Time-Dependent Changes in Fresh-Frozen Bone Block Grafts: Tomographic, Histologic, and Histomorphometric Findings

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ABSTRACT

Background: Bone allografts have shown satisfactory clinical results in alveolar ridge reconstructions. However, the process of incorporation and the resorption rates of these grafts are not yet fully understood.

Purpose: The aim of this study was to use computed tomography (CT), histology, and histomorphometry to assess the time-dependent rates of resorption and incorporation of fresh-frozen bone allografts.

Materials and Methods: Twenty-four patients underwent alveolar ridge reconstruction with bone block allografts and were randomly allocated to three groups with different graft healing periods (4, 6, or 8 months) before implant placement. To assess the resorption rates, CT scans were acquired within 7 days after bone graft surgery and at the end of the period. Graft samples were collected and sent for histological and histomorphometric analyses.

Results: The graft resorption mean rates were 50.78% ± 10.43, 32.77% ± 7.84, and 13.02% ± 3.86 for the 4-, 6-, and 8-month groups, respectively, and were significantly different among the three groups. Newly formed bone with osteocytes near the grafted bone was observed in all three groups. The number of osteocytes was significantly lower at 4 months. Grafted bone remains were significantly higher in the shortest period of time. All of the grafts showed large amounts of calcified tissue.

Conclusions: All three groups showed new bone formation and different bone resorption rates. Graft healing periods of 4 months showed less graft resorption and seemed to be the most favorable for implant placement. Healing periods of 8 months showed the largest rate of graft resorption, which could render the grafts unfavorable for implant placement.

KEY WORDS: allograft, alveolar ridge reconstruction, atrophic maxilla, bone grafting

INTRODUCTION

Dental implants are considered a predictable modality of treatment, showing high success rates in totally and partially edentulous patients. Although available techniques allow clinicians to address most complex cases successfully, extremely atrophic maxillary ridges are still a challenge in implantology. The rehabilitation of alveolar ridges with implants requires bone quantity and quality sufficient to achieve aesthetic and stable results.[1,2]

Several bone substitutes and surgical techniques are currently available for the reconstruction of resorbed ridges. These reconstructions are very often required, as current concepts have determined that implants should be placed in accordance with prosthetic planning and not bone availability.[3,4]

Reports are common in the literature on the use of autogenous bone (AB) grafts in alveolar ridge reconstructions with high success rates.[5–7] However, the use of AB can result in increased morbidity due to harvesting

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surgery. Similarly, a limited availability of donor sites from the patient and the potential risk of paresthesia, injury to vital structures, excessive swelling, and complications associated with surgical access have stimulated the demand for alternative bone substitutes.[8–12]

The use of fresh-frozen bone (FFB) allografts from human donors for the reconstruction of large bone defects in orthopedics has been reported often in the literature. In fact, orthopedic surgeons routinely transplant not only bony tissue but also ligaments, menisci, and articular surfaces with success.[13–17] FFB grafting appears to present satisfactory results in alveolar ridge reconstruction, but the data available are mostly from case reports and case series with only short follow-ups.[18–26]

Apparently, the optimal graft healing time for the second stage of surgery (implant placement) has not received a consensus in the literature. It is assumed that the longer the waiting time is, the greater the degree of graft incorporation will be, which would be beneficial for implant placement. However, the extent of the waiting period for the second stage also appears to result in increased bone graft resorption, which in extreme cases can result in insufficient bone quantity for implant placement.[27]

The time for incorporation and the resorption rates of FFB allografts have not yet been defined, and studies on these topics are scarce in the literature.

From this point of view, our hypothesis is that there is a time-related intersection between the lowest graft resorption rate and best incorporation possible that could lead to implants osseointegration. The aim of this study was to evaluate the incorporation and resorption rates of FFB block allografts using different graft healing periods before implant placement.

MATERIALS AND METHODS

Clinical Protocol

This study protocol was approved by the Ethics Committee of University Hospital Pedro Ernesto (CEP-HUPE, 2762/2010). All of the eligible patients signed informed consent forms.

From July 2010 to July 2011, 24 consecutive patients were included in this study (6 men and 18 women, mean age: 53.8 ± 8.4 years old, ranging from 47 to 68 years) from the Department of Implantology at Rio de Janeiro Catholic University, who had indications for bone block reconstruction prior to implant placement. The exclusion criteria were smoking, systemic diseases, current or previous therapy with oral or intravenous bisphosphonates and irradiation in the past 5 years.

