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Quantification of bone mass gain in response to the application of biphasic bioceramics and platelet concentrate in critical-size bone defects

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Abstract Biphasic bioceramics have been widely indicated for bone reconstruction; however, the real gain in bone mass due to the presence of such biomaterials has not been established yet nor the advantages of its association with platelet concentrate. This study aims at quantifying the volume of bone matrix, osteoblasts, osteocytes, blood vessels and adipose tissue after the application of a biphasic bioceramics composed of 65% hydroxyapatite and 35% β-tricalcium phosphate. Critical-size bone defects were produced in rabbit femora and reconstructed with bioceramics only, with bioceramics combined with platelet concentrate, with platelet concentrate alone, and with no treatment (blood clot). The quantitative evaluation was performed on histological sections using histomorphometry. Our data provide original evidence that consolidates the indication of bioceramics for clinical bone loss reconstruction. The application of biphasic bioceramics alone led to major bone mass gain and was followed by its association with platelet concentrate. On the other hand, platelet concentrate can contribute to the augmentation and maintenance of the adipose tissue, representing a new field for future applications in plastic surgery.

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1 Introduction

The search for biomaterials for bone reconstruction is a constant quest. Different biomaterials have been widely used to combine the advantages of bone grafts without their disadvantages. Among them, calcium phosphate bioceramics are the most widely used due to their biocompatibility, more predictable reabsorption rates, good mechanical properties and the fact that they do not require a second surgery to be performed [1–13]. Some types of bioceramics are also bioactive, allow osteoconduction and osteoinduction at different rates, and can work as a drug delivery system, releasing them in different patterns [5, 6, 8, 14–16].

The first studies on biphasic bioceramics with varying hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) ratios were reported by LeGeros et al. [18]. One of their advantages is the establishment of an optimum balance of the more stable phase of HA and more soluble TCP leading to its controlled dissolution. As the biomaterial solubilizes in the host tissue, it releases calcium and phosphate ions into the biological medium which in conjunction with the physical structure of the biomaterial, favor the bone ingrowth at the expense of the bioceramics [18, 19].

To enhance bone repair, platelet-rich plasma (PRP) and fibrin glue have been applied in association with autologous bone grafts, allografts, xenologous bone grafts, and some biomaterials [6, 20–26] but with controversial results. The platelets mark the beginning of the bone repair. When platelets are in contact with collagen, they are activated,

leading not only to the formation of fibrin clots and blood clots, but also to the release of very important growth factors such as TGF-β1, TGF-β2, VEGF, PDGFaa, PDGFbb, PDGFab, EGF, IGF, VEGF, PDEGF, PDAF and PF-4 [6, 24, 25, 27–31]. This cocktail influences chemotaxis, cell proliferation and differentiation, angiogenesis, removal of debris and extracellular matrix deposition [28, 31, 32].

A biphasic calcium phosphate bioceramics (Osteosynt®), composed of 65% HA and 35% β-TCP with intercommunicating micro and macropores, has been clinically applied with great success in neurosurgeries, orthopedics surgeries, plastic surgeries and maxillofacial surgeries over the last few years [33]. There is no data, however, related to the exact volume of the new bone formed into this biomaterial. Moreover, the effect of its association with platelet concentrate on bone formation has not yet been analyzed. Such knowledge will provide new information regarding its current clinical benefits and will contribute to the development of new strategies for the use of such biomaterial in bone reconstruction.

This research aims at quantifying the volume of the new bone tissue (bone matrix, osteoblasts, osteocytes, blood vessels and adipose tissue) formed inside critical-size bone defects induced in rabbit femora and reconstructed through biphasic HA and β-TCP associated or not with autologous platelet concentrate.

2 Material and methods

2.1 Surgical procedures

This study was approved by the local ethical committee for the care and use of laboratory animals. Thirty adult male *New Zealand* rabbits were used with critical-size femur defects produced in all of them. They received 200 g of food daily and water ad libitum.

