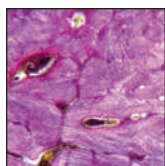


Comparison of Three Biphasic Calcium Phosphate Block Substitutes: A Histologic and Histomorphometric Analysis in the Dog Mandible



Octavi Ortiz-Puigpelat, DDS, MS, PhD^{1*}
 Basel Elnayef, DDS, MS, PhD^{2*}
 Marta Satorres-Nieto, DDS, MS, PhD²
 Jordi Gargallo-Albiol, DDS, PhD, MSc, EBOS²
 Federico Hernández-Alfaro, MD, DDS, PhD, FEBOMS³

The purpose of this animal study was to determine which ratio of hydroxyapatite (HA) and tricalcium phosphate (TCP) is the most appropriate in the composition of alloplastic biphasic block grafts, in terms of bone density and bone formation, for the regeneration of alveolar defects. Different concentrations of HA/TCP were used for the alloplastic block grafts: 100/0 (HA100 group), 79/21 (HA75 group), and 57/43 (HA50 group); the control treatment filled the defect with a collagen plug. All control and test sites were covered with a resorbable collagen membrane. Sacrifices were performed at 4, 12, and 24 weeks after grafting. Microcomputed tomography and histologic and histomorphometric analyses were performed to determine bone density and the characteristics of the regenerated bone as well as the percentages of newly formed bone (NB), residual material (RM), and connective tissue (CT). Bone density increased significantly over time ($P < .001$), with stabilization between 12 and 24 weeks ($P = 1.000$). No differences in density were observed between the different test blocks ($P = .813$). The percentage of NB increases over time, independent of the concentration ($P < .001$). At 12 weeks, the control group exhibited more NB than the HA100 group ($P < .001$). At 24 weeks, the HA50 group exhibited more NB than the HA100 ($P < .001$) and control ($P = .066$) groups. At 24 weeks, the HA100 and HA75 groups showed high RM percentages. The HA50 group exhibited an increased tendency of less RM percentage compared with the HA100 and HA75 groups. Although slight differences were found, the HA50 group's HA/TCP ratio seems the appropriate concentration when taking into account the bone density and percentage of NB and RM at 12 and 24 weeks of healing. *Int J Periodontics Restorative Dent* 2019;39:315–323. doi: 10.11607/prd.3837

¹Department of Oral and Maxillofacial Surgery, Universitat Internacional de Catalunya; Clínica Dental Ortiz-Puigpelat, Barcelona, Spain.

²Department of Oral and Maxillofacial Surgery, Universitat Internacional de Catalunya, Barcelona, Spain.

³Department of Oral and Maxillofacial Surgery, Universitat Internacional de Catalunya; Instituto Maxilofacial, Teknon Medical Center, Barcelona, Spain.

*Both authors contributed equally to this work.

Correspondence to: Dr Octavi Ortiz-Puigpelat, Universitat Internacional de Catalunya, C/Josep Trueta s/n 08195 St. Cugat del Vallès, Barcelona, Spain.
 Phone: +34 935042000. Email: octavi_ortiz@hotmail.com

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Calcium phosphates (CP) are usually found in the form of hydroxyapatite (HA) and tricalcium phosphate (TCP). Generally, HA bone substitutes are considered to be nonresorbable, while TCP is a highly resorbable material.¹ Biphasic calcium phosphates (BCPs) contain different concentrations of HA and TCP and offer significant advantages over other calcium-phosphate ceramics due to controlled bioactivity and a balance between resorption and nonresorption, which favors the stability of the biomaterial while also promoting new bone formation.^{2,3} Depending on the concentration of HA and TCP, it is possible to obtain a BCP ceramic that can be applied to large bone defects, where the mechanical stability of the graft and new bone formation become sustained over a long period of time.⁴

Several studies have evaluated different HA/TCP concentrations of particulate BCP, with different histomorphometric and histologic results.^{5–10} However, most of these studies confirm that BCP with a high TCP content tends to result in important graft volume reduction.^{6–9,11} On the other hand, particulate grafts in large defects are technically sensitive and have a tendency to collapse during the healing interval.^{12–14} In this regard, the use of block grafts appears to overcome these deficiencies.¹¹ Scientific



Fig 1 Visual aspect of the alloplastic BCP test blocks (Osteon 3, Dentium).