All of the patients underwent cone-beam computerized tomography (CBCT) (I-Cat® Image Sciences International, Hatfield, PA, USA) scanning to confirm the indication of the procedure and the surgery planning. The number and size of the bone blocks were determined using linear measurements on the CT scans. FFBs were acquired for each patient, according to the Brazilian National Transplant System policy from the Musculoskeletal Tissue Bank of the National Institute of Traumatology and Orthopedics (INTO). The grafts used were fragments of cortico-cancellous proximal tibia, iliac crest and femur bones.

All of the procedures followed the same protocol: the bone grafts were left in sterile saline for approximately 30 minutes before to thaw them; then they were sculpted into block shapes to fit the defects appropriately (Figure 1, A and B). Local anesthetic, consisting of lidocaine 2% with epinephrine 1:100,000 (DFL®, Rio de Janeiro, RJ, Brazil), was administered, and a full-thickness mucoperiosteal flap was elevated. The recipient site was prepared using a surgical drill at a low speed to create a "box" of approximately 0.5 mm in depth to increase the contact area and stabilize the bone blocks. Microperforations were created with a 1.1 mm twist drill until it reached the medullary region of the recipient site, to facilitate vascularization of the grafts (Figure 2). The blocks were then adapted appropriately to the recipient bed and were fixed with titanium screws of 1.6 mm in diameter and 10, 12, or 14 mm in length, depending on the needs of each region (Figure 3). Each block was fixed with at least two screws to ensure the mechanical stability of the grafts. The flaps were repositioned, ensuring primary closure of the wounds. To close, 4-0 silk sutures were used (Ethicon Inc.®, Somerville, NJ, USA).

The patients were medicated with 500 mg azithromycin once daily for 3 days, and nonsteroidal anti-inflammatory drugs (NSAIDs) for postoperative pain control and chlorhexidine 0.12% mouth rinse twice per day for 15 days. Sutures were removed 7–14 days after surgery.

All of the patients underwent a CBCT scan at a maximum interval of 7 days after graft surgery. The patients were then randomly assigned to one of three

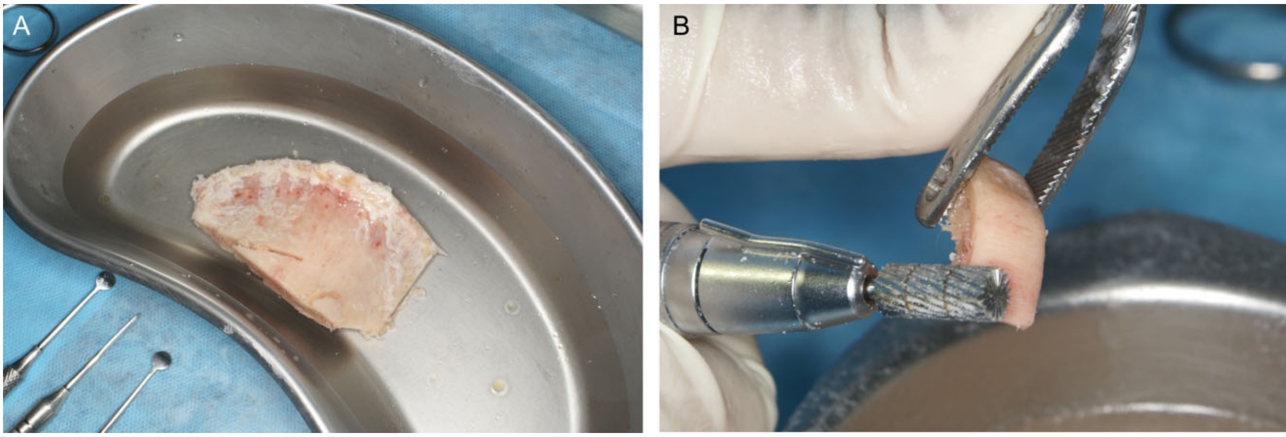

Figure 1 *A*, Bone graft in sterile saline to thaw. *B*, Bone block being sculpted to fill the defect.

groups as follows: Group 1 – patients who waited 4 months for implant placement; Group 2 – patients who waited 6 months for implant placement; and Group 3 – patients who waited 8 months for implant placement.

After the graft healing period for each group, all of the patients underwent a new CBCT scan at a maximum interval of 7 days before the day planned for implant placement surgery.

The patients were scheduled for implant placement procedures according to the times determined for each group. All of the procedures followed the same routine: local anesthesia was administered using lidocaine 2% with epinephrine 1:100,000, and a full-thickness mucoperiosteal flap was elevated. The screws used to fix the blocks were removed, and a graft sample was collected with a trephine drill 3.0 mm in diameter applied to a bucco-palatal depth of 10 mm (Figure 4, A and B). The fragments were preserved in 10% buffered formalin. The implants were placed using implant system drills (International Intralock®, Boca Raton, FL, USA) according to the manufacturer's specifications. The flaps were repositioned and closed with 4-0 silk sutures.