The animals were weighed and anesthetized with an intravenous injection of 0.2 mg/kg levomepromazine chlorhydrate (Neozine®, Rhodia Farma). After 15–20 min they received an intramuscular injection of ketamine chorhydrate (Vetaset®, FORT DODGE Saúde Animal Ltda.) associated with xylazine hydrochloride (Rompun®, Bayer) (6–10 mg/kg and 0.1 ml/kg, respectively). The lateral regions of both posterior limbs were tricotomized and the surgical area was prepared. These procedures were followed by skin incisions and dissections following the muscle plane to allow access to the femur. The periosteum was incised and reflected. A neutralizing plate (4 holes and 2.0 mm diameter) was adapted to the bone and the two central holes demarked to determine the correspondence of the middle portion of the plate with the middle portion of the diaphysis of the femur. The plate was then removed and a 5-mm-diameter trephine, operated with a low rotation motor, was used to produce the bone defects with abundant irrigation. The plates were positioned again, and each of them was fixed with four screws (6 mm in length and 2.0 mm in diameter) in order to obtain the required mechanical stability to the region. The outer portion of the cortical bone and the entire medullar region were removed.

The defects were reconstructed as follows: with bioceramics, bioceramics + platelet concentrate, with a platelet concentrate alone, and with no reconstruction (allowed to be filled with natural blood clot) (Fig. 1). Samples were also obtained from bones without defects (sham group, in which the only procedure done was the incision and reflection of the periosteum). These samples in conjunction with the ones without reconstruction (filled naturally with blood clot) represented our two control groups. The animals were sacrificed 15, 30, and 90 days after the surgery.

The bioceramics used was the Osteosynt® (EINCO Biomaterial Ltda., Belo Horizonte, Minas Gerais, Brazil), containing 65% hydroxyapatite and 35% β-tricalcium phosphate, with intercommunicating micro and macropores in 40–60 mesh granules. The rigid internal fixation of the

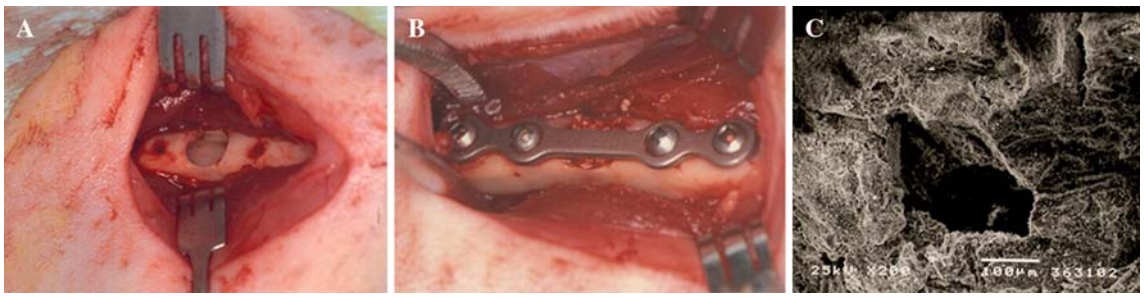

Fig. 1 Critical-size bone defects in rabbit femora. **a** The femoral diaphysis from where the cortical and medullar bone were removed. **b** The defect reconstructed with biphasic bioceramics (Osteosynt®). The femur was stabilized with a titanium plate and four screws. **c** SEM of a bioceramics granule with 40–60 mesh, showing a macropore

femora was performed with the use of titanium plates and screws from MDT/Biotechnology (Rio Claro, São Paulo, Brazil).

2.2 Platelet concentrate

The protocol described by SONNLEITNER, HUEMER & SULLIVAN (2000) [26] was followed. Nine ml of venous blood were obtained from each animal using a syringe containing 1 ml of sodium citrate. The blood was then transferred to another tube and centrifuged for 20 min at 1,200 rpm. This resulted in an opaque red substance composed basically of red blood cells, leucocytes and platelets, in the lower part of the tube, and a transparent yellowish substance composed of plasma and platelets, in the upper part of the tube. The 6–8 mm located immediately below the division line of the two fractions correspond to the highest concentration of the platelets. The entire upper fraction and the 6–8 mm previously mentioned were transferred to another tube and centrifuged again for 15 min at 2,000 rpm. Finally, the transparent platelet-poor upper portion was discarded. The lower portion, having a platelet concentration of 350–400% was used, with or without the bioceramics. For each 0.5 g of bioceramics, 0.8 ml of platelet concentrate were used. The term "platelet-rich plasma (PRP)" refers to the complex where the thrombin has been used. In this study we used the term "platelet concentrate" to describe its liquid form, which avoids the use of any homologous or heterologous product such as bovine thrombin.