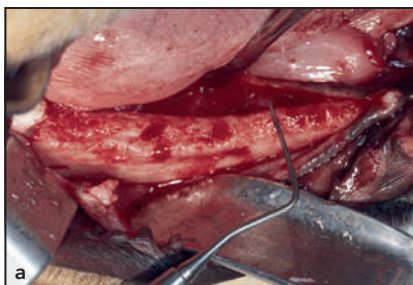


Fig 2 (a) Alveolar ridge condition 3 months after extractions. (b) Creation of three critical-size defects in each hemi-mandible.



evidence of the clinical efficacy of block alternatives, such as allogenic, xenogenic, and alloplastic grafts, remains controversial.^{9,11,15}

Alloplastic BCP block grafts have been used in several animal studies.^{1,11,16–18} Some of them conclude that BCP blocks perform better than monophasic blocks of HA or TCP alone, since unevenness between bone formation and volume maintenance occurs.^{5,16,17} However, the infiltration of newly formed bone into alloplastic BCP blocks has been questioned.^{9,11} Such infiltration is dependent on the pore structure and interconnectivity of the block^{11,19}; consequently, the process whereby alloplastic BCP blocks are made is crucial.¹¹

A recent article published by Lim et al¹¹ used BCP block grafts with different HA/TCP ratios of 8:92, 48:52, and 80:20 in rabbit skull defects. Such blocks were manufactured using a modified extrusion process to afford a more convenient pore size and increased pore interconnectivity. The authors concluded that such blocks exhibit limited osteoconductive capacity. However, further research is needed in relation to the manufacturing process of

BCP blocks and the impact of different HA/TCP ratios upon bone turnover.^{9,11}

The present study was carried out to analyze and compare the bone density and bone-forming capacity of BCP blocks with different HA/TCP ratios, based on microcomputed tomography and histologic and histomorphometric analyses in a canine model.

Materials and Methods

The study sample consisted of nine beagle dogs. The study protocol was approved by the Ethics Committee of the Universitat Internacional de Catalunya (Spain) and the Ethics Committee for Animal Research of the Universidad de Murcia (Spain), following the European Community guidelines. In each dog, six critical-size defects were made to test BCP blocks (Osteon 3, Dentium) with three different HA/TCP concentrations: 100/0 (HA100), 79/21 (HA75), and 57/43 (HA50). The pore size of the blocks was between 200 and 400 μm , with a total porosity of 80% and a crystallinity of 95% (Fig 1). The manufacturing method

involved the direct foaming technique. Healing observations were made at 4, 12, and 24 weeks.

Surgical Procedure

The animals were premedicated with an intramuscular injection of 10% Zolazepam 0.10 mL/kg, 0.12% acepromazine maleate (Calmo Neosan, Pfizer) administered 10 minutes before general anesthesia with butorphanol 0.2 mg/kg (Torbugesic, Zoetis) and 35 mg/kg medetomidine (Medetor, Virbac).

Intrasulcular incisions were made, followed by removal of premolars (P2, P3, P4) and the first molar (M1) in the mandibles of each dog.