The patients were medicated with 500 mg azithromycin once daily for 3 days, and NSAIDs for postoperative pain control and chlorhexidine 0.12% mouth rinse twice per day for 15 days. The sutures were removed 7 days after surgery.

All implants placed were allowed to heal submerged for 3 months. After this period, the patients underwent the second stage implant surgery and the osseointegration was evaluated clinically in order to assess the short-term cumulative survival rate (CSR).

Tomographic Analysis

Tomographic measurements were taken using computer software (Dental Slice Converter© BioParts, SP, Brazil)

Figure 2 Recipient site perforated to facilitate the grafts vascularization.

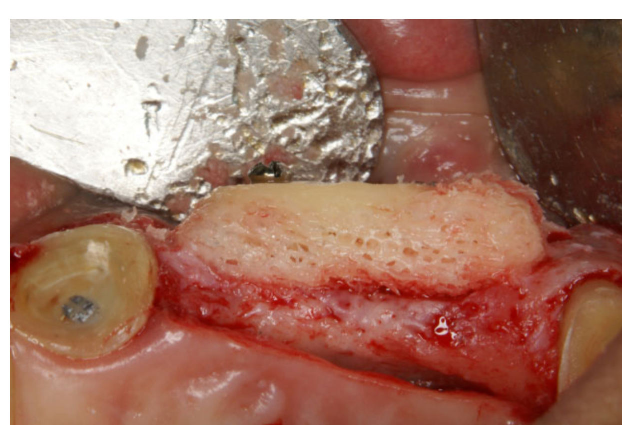

Figure 3 The bone block adapted appropriately to the recipient bed ready to be fixed.

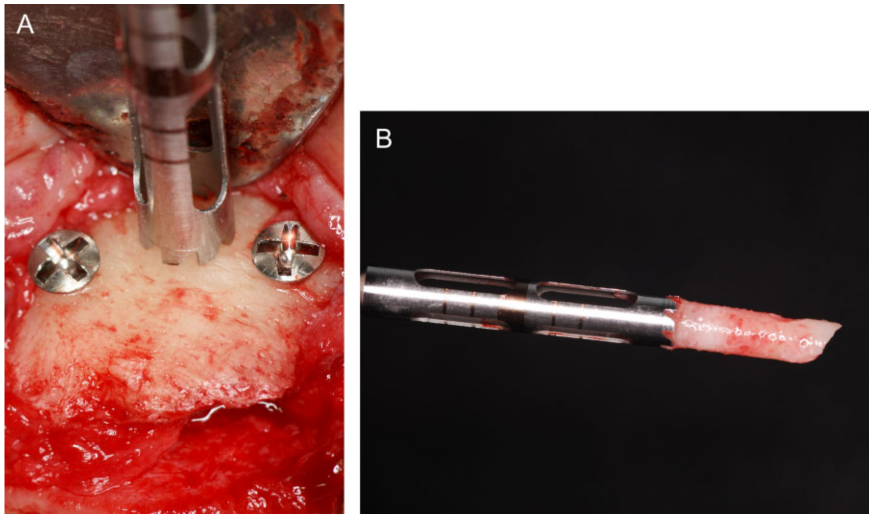

Figure 4 *A*, Graft sample being collected with a trephine drill. *B*, Sample removed to be sent to histological analysis.

and were performed by a single operator. Bone blocks were virtually isolated on CT images using the grayscale threshold tool selection. Gross selection errors were corrected manually by the operator. The volume (cm^3) for each block was calculated automatically by the software.

The accuracy of the CT measurements using this method was validated in a previous in vitro pilot study (data not shown). The intraexaminer calibration was performed using three CT scans of patients not included in the study, acquiring two measurements for each test with an interval of 1 week between them. The Kappa index was 0.98.

The volumetric changes were determined for each graft using the following formula:

$$\text{Volume reduction (\%)} = -\frac{\text{Final volume} - \text{Initial volume}}{\text{Initial volume}} \times 100.$$

Thus, the final volume is given as a percentage of the reduction compared with the initial volume because the size of the grafts differed depending on the needs of each patient.

A statistical analysis was performed to compare the mean percentage reduction in volume among the three groups. The normality of the sample was tested using the Shapiro–Wilk test. The comparison among groups was performed using one-way analysis of variance (ANOVA). Significance was determined at a threshold of 5% ($p < .05$). Discrepancies within each group were evaluated using the Friedman test.

Histological Analysis

Samples of the grafts taken during implant placement were subjected to histological analysis.