2.3 Histological analysis

The samples were fixed with a Karnovsky solution (50 ml of 0.8% paraformaldehyde, 500 ml of 10% glutaraldehyde, and 100 ml of 0.2 M phosphate buffer) for 2 days, and decalcified in a Perenyi solution (15 ml of 1% chromic acid, 40 ml of 10% nitric acid, 30 ml of 95% etilic alcohol, and 15 ml of distilled water) for 8 days. After decalcification, plates and screws were removed and the central portion of the region corresponding to the defect was prepared for histological analysis. The samples were embedded in methacrylate, Technovit® 7100 (Kulzer, Wehrheim, Germany), 4-μm serial sections were obtained, and one in each group of ten was chosen for analysis. Toluidine blue staining was used.

2.4 Histomorphometric analysis

Quantitative histomorphometry was performed by superimposing grids of 400 points over the images of the sections. Photographs of histological sections amplified 250 times were made using an Olympus BX50 microscope, with a coupled camera, and translated into black-and-white mode. They were scanned, amplified by 30%, and then analyzed [34, 35]. Three photographs from each section were evaluated: one of the medial cortical region, one of the lateral cortical region, and one of the medullar region, resulting in an analysis of 900 images during the study. Bone matrix, osteoblasts, osteocytes, blood vessels, bioceramics and adipose tissue were quantified. Other tissues or cells were classified in other groups.

2.5 Statistical analysis

The *Kruskal–Wallis* test was used to compare 5 treatments (including sham) and 3 periods of sacrifice. All the results were considered relevant at a level of 5% significance ($P < 0.05$). The data was analyzed by the Statistica Program, version 5.

3 Results

3.1 Histological analysis

3.1.1 Sham group

The samples showed the pattern of Havers' channels and osteocytes longitudinally exposed at the outer layer of the cortical bone, while the Havers' systems were well defined at the inner layer. The medullar tissue was completely composed of adipose tissue.

3.1.2 No reconstruction group

After 15 days post surgery, defects naturally filled with normal blood clots showed only islands of bone tissue without a defined orientation at the cortical region; however, many blood vessels could be seen inside the adipose tissue at the medullar region. After 30 days post surgery, the cortical zone presented an uninterrupted layer of bone tissue. This pattern was maintained for 90 days, with the adipose tissue occupying part of the cortical region (Fig. 2a, b).

3.1.3 Bioceramics group

On day 15, bone tissue could be observed around and inside the granules of ceramics both at the cortical and medullar regions. One month after surgery, samples showed a better organized pattern of compact bone tissue at the cortical zone. At the medullar region, the bone tissue maintained the same pattern of distribution (around and inside the granules) despite the fact that some