After 3 months of healing, a midcrestal incision was made from the second molar (M2) to the first premolar (P1), and the flap was raised to expose the entire surgical area (Fig 2). Six critical-size, two-wall box-type bone defects measuring 5 mm in depth and 6 mm in length, with a 6-mm separation between them, were performed in each dog with a handpiece at 40,000 rpm under saline irrigation (Fig 2). A total of 54 bone defects were made. After

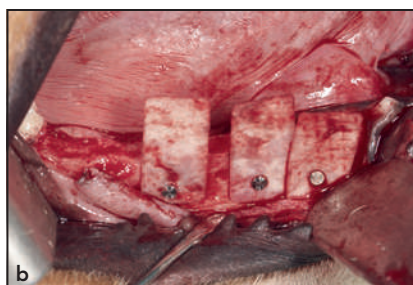
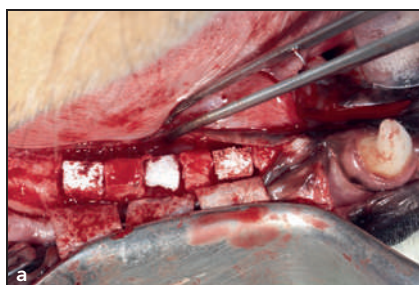


Fig 3 (a) Insertion of the alloplastic test blocks into the defects. (b) Fixation of the collagen membrane at the vestibular site in all test and control sites.

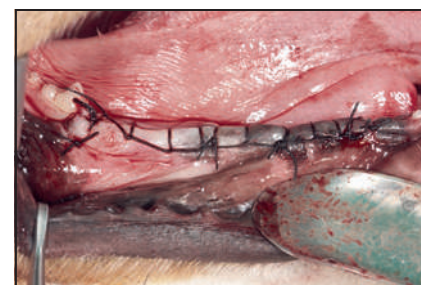


Fig 4 The flap is closed with non-interrupted sutures.

defect preparation, the study blocks were randomly assigned into each defect (Fig 3). One defect in each dog was left for control site.

The blocks and control sites were covered by a cross-linked collagen membrane, measuring 10 × 20 mm in size (Collagen Membrane, Dentium), and fixed with tacks (Fig 3). Flaps were closed with continuous nonresorbable sutures (Silk, Lorca Marin) (Fig 4).

Three dogs were sacrificed at each healing time of 4, 12, and 24 weeks after block insertion by means of an injection of Sodium Pentothal (Abbott) into the carotid artery, with a mixture of 5% glutaraldehyde and 4% formaldehyde. The mandibles were extracted and fixed in 10% buffered formalin solution for 10 days.

Microcomputed Tomographic Analysis

Block sections, including the grafted and control sites and surrounding tissue, were analyzed by microcomputed tomography (micro-CT) using multimodal scans (SPECT/CT Albira II ARS, Oncovision). Image

acquisition parameters were 45 kV, 0.2 mA, and a voxel size of 0.05 mm. Bone density was measured using AMIDE (Amide's a Medical Image Data Examiner) software. Three regions of interest (ROI) of 1 mm³ in volume were obtained from each defect area (Fig 5). The three ROIs were obtained at the middle portion of the defect sites. Hounsfield units obtained from the ROI were used to calculate bone density values and correlate them to the different bone density types.²⁰

Histologic and Histomorphometric Analyses

Following micro-CT analysis, the samples were processed according to the method described elsewhere.²¹ The specimens were embedded in methacrylate glycol resin (Technovit 9100 VLC, Kulzer) in order to obtain buccolingual sections with a thickness of 15 µm using diamond discs (Exakt Apparatebau). The sections were then stained with toluidine blue and fuchsin, followed by examination under a light microscope (Eclipse E200, Nikon). The following param-

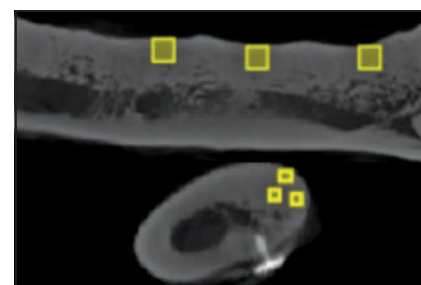


Fig 5 Positioning of the different ROIs at the middle portion of the defects in the test and control sites.

eters were measured for histomorphometric analysis: the area of newly formed bone (NB), the area of residual graft material (RM), and the area of connective tissue (CT).