The samples were routinely processed, decalcified in 5% nitric acid, embedded in paraffin and stained with hematoxylin and eosin following the standard protocol of the Laboratory of Oral Pathology, School of Dentistry, State University of Rio de Janeiro.

The samples were examined by optical microscopy at 10×, 20×, and 40× magnification.

The analysis was descriptive, without comparison between the groups. The parameters described were the presence of newly formed bony tissue, the presence of osteoblasts/osteocytes, and the presence of vessels. For comparative purposes, the histomorphometry method was used.

Histomorphometric Analysis

The slides were evaluated by a single examiner in 10 fields of 40× per slide. The criteria for the assessment of the incorporation of the grafts were osteocyte count, vessel count, remaining bone graft, and proportions of calcified tissue/noncalcified tissue.[28] To quantify the remaining bone graft, scores were used according to the percentage occupied in each field, as follows: 1 – less than 33% of the visual field filled with the remaining graft; 2 – between 34% and 66% of the visual field filled with the remaining graft; and 3 – more than 67% of the visual field filled with the remaining graft. To assess the proportions of calcified tissue/noncalcified tissue, the following scores were used: 1 – less than 33% of the visual field filled with calcified tissue; 2 – between 34% and 66% of the visual field filled with calcified tissue; and 3 – more than 67% of the visual field filled with calcified tissue.

Intraexaminer calibration was performed for all four criteria using 10 fields with 40× magnification, with a 1-week interval between them, for three slides not included in the study. For the osteocyte and vessel counts, the Kappa index was 0.95. For the remaining grafts and proportions of calcified tissue/noncalcified tissue, the Kappa index was 0.80.

The means of the osteocyte count and vessel count for each group were statistically compared among the groups using the Kruskal–Wallis H test. The median of the remaining graft scores and proportions of calcified tissue/noncalcified tissue for each group were also compared statistically among the groups using the Kruskal–Wallis H test. Discrepancies within each group for all histomorphometric evaluations were evaluated using the Friedman test.

RESULTS

Of the 24 subjects initially included in this study, one patient developed an infection at the surgery site and required graft removal. Another patient did not comply with the correct interval for CT scans. These two patients were excluded from the study.

During reopening, all of the blocks proved mechanically stable and clinically well incorporated into the recipient beds. There was no displacement of the grafts at drilling and placement of the implants. All of the grafts showed evidence of vascularization from a clinical standpoint, verified by intraosseous bleeding.

A total of 75 implants were placed in the regions grafted in the 22 patients in the study. In all of the cases, the implants achieved proper primary stability with satisfactory insertion torque (>35 N).

Of the implants placed, 1 (1.33%) was lost early, although there was no clinically apparent involvement of the graft. The CSR was 98.67%.

The resorption patterns of the grafts were clearly not uniform, for example, the areas of the grafts that were more or less resorbed varied for each patient and for each block individually. Apparently, the cancellous bone regions showed more resorption than the cortical regions, although this difference was not evaluated in the study.

Tomographic Assessment

There was a clear difference in graft resorption at different time intervals, indicating that the shorter intervals – 4 and 6 months – resulted in significantly smaller reductions in size (Table 1).

A comparison of the groups showed a statistically significant difference in the volume reduction of the grafts among the three groups (Figure 5). The results of the one-way ANOVA test are summarized in the Table 2.

No discrepancy among the groups was found with regard to the rate of resorption, as confirmed by the Friedman test. Thus, homogeneity was observed within each group.

Histological Assessment

The histological analysis showed the presence of osteoblasts at the margins of the calcified regions. Superficial regions of the biopsies, equivalent to the cortical portions of the grafts, presented several osteocyte spaces with no cellular content – a feature of the graft remains. Newly formed bone was also commonly observed, populated by osteocytes near the regions of grafted bone (Figure 6, A and B). Vessels were also found abundantly in the biopsies.

Histomorphometric Assessment

Statistically significant differences among the groups were found after the histomorphometric analysis.

The osteocyte number was significantly lower in Group 1 compared with Groups 2 and 3, as evidenced by the Kruskal–Wallis H test (Figure 7). Similarly, the vessel

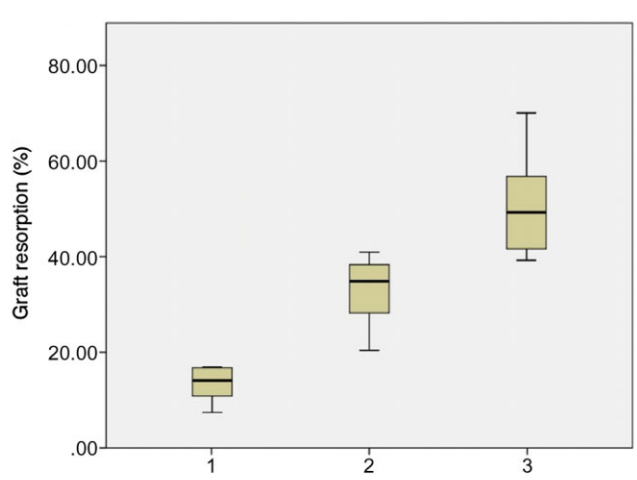

Figure 5 Boxplot of the grafts resorption in the groups.