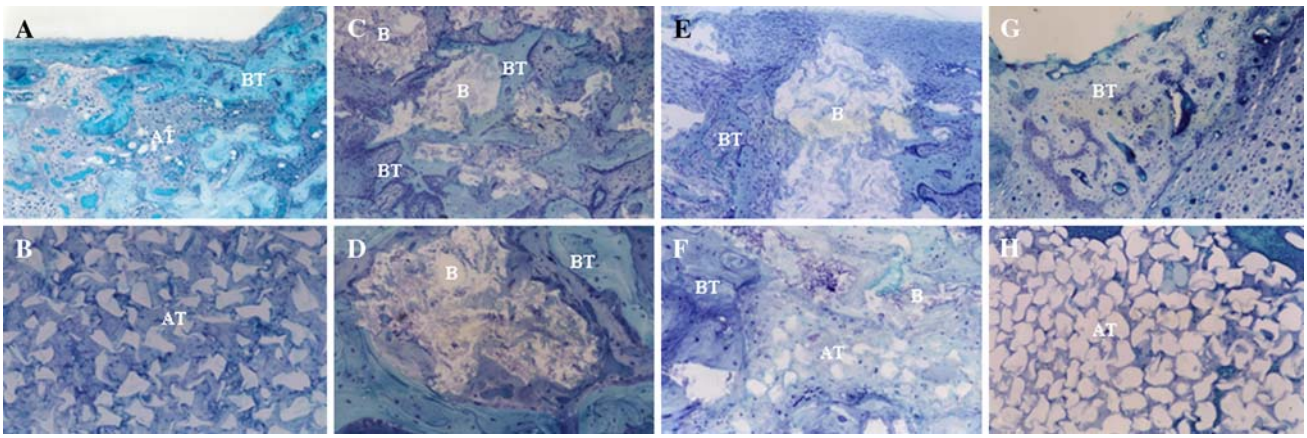

Fig. 2 Histological analysis of samples with no reconstruction (**a** and **b**), reconstructed with bioceramics (**c** and **d**), bioceramics + platelet concentrate (**e** and **f**), and only with platelet concentrate (**g** and **h**). Thirty days post surgery. **a**, **c**, **e** and **g**: cortical regions, 250×. **b**, **d**, **f** and **h**: medullar regions, 500×. Toluidine blue staining. AT: adipose tissue, BT: bone tissue, B: bioceramics

adipose cells could be seen surrounding this complex. Ninety days after surgery, more fat tissue at the medullar zone was seen but, nevertheless, well organized bone tissue was observed around and inside the whole granules (Fig. 2c, d).

3.1.4 Bioceramics + platelet concentrate group

Samples with 15 days of reconstruction showed bone tissue around and inside the biomaterial at both the medullar and cortical regions. Fat tissue was also observed at the medullar zone. After 30 days, polimorphonuclear and adipose tissue were observed at the medullar zone. After 90 days, the pattern of lamellar bone in the bioceramics sites, both at the cortical and medullar regions, was maintained although more fat tissue was observed at the medullar zone (Fig. 2e, f).

3.1.5 Platelet concentrate group

On day 15, bone and cartilage were observed among the conjunctive tissue at the cortical zone, while adipose tissue completely filled the medullar zone. On day 30 and day 90, osteocytes surrounding Havers' channels could be seen in the cortical region, while the medullar zone was reestablished with fat tissue (Fig. 2g, h).

3.1.6 Histomorphometric analysis

Day 15 cortical samples showed higher bone matrix synthesis and a larger quantity of osteoblasts around the defects treated only with bioceramics. The number of osteocytes was higher in the bioceramics and bioceramics + platelet concentrate group, and between them, there was no statistically relevant difference. A higher number of blood vessels and a greater amount of adipose tissue were observed in the group which did not undergo reconstruction (maintained with blood clots) (Table 1). In the medullar region, the animals treated with bioceramics showed the highest values of bone matrix and osteoblasts. Those treated with bioceramics and bioceramics + platelet concentrate showed a corresponding quantity of osteocytes, however larger than in the other groups. The lowest volume of adipose tissue was found in the bioceramics samples (Table 2).

Thirty days after surgery, the cortical region showed the highest quantity of bone matrix in the ceramics samples. There were no statistically relevant differences regarding the number of osteoblasts between the groups analyzed, however, the largest quantity of osteocytes was found in the bioceramics + platelet concentrate samples. The two samples treated with the platelet concentrate also showed not only large percentages of adipose tissue (although lower than the group without reconstruction) but also the largest quantity of other tissue (Table 3). The highest percentage of bone matrix in the medullar zone was observed in the defects treated with bioceramics alone. This group and the group treated with bioceramics + platelet concentrate showed higher quantities of osteoblasts, osteocytes and other tissues than the other groups. The highest number of blood vessels was seen in the defects without reconstruction (with blood clot) (Table 4).

The lowest number of osteoblasts and the highest percentage of adipose tissue were found in the cortical zone of 90-day samples in the groups that received no reconstruction. All the other experimental groups showed no statistically relevant differences between these features. The animals treated with bioceramics and those whose defects were filled with blood clots alone (no

Table 1 Quantification of bone matrix, osteoblasts, osteocytes, blood vessels, bioceramics, adipose tissue and others, at cortical region, 15 days after surgery