Statistical Analysis

Statistical analysis was performed using the SPSS 15.0 and R.3.0.2 softwares. A descriptive analysis was made of both bone density and the histomorphometric parameters. Since a small sample was studied, nonparametric Brunner-Langer models were used. Inferential statistics were applied to determine possible significant differences in density according to the

Table 1 Bone Density Results (in Hounsfield units) of the Control and Test Groups at Different Time Points After Microcomputed Tomography

	Control	HA100	HA75	HA50
Bone density				
4 w	25.5 ± 161.11	597.05 ± 145.47	572.72 ± 173.86	477 ± 114.50
12 w	909.64 ± 57.00	848.86 ± 49.33	884.30 ± 28.00	860.03 ± 28.00
24 w	861.57 ± 35.62	886.01 ± 24.50	849.30 ± 72.23	880.55 ± 70.25

Table 2 Percentages of New Bone Formation, Residual Graft Material, and Connective Tissue of Each Group at All Sacrifice Times

	Control	HA100	HA75	HA50
NB				
4 w	45 ± 41	25 ± 15.40	48 ± 18.20	36 ± 6.50
12 w	76.70 ± 5.80	72 ± 4.50	73 ± 9.10	68 ± 16.40
24 w	75 ± 5	71 ± 11.4	72 ± 16	83 ± 2.70
RM				
4 w	0	12 ± 5.7	5 ± 3.5	8 ± 2.7
12 w	0	6 ± 2.2	4 ± 2.2	6 ± 2.2
24 w	0	6 ± 2.2	5 ± 3.5	2 ± 2.7
CT				
4 w	55 ± 41	63 ± 15.70	47 ± 17	56 ± 6.50
12 w	23.30 ± 6	22 ± 3	23 ± 8	26 ± 14.30
24 w	25 ± 5	23 ± 11	23 ± 14.4	15.00

NB = newly formed bone; RM = residual graft material; CT = connective tissue.

concentration used and the different time intervals. In relation to the histomorphometric parameters, the authors explored possible significant differences in NB, RM, and CT distributions according to the HA/TCP ratio used and the different time intervals (4, 12, and 24 weeks).

Results

A total of 54 observations were made. In general, no major complications were reported during the healing process. No exposure of the blocks was found. A maintained

volume at the defect site could be observed in the test groups, whereas in the control group, a collapse of the vertical height in the middle portion of the defect was observed. However, some blocks were partially resorbed independent of the sacrifice times.

Microcomputed Tomographic Analysis

Bone density results obtained in the control and test sites are displayed in Table 1. At 4 weeks, the concentrations of the HA100 and HA75

groups showed significantly higher density than in the group control ($P = .003$ and $P = .008$, respectively). The density in the HA50 group was found to be significantly lower than that of the HA100 group ($P = .008$).

Bone density increased significantly over time ($P < .001$), with stabilization between 12 and 24 weeks ($P = 1.000$). No differences in density according to the different test blocks were observed ($P = .813$); this applied to all time points ($P = .324$). The micro-CT also revealed that, generally, the blocks were more resistant to soft tissue collapse than control groups.