TABLE 1 Initial Volume, Final Volume, Percentual Volume Reduction, and Mean Volume Reduction for Each Group

Group	Patient No.	Gender	Age	Initial Volume (mm^3)	Final Volume (mm^3)	Volume Reduction (%)	Mean Volume Reduction (% ± SD)
Group 1 (4 months)	1	F	47	882.7	804.95	8.81	13.02 ± 3.86‡
	2	F	62	768.54	662.81	13.76	
	3	F	48	848.25	709.71	16.33	
	4	M	37	668.81	587.51	12.16	
	5	M	48	462.54	386.34	16.47	
	6	F	61	328.12	304.88	7.08	
	7	F	55	780.55	651.54	16.53	
	8	F	49	1,492.42	972.29	34.85	
Group 2 (6 months)	9	F	64	2,151.64	1,444.11	32.88	32.77 ± 7.84*
	10	F	48	995.2	587.7	40.95	
	11	F	47	844.08	519.48	38.46	
	12	M	50	1,124.77	858.9	23.64	
	13	F	52	479.81	296.42	38.22	
	14	F	60	761.1	605.69	20.42	
	15	F	62	456.35	276.36	39.44	
Group 3 (8 months)	16	M	42	986.14	513.3	47.95	50.78 ± 10.43†
	17	F	58	1,525.79	877.6	42.48	
	18	M	53	637.38	285.94	55.14	
	19	F	62	794.2	236.07	70.28	
	20	F	68	305.31	149.81	50.93	
	21	F	45	1,491.23	613.72	58.84	
	22	F	66	726.83	427.44	41.19	

*Statistically different from groups 1 and 3.
†Statistically different from groups 1 and 2 ($p < .05$).
‡Statistically different from groups 2 and 3.

number also showed significant differences among the groups (Figure 8). However, in the vessel evaluation, Group 2 proved to be different from Groups 1 and 3. Groups 1 and 3 showed no statistically significant differences between them. The results of the Kruskal–Wallis H test are summarized in Tables 3 and 4 for the osteocyte and vessels count, respectively.

The assessment of the remaining grafts in the biopsies showed significant differences between Group 1 and the other groups using the Kruskal–Wallis test. Results

TABLE 2 Comparison among the Groups Using One-Way ANOVA Test

Group a	Group b	Difference a – b	Standard Deviation	*p* Value	Confidence Interval Inferior	Confidence Interval Superior
1	2	−19.75	4.28	.001	−29.34	−10.16
	3	−37.76	4.15	<.001	−49.17	−26.34
2	1	19.75	3.30	.001	10.16	29.34
	3	−18.01	4.73	.007	−30.90	−5.12
3	1	37.76	3.97	<.001	26.24	49.17
	2	18.01	4.73	.007	5.12	30.90