Variable	Treatment*	Minimum	Maximum	Median	Mean	SD**	*P* value
Bone matrix	1	66.4	83.6	76.6	75.1	8.0	<0.001
	2	11.5	34.9	21.1	22.8	9.3	
	3	45.4	49.9	48.3	47.9	1.9	1 > 3 > (4 = 5) > 2
	4	36.8	46.3	44.6	43.4	4.0	
	5	37.5	48.2	40.2	41.8	4.6	
Osteoblasts	1	0.3	4.2	2.3	2.3	1.6	0.031
	2	0.3	7.2	6.6	5.3	2.9	
	3	2.9	10.1	9.0	7.6	3.0	3 > 2 > 5 > (4 = 1)
	4	1.7	5.1	2.9	3.1	1.2	
	5	2.6	4.1	4.0	3.7	0.6	
Osteocytes	1	9.0	12.6	11.0	10.7	1.4	<0.001
	2	2.0	6.6	6.5	5.4	1.9	
	3	14.5	16.7	15.8	15.6	0.8	(4 = 3) > 5 > 1 > 2
	4	13.8	17.8	16.1	16.0	1.8	
	5	11.5	15.7	13.6	13.4	1.6	
Blood vessels	1	3.6	5.7	5.1	4.8	0.8	0.005
	2	2.1	9.8	5.2	5.6	2.8	
	3	0.1	4.8	0.6	1.5	1.9	(2 = l) > 5 > (3 = 4)
	4	0.4	0.7	0.5	0.5	0.1	
	5	1.6	6.1	3.4	3.7	2.0	
Bioceramics	1	0.0	0.0	0.0	0.0	0.0	<0.001
	2	0.0	0.0	0.0	0.0	0.0	
	3	8.8	15.1	11.2	11.9	2.4	4 > 3 > (1 = 2 = 5)
	4	13.3	18.6	16.3	16.2	1.9	
	5	0.0	0.0	0.0	0.0	0.0	
Adipose tissue	1	0.0	11.9	0.0	2.4	5.3	0.001
	2	40.9	84.1	59.2	60.6	16.1	
	3	0.0	1.2	0.0	0.3	0.5	2 > 5 > (4 = 1 = 3)
	4	0.0	16.1	0.3	3.8	7.0	
	5	11.2	38.8	31.2	29.8	11.2	
Others	1	0.0	13.1	4.2	4.6	5.3	0.002
	2	0.0	0.8	0.0	0.3	0.4	
	3	11.9	18.7	14.5	15.2	3.2	(4 = 3) > 5 = 1 > 2
	4	14.6	21.1	16.2	17.0	2.5	
	5	0.3	16.8	5.9	7.6	6.1	

* Treatment groups: 1—sham, 2—no reconstruction, 3—bioceramics, 4—bioceramics + platelet concentrate, 5—platelet concentrate

** SD: standard deviation

reconstruction) showed the highest quantities of blood vessels, while the defects treated with a platelet concentrate alone showed the highest number of osteocytes and the largest volume of adipose tissue (Table 5). In the medullar zone, the highest percentage of bone matrix, osteoblasts, and osteocytes was observed in the bioceramics samples while the experimental groups that received the platelet concentrate contained the largest volume of adipose tissue (Table 6).

4 Discussion

Critical-size bone defects were produced in *New Zealand* rabbit femora. The quantity of bone matrix, osteoblasts, osteocytes, adipose tissue, blood vessels and other tissues and cells were measured by histomorphometry after the application of a biphasic HA/β-TCP alone or in association with platelet concentrate. This is the first study that describes such quantification. The measures were obtained

Table 2 Quantification of bonematrix, osteoblasts, osteocytes, blood vessels, bioceramics, adipose tissue and others, at medullar region, 15 days after surgery