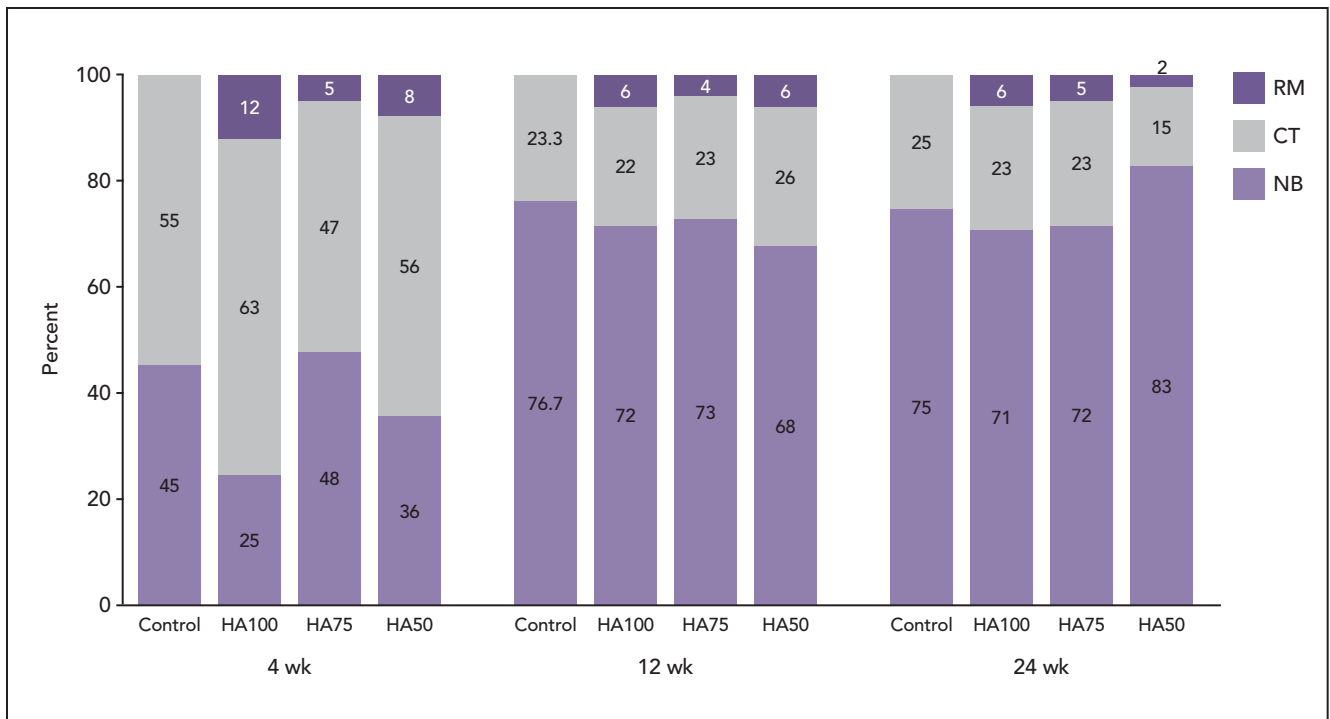


Fig 6 NB, RM, and CT percentages in the different groups at each point of sacrifice.

Histologic and Histomorphometric Analysis

The different percentages of NB, RM, and CT obtained after histomorphometric analysis are shown in Table 2. In order to help visualize the results, a graphical representation of the percentages of the different HA/TCP ratios at the different time points was elaborated (Fig 6).

Four Weeks

The defects were collapsed at the coronal portion in the control groups. In one sample, the membrane was not maintained during the first 4 weeks of healing, and the defect was seen to have partially collapsed with the presence of abundant granulation tissue. In the test

sites, the biomaterial was clearly distinguishable, bordered by immature bone, and with abundant residual material surrounded by fibroblasts and osteoblasts. However, no osteons were observed in the most coronal portion of the graft (Fig 7). Examining the effects of different HA/TCP concentrations on the percentage of NB, the differences did not reach statistical significance in any of the comparisons. The greatest percentage of RM and lowest percentage of NB corresponded to the HA100 group. On the other hand, the highest percentage of NB (48%) and lowest percentage of RM (5%) were observed in the HA75 group. In the control group, 45% of NB was observed, higher than the HA100 and HA50 groups (Fig 6).