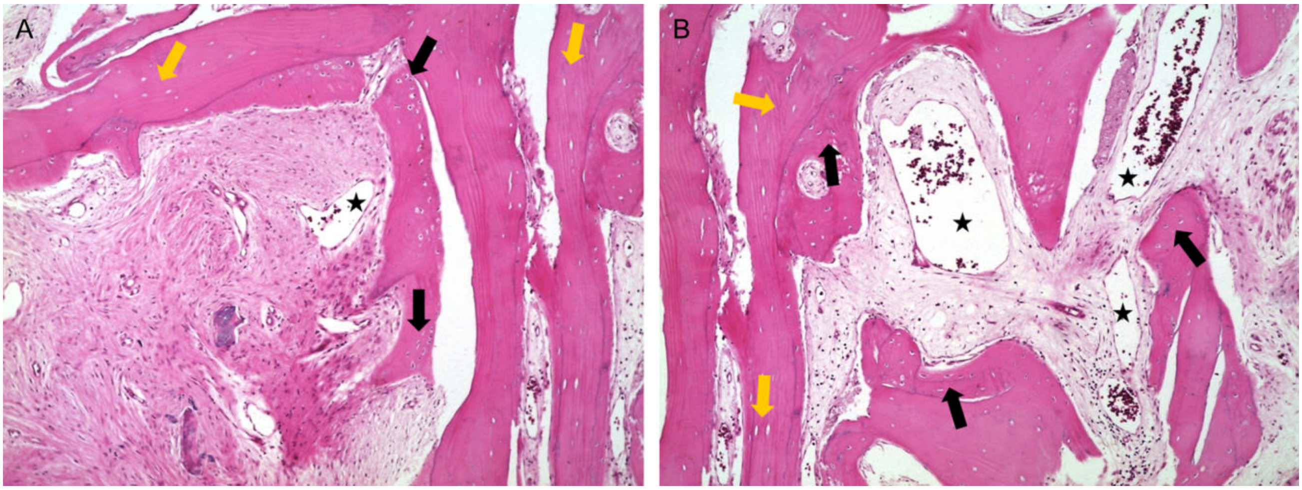

Figure 6 *A* and *B*, Newly formed bone with the presence of osteocytes (black arrows) near the regions of remaining grafted bone showing osteocyte spaces with no cellular content (yellow arrows) and vessels (Stars).

are presented in Table 5. There were no differences between Groups 2 and 3 (Figure 9). There were no discrepancies within each group, as confirmed by the Friedman test.

There were no significant differences among the groups regarding the proportions of calcified/noncalcified tissue (Figure 10 and Table 6). As in previous evaluations, the groups were homogeneous, as confirmed by the Friedman test.

DISCUSSION

Over the past 10 years, an increasing number of studies have been published using bone allografts in dentistry. The search for biomaterials that present satisfactory results, coupled with predictability, availability, less surgery time, morbidity, and invasiveness, has been a major focus of implantology.[18–26,29,30]

In any modality involving bone grafting with two stages, there is a concern with regard to dimensional

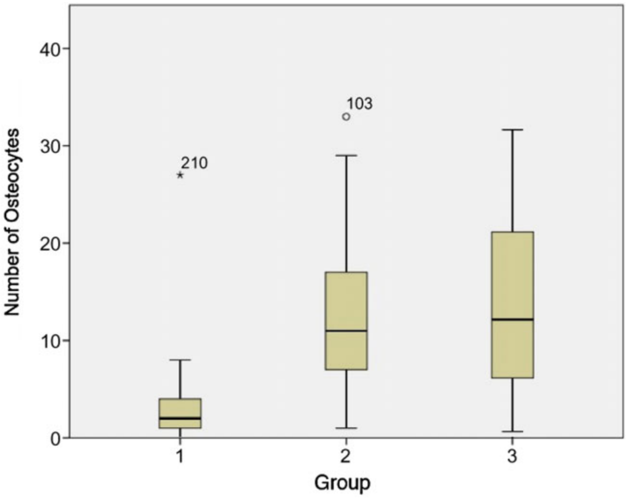

Figure 7 Boxplot of the number of osteocytes in the groups.

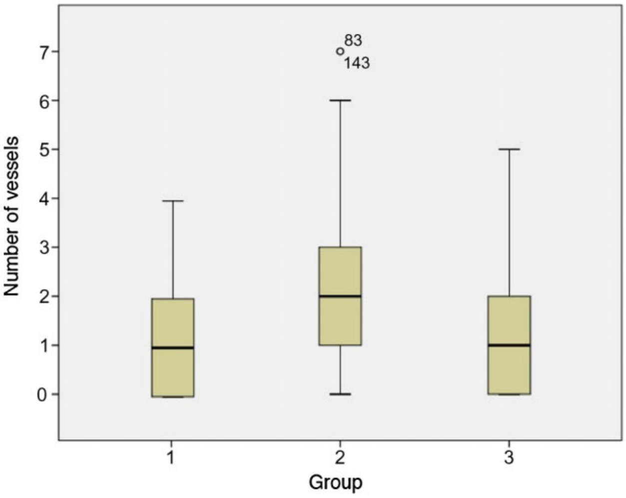

Figure 8 Boxplot of the number of vessels in the groups.

