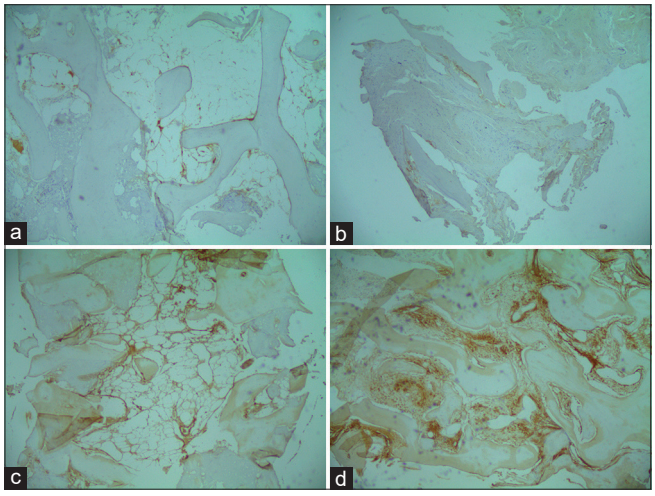

Figure 4: (a) Photomicrograph of a histological section from the control group immunostained for Runt-related transcription factor 2 (Runx2, ×100). (b) Photomicrograph of a histological slide from the test group immunostained for Runx2 (×100). (c) photomicrograph of a histological section from the control group immunostained for osteocalcin (OC, ×100). (d) Photomicrograph of a histological slide from the test group immunostained for osteocalcin (×100)

A number of factors can influence bone development, growth, and repair. The mediators involved in osteogenesis include transcription factors, growth factors, cytokines, metabolites, hormones, mechanical loading, and aging.[12-14] Osteogenic transcription factor Runx2/Cbfa1 has been shown to increase osteogenic gene expression synergistically, including that of OC, bone sialoprotein (BSP), alkaline phosphatase, and biological mineral deposition in primary dermal fibroblasts.[15-18] Runx2 is best known as the master regulator of osteoblast differentiation, osteoblast function, and osteoblast marker gene expression. In fact, the osteogenic activity of bone marrow stromal cells has been reported to be enhanced by Runx2 overexpression, both *in vitro* and *in vivo*.[19] In osteoblast biology, Runx2 regulates the process of osteoblast differentiation at different stages, albeit more accurately in the earlier stages.[19] In addition, regulation by Runx2 takes place in a positive manner at the early stages of differentiation, whereas Runx2 inhibits the process at later stages. Runx2 is involved in the expression of the Col1, osteopontin, BSP, and OC bone matrix genes, all of which are involved in the process of osteoblast differentiation. Runx2 expression has to be downregulated before further bone maturation can occur.[20,21] In the present study, the Runx2 expression was higher in the TG ($P < 0.05$); however, when the absolute values in both groups were critically analyzed, it was noticed that they were very close to zero. This indicates that there was no relevant Runx2 expression in either group. We speculate that this low level of Runx2 in both groups is related to the time period elapsed before reopening the grafted site for implant placement (i.e., 4 months), since its expression is higher at earlier stages of osteoblast differentiation.[19]

OC, also known as bone γ-carboxyglutamic acid-containing protein, is preferentially expressed by osteoblasts and is the most abundant noncollagenous bone matrix protein, often used as a late marker for bone formation.[22] This protein is commonly produced by mature osteoblasts and is deposited in the extracellular matrix, signaling the occurrence of bone repair. The results of the present study evidenced that OC expression was higher in the TG than in the CG ($P < 0.05$), indicating that the bone tissue in the group where BMAC was associated with the graft had a higher level of maturity.[23,24] It should be borne in mind that the bone specimens were harvested 4 months after grafting, and it is well known that the average time for bone regeneration to occur is 6–8 months. Accordingly, most authors have reported an average of 6–8 months between grafting for horizontal augmentation and harvesting of specimens; thus, it would be interesting to investigate whether the maturity level of the bone tissue would also be impacted by associating BMAC with the bone xenograft material. As stated earlier, a previous study[6] showed a tendency

Table 1: Immunohistochemical analysis

Group	Mean	SD (±)	Median	Minimum	Maximum	*n*
OC expression						
CG	11.40	1.34	12.00	10.00	13.00	5
TG	27.40	2.70	28.00	23.00	30.00	5
Runx2 expression						
CG	0.40	0.55	0.00	0.00	1.00	5
TG	2.80	0.84	3.00	2.00	4.00	5

SD: Standard deviation; OC: Osteocalcin; CG: Control group; TG: Test group; Runx2: Runt-related transcription factor 2

Table 2: Intergroup analysis

Group	*P*
OC	
Control group versus test group	0.0086*
Runx2	
Control group versus test group	0.0076*

*Statistically significant (Wilcoxon Mann–Whitney U test, $P<0.05$). OC: Osteocalcin; Runx2: Runt-related transcription factor 2

toward a greater amount of MT in the TG compared with the CG. However, in the present study, an increase in OC expression was observed in the TG, when the study groups were analyzed immunohistochemically. This result could indicate that the presence of osteogenic cells in the BMAC leads to greater maturity of the bone tissue in a shorter time interval, and this may influence the decision-making process involved in establishing the period required for bone development (after bone grafting and before installing dental implants) when using this technology.

Conclusion

The use of BMAC associated with a bone xenograft resulted in a higher expression of both OC and Runx2.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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