Twelve Weeks

The control group showed mature tissue with abundant osteons and scarce presence of connective tissue, little immature bone, and an abundant trabecular tissue. Regarding the histomorphometric results at 12 weeks, the control group generated significantly more percentage of NB than the HA100 group ($P < .001$), and also had a certain superior tendency than the HA75 and HA50 groups, although without reaching statistical significance ($P = .083$). The HA75 and HA50 groups continued to show residual material, with the presence of NB. The HA100 and HA50 groups showed a significantly greater presence of RM than the HA75 group ($P = .046$). The residual graft particles were integrated

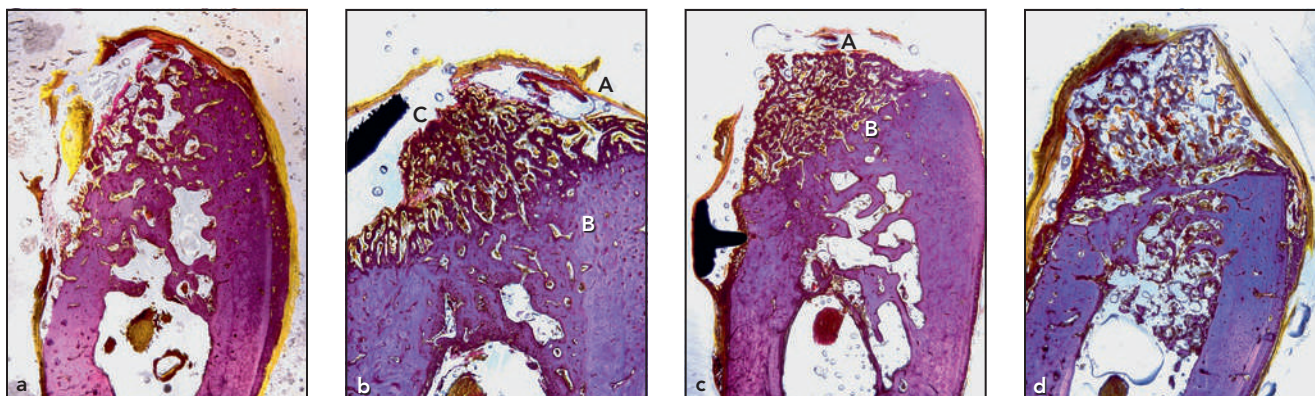


Fig 7 Histologic images of the different groups at 4 weeks. (a) Control site. Notice the initial bone formation, but some vertical collapse has occurred. (b) HA100 group. Notice the presence of (A) the resorbable membrane, (B) the mineralized bone tissue, and (C) some separation between the membrane and regenerated area. (c) HA75 group. The (A) connective tissue and (B) interface between residual and regenerated bone can be distinguished. (d) HA50 group. The interface between the block and native bone is noticeable.

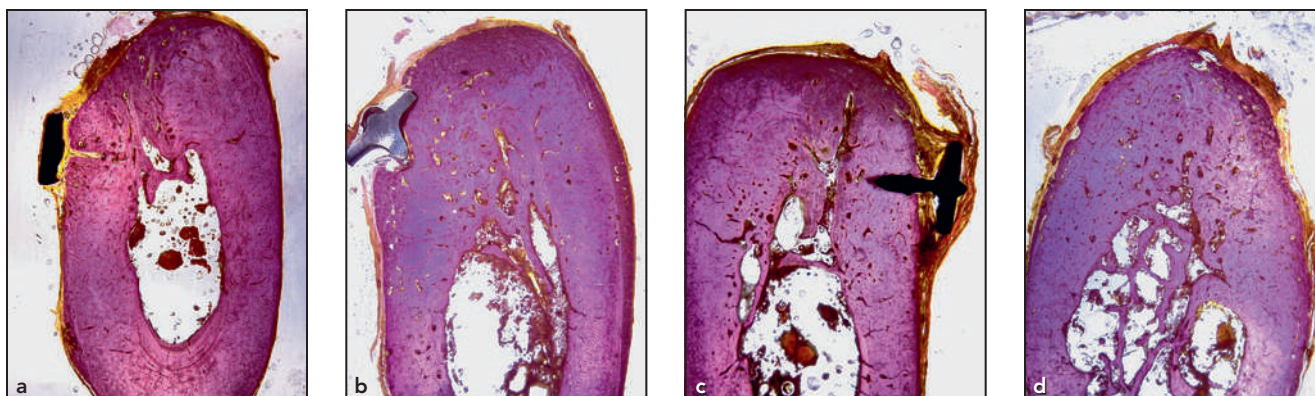


Fig 8 Histologic images of the different groups at 12 weeks. (a) Control site. Notice the partial regeneration of the defect. (b) HA100 group. Some residual particles were engulfed by NB. (c) HA75 site. Homogenous NB. (d) HA50 site. Homogenous pattern of mineralized tissue.

within mature bone, with medullary tissue in the formation process (Fig 8).