TABLE 3 Result of Kruskal–Wallis H Test of the Osteocyte Count

	Value
Chi^2	100,922
df	2
p Value	<.001

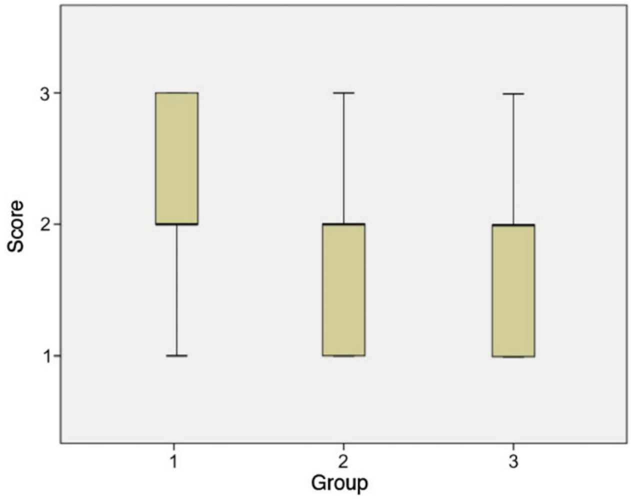

Figure 9 Boxplot of the score of remaining grafts in the groups.

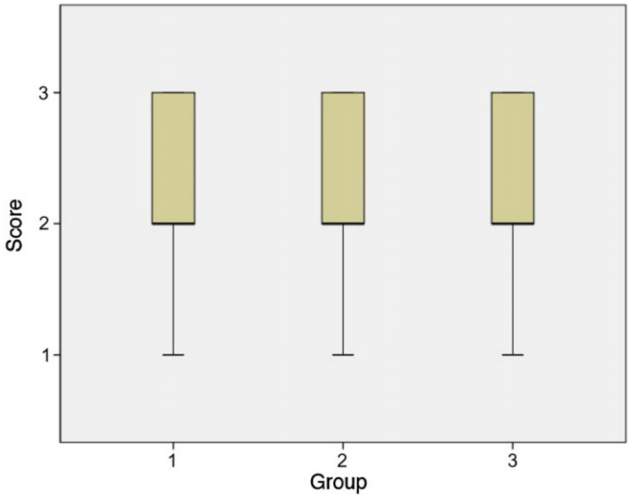

Figure 10 Boxplot of the scores of the proportion between calcified/noncalcified tissue in the groups.

changes that occur during the incorporation process. Available studies evaluating the amount of graft resorption have not used standardized methodologies and mostly have not compared different time intervals.[27,31,32] Moreover, several studies have used two-dimensional measurements, using either a reference point (dimensional reduction relative to the head of the screw) or linear measurements on radiographs or CT scans. It is known that the resorption of bone grafts occurs in a three-dimensional and nonuniform pattern. Linear measurement techniques for the assessment of bone graft resorption can easily under- or overestimate the process, depending on the reference point and the regions used. Thus, three-dimensional evaluation of morphological changes is an important tool in the analysis of the resorption of bone grafts.

Few studies have addressed the behavior of FFB allografts for alveolar ridge reconstruction. The evidence for using this type of biomaterial is now based on case reports and case series.[18–26,33] Although some studies have presented short-term follow-ups, implant success rates, and histological and histomorphometric analyses, the available data are still descriptive.[23–26,29] To date, there have been no randomized prospective studies to evaluate the time-dependent characteristics of these grafts.

Transmission of infectious diseases as a result of grafting procedures using bone allografts are discussed in the literature.[34,35] However, modern bone bank processing protocols have proven to be extremely safe regarding the infection control. In fact, with the screening for infectious diseases and the donor eligibility done by bone banks, the estimated risk of a graft to be infected with HIV is smaller than 1 in 8 million.[36,37] Thus, FFB allograft is currently considered safe from the immunological aspect.

In addition to clinical data from longitudinal studies, it is necessary to evaluate the grafts' short-term

TABLE 4 Result of Kruskal–Wallis H Test of the Vessels Count

	Value
Chi^2	15,902
df	2
p Value	<.001

TABLE 5 Result of Kruskal–Wallis H Test of the Remaining Grafts

	Value
Chi^2	26,571
df	2
p Value	<.001

TABLE 6 Result of the Kruskal–Wallis H Test of the Proportion of Calcified/Noncalcified Tissue

	Value
Chi^2	0.511
df	2
p Value	.775

behavior to establish guidelines for the use of fresh-frozen human bone tissue. To our knowledge, this is the first study analyzing the process of resorption and incorporation of block allografts at different time intervals.

The best time to reenter grafted sites is a matter of debate, not only for allogenous bone.[23,29] Ideally, the best time for the second stage of surgery would be at the intersection between the lowest resorption rate and best incorporation able to promote osseointegration.

Studies evaluating the volume reduction of autografts in animal models over 70 days have found rates between 28% and 68%, depending on the type of bone grafting (iliac or calvaria, respectively).[38] The results from human studies have shown slightly lower rates of resorption for autografts also from the calvaria and iliac (16.2% and 47%, respectively, at 6 months).[31] Although we have used grafts from three different donor sites (tibia, iliac crest, and femur), the homogeneity intragroup was statistically evaluated in order to avoid discrepancies in resorption rates caused by the graft origin.