Twenty-four Weeks

The control group showed a Homogenous pattern with a bone matrix distributed in the form of tissue laminas with abundant osteons and the identification of Haversian systems. The HA50 group was char-

acterized by compact tissue along the entire section of the blocks, with less cellular activity and larger number of osteons composed of Haversian canals, surrounded by lamellae and canaliculi. Also, the HA50 group generated significantly more NB than the HA100 group ($P = .004$), with a superior tendency than the control group without reaching statistical significance ($P = .066$).

The HA75 group presented compact bone tissue, with osteocytes forming osteons surrounding scarce remaining biomaterial particles. The HA100 group in turn showed HA particles integrated into mature bone with visible osteons and a lesser amount of RM. The HA100 and HA75 groups showed significantly more RM than the control and HA50 groups ($P < .001$) (Fig 9).

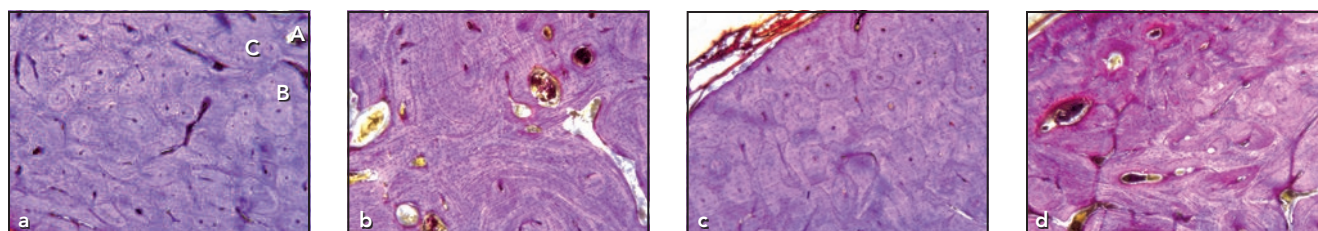


Fig 9 Histologic images of the different groups at 24 weeks with homogenous patterns of mineralized bone. (a) Control site with (A) Haversian channels and (B and C) osteons. (b) HA100 site. Mature mineralized tissue with osteocytes. (c) HA75 site. Osteons can be observed. (d) HA50 site. Homogenous bone with osteocytes.

Discussion

The small sample size and the canine model used in the present study made it so that comparisons with other studies may prove to be difficult. In terms of complications, some blocks proved difficult to fix into the defects during surgery, and this could explain why some blocks were partially resorbed at some test sites, independent of the healing period time points. Nevertheless, test groups exhibited more volume maintenance at defect sites compared with control sites. These findings are in agreement with other studies where BCP blocks were analyzed.^{1,11}

The results of the micro-CT analysis revealed that bone density increased over time with almost no statistical differences between test groups. However, some differences between density values were observed at 4 weeks: higher values in the HA100 and HA75 groups and lower values in the HA50 and control groups. This is because the HA100 and HA75 groups have a higher concentration of HA particles, which are more resistant to resorption at the initial phases of bone regeneration.

In the present study, the test blocks had a pore size between 200 and 400 μm , total porosity of 80%, and pore interconnectivity. These features can influence fluid and cell penetration and therefore affect the osteoconduction capacity of alloplastic grafts.²² The abovementioned method differs from the one used in the study by Lim et al,¹¹ where an extrusion technique was applied in order to increase pore interconnectivity. In their study, BCP blocks had a pore size of 140 to 170 μm and a total porosity of 80%. Despite the different block fabrication method and animal model used, they used similar HA/TCP concentrations. In their study, HA/TCP concentrations of 80/20 (HA80), 48/52 (HA48), and 9/92 (HA8) were analyzed histomorphometrically. However, different results were obtained when comparing both studies. In the present study, a high percentage of NB was found at 4 weeks ($25\% \pm 15.4\%$ HA100; $48\% \pm 18.2\%$ HA75; and $36\% \pm 6.5\%$ HA50), 12 weeks ($72\% \pm 4.5\%$ HA100; $73\% \pm 9.1\%$ HA75; and $68\% \pm 16.4\%$ HA50), and 24 weeks ($71\% \pm 11.4\%$ HA100; $72\% \pm 16\%$ HA75; and $83\% \pm 2.7\%$ HA50).

However, in Lim et al's study,¹¹ a low percentage of NB was found in the test groups at 2 weeks ($16.87\% \pm 7.24\%$ HA8; $17.28\% \pm 4.66\%$ HA48; and $17.32\% \pm 2.17\%$ HA80) and 8 weeks of healing ($11.55\% \pm 3.92\%$ HA8; $17.32\% \pm 2.17\%$ HA48; and $16.03\% \pm 9.20\%$ HA80), whereas the control group showed a significantly greater percentage of newly formed bone ($28.38\% \pm 10.10\%$ and $28.92\% \pm 9.66\%$ at 2 and 8 weeks, respectively). A possible explanation for such differences between studies is that the bone blocks in the present study were covered with a resorbable collagen membrane, which could limit soft tissue growth and allow bone infiltration from the surrounding walls of the defects.²³ Also, according to the histologic analysis, bone ingrowth was observed not only in the middle (center) portion but also in the most crestal portion of the blocks. In the study of Lim et al,¹¹ this bone infiltration could be observed only in the middle lower portion of the test blocks, reinforcing the idea that barrier membranes are needed to cover alloplastic bone blocks despite their space-maintaining ability.¹¹

Analyzing the results of the histomorphometric study, it was revealed that the different ratios of HA/TCP generally do not significantly influence the percentage of NB. It was observed that the percentage of NB increased significantly over time ($P < .001$), especially between 4 to 12 weeks, then stabilized. Additionally, the following observations were made: at 4 weeks, no statistically significant differences were observed among the different groups; at 12 weeks, the control group exhibited a greater percentage of NB than the HA100 group ($P < .001$); and at 24 weeks, the HA50 group exhibited more NB than the HA100 ($P < .001$) and control ($P < .066$) groups, but at the same level of the HA75 group. The limited bone ingrowth in the test sites between 4 and 12 weeks suggests a delay in early healing compared with the control sites.

When analyzing the percentages of RM, some differences between groups appear, especially at 24 weeks, where the HA100 and HA75 groups exhibited a greater percentage of RM than the HA50 group ($P = .054$). These results reinforce the idea that low resorption particles, such as HA, remain unaltered over time. Similar results were also found in the study by Lim et al,¹¹ in which BCP blocks with higher percentages of HA had a greater percentage of RM. The HA50 ratio seems the most interesting, since it was the one with a higher percentage of NB and a maintained percentage of RM at 24 weeks. These results are also comparable to Lim et al,¹¹ where the test group with an

HA/TCP ratio of 48/52 was the one that obtained more favorable histomorphometric values (NB and RM percentages) at 2 and 8 weeks.

Conclusions

Within the limitations of the present study, it can be concluded that BCP blocks performed better than control sites in terms of volume maintenance. The fabrication method used for the test blocks allowed adequate pore size and interconnectivity to facilitate bone infiltration. Although a few differences were found between the test blocks, the HA50 group exhibited promising results in terms of NB percentage and presence of RM; it had no significant differences with the HA75 test group. Block coverage with a resorbable membrane seems an effective way to promote bone infiltration at the outer portions of synthetic blocks.

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Conflict of interest: Authors state that they do not have any financial or personal interests that may inappropriately influence (bias) his or her actions during the elaboration of the present article.

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