Comparing AB and allogenous block grafts in humans using CT analysis, a significantly greater amount of resorption was found in the allograft groups at a 6-month interval.[25,30] Despite the volume difference, both the autograft and allograft sites were favorable to the implant placement and showed significant increases in the alveolar ridge dimensions.

Within the limits of our work, the shorter time interval (4 months) showed significantly less volume resorption than the other groups (13.02% $\pm$ 3.86). The interval of 8 months showed up to 70.28% total graft volume resorption and was extremely unfavorable for implant placement.

There were no complications related to incorporation of the grafts, such as block graft detachment of the recipient site. All of the bone blocks were firmly attached to the recipient beds at all of the time intervals and during the second surgical intervention. All of the implants showed good primary stability. The results of this study are consistent with previously published reports.[18,22,23,26,29,33]

The histological analysis of allografts used in hip and knee surgeries, after follow-up periods of up to 4 years, showed graft remains in the remodeling process. In fact, these grafts were functional after long periods of time and still presented histological graft remains.[39–41] It is noteworthy that orthopedic grafts are subjected to high levels of mechanical load, and this type of structural integrity is highly demanded. For these authors, the maintenance of the mechanical integrity of the grafts could be responsible for clinical success in long-term results.[40] Spin-Neto and colleagues[26] found more graft remains at sites grafted with allogenous bone compared with autologous bone at a 7-month interval. This finding was attributed by the authors to a slower rate of allograft remodeling, although it did not impair the implant placement outcome. From this point of view, histological findings alone did not seem to be good predictors of the success of grafts over the long term. The remaining presence of "nonviable bone" or "necrotic bone" might not have clinical significance regarding long-term implant success.

In our study, all of the groups showed remains of grafted tissue at the second stage. The 6- and 8-month groups showed no significant differences between them, but the 4-month group showed significantly greater amount of graft remains. These data suggest that the remodeling process is dynamic and that it possibly occurs at a slower rate within 6 months. The clinical implications of these data remain to be confirmed, although these differences did not influence the short-term results of the implants placed in the present study.

Clinically, all of the grafts exhibited bleeding at perforation, demonstrating the presence of vascularization. Histomorphometrically, our data indicated a significant difference between Group 2 and the other groups, but there was not a significant difference between Group 1 and Group 3. This finding suggests that vascularization of the grafts is influenced by variables other than time. Some authors have suggested that the microarchitecture of the grafts might have an impact on angiogenesis.[32] Notwithstanding these variations, all of the groups showed histological evidence of vascularization.

In the present research, at all time intervals, we noted the presence of mineralized tissue containing osteocytes in the graft biopsies. Superficial layers of bone blocks presented more graft remains, as evidenced by mineralized tissue with osteocyte spaces and with no cellular content. Deeper layers showed trabecular vascularized bone, with the presence of osteoblasts, osteocytes, connective tissue, and different amounts of graft remains in all of the groups. The presence of osteoblasts near osteocytes, trapped in newly calcified tissue, suggests new bone formation activity. These findings are consistent with previous reports of allograft biopsies.[23,25,26,29] Histological analyses of other bone substitutes, including AB, have reported similar findings.[42–45] Discrete or no inflammatory infiltrate was found in all of the samples, corroborating the results of previous articles[22,23,25,26,29] but disagreeing with Lumetti and colleagues'[30] work. The presence of residual bone marrow was associated with the inflammatory response by the authors and might differ from that of bone banks due to variables in graft processing.

According to our data, the group that waited for a 4-month interval presented significantly fewer osteocytes than the other groups. Groups 2 and 3 showed no differences between them. This finding suggests an increase in the recruitment of osteoblasts and osteocytes with time.

All of the groups showed large amounts of calcified tissue, as demonstrated by histomorphometry. There were no differences among the three groups regarding this variable. The presence of a sufficient amount of calcified tissue at the time of implant placement seems to influence the implant's primary stability directly.

Of the 75 implants placed, one (1.33%) was lost. The histomorphometric differences among the groups had no impact on the clinical responses of the implants on a short-term basis. This finding suggests that the maturation and incorporation of the grafts over 4 months of healing period might be sufficient for osseointegration. The CSR of implants in this study (98.67%) is consistent with that found in other FFB allograft trials and with the data from other reconstructive modalities.[3,19,20]

CONCLUSIONS

Within the limitations of this study, we conclude that a 4-month period is the most favorable time interval for the second stage of surgery after FFB grafting.

More studies are needed to confirm these results and to establish treatment protocols for the use of FFB block allografts in alveolar ridge reconstruction.

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