Variable	Treatment*	Minimum	Maximum	Median	Mean	SD**	*P* value
Bone matrix	1	0.0	0.0	0.0	0.0	0.0	0.001
	2	0.0	27.4	0.0	8.7	12.6	
	3	43.4	49.3	48.4	47.4	2.4	3 > 4 > 5 > (2 = 1)
	4	33.3	47.4	34.9	37.0	6.0	
	5	0.0	45.3	22.3	22.7	16.6	
Osteoblasts	1	0.0	0.0	0.0	0.0	0.0	0.001
	2	0.0	0.4	0.0	0.1	0.2	
	3	0.9	10.5	5.8	6.2	3.0	3 > 4 > 5,2e1
	4	1.4	2.9	2.7	2.5	0.7	5 > 1
	5	0.0	2.9	0.0	0.0	1.3	
Osteocytes	1	0.0	0.0	0.0	0.0	0.0	0.001
	2	0.0	0.7	0.0	0.1	0.3	
	3	10.0	15.6	12.6	12.0	1.7	(3 = 4) > 5 > (l = 2)
	4	10.3	14.1	13.8	12.8	1.7	
	5	0.0	15.5	6.9	7.2	6.0	
Blood vessels	1	0.0	31.9	3.5	8.7	13.1	0.086
	2	0.0	7.6	1.3	3.4	3.5	
	3	0.3	3.6	0.9	1.3	1.3	1 = 2 = 3 = 4 = 5
	4	0.0	2.8	0.4	1.0	1.1	
	5	3.0	13.5	5.8	6.3	4.2	
Bioceramics	1	0.0	0.0	0.0	0.0	0.0	<0.001
	2	0.0	0.0	0.0	0.0	0.0	
	3	10.9	20.9	14.3	16.0	4.3	4 > 3 > (1 = 2 = 5)
	4	19.2	23.6	22.8	21.8	1.8	
	5	0.0	0.0	0.0	0.0	0.0	
Adipose tissue	1	68.1	100.0	96.5	91.3	13.1	0.001
	2	72.6	98.7	92.4	87.8	3.5	
	3	0.0	1.8	0.0	0.4	0.8	(1 = 2) > 5 > 4 > 3
	4	1.8	12.8	9.9	8.6	4.8	
	5	30.4	97.0	55.5	61.6	25.0	
Others	1	0.0	0.0	0.0	0.0	0.0	<0.001
	2	0.0	0.0	0.0	0.0	0.0	
	3	9.1	25.1	13.8	15.9	7.3	(4 = 3) > (5 = 1 = 2)
	4	13.6	23.3	13.9	16.4	4.2	
	5	0.0	3.9	0.0	1.3	1.9	

* Treatment groups: 1—sham, 2—no reconstruction, 3—bioceramics, 4—bioceramics + platelet concentrate, 5—platelet concentrate

** SD: standard deviation

not only in the earlier periods of bone formation but also in more advanced stages of the process. Samples that were reconstructed only with bioceramics showed the highest quantity of bone mass gain. The advantage showed by the bioceramics + platelet concentrate group regarding bone tissue was relative to its maturation, which could be observed by the number of osteocytes on day 30 and day 90 post-operatively. On the other hand, this group also showed high quantities of inflammatory cells and fibrous tissue, which were classified as other tissue. It is well known that the inflammatory process is essential for the wound healing and will always exist independently of the procedure of choice; however, the modulation of this process is extremely important.

The efficacy of PRP has been very controversial, varying from no efficacy when used in conjunction with allografts [30], bioglass [22], and dense HA/β-TCP ceramics [25] to good results when PRP is applied in conjunction with enriched fibronectin and laminin allografts and bone marrow aspirate [20] or with demineralized

Table 3 Quantification of bonematrix, osteoblasts, osteocytes, blood vessels, bioceramics, adipose tissue and others, at cortical region, 30 days after surgery

Variable	Treatment*	Minimum	Maximum	Median	Mean	SD**	*P* value
Bone matrix	1	66.3	90.2	74.0	75.7	92	<0.001
	2	30.2	45.9	34.8	36.8	7.0	
	3	60.1	68.3	66.8	65.6	3.4	1 > 3 > (4 = 5) > 2
	4	43.9	56.9	48.1	48.9	4.8	
	5	33.4	49.6	42.5	43.4	6.5	
Osteoblasts	1	0.0	4.2	0.3	1.2	1.8	0.225
	2	0.0	8.8	4.9	4.8	3.1	
	3	0.8	7.6	2.1	3.3	2.7	1 = 2 = 3 = 4 = 5
	4	0.6	5.1	3.3	2.7	2.0	
	5	0.6	4.1	3.5	2.5	1.8	
Osteocytes	1	4.3	19.2	10.8	11.9	5.6	0.009
	2	1.4	8.9	6.8	5.5	3.2	
	3	4.8	12.0	8.9	8.1	3.1	4 > (P5) > 3e2
	4	11.8	16.2	12.4	13.1	1.8	
	5	9.2	11.7	11.6	10.7	1.3	
Blood vessels	1	1.8	6.7	4.3	4.0	1.8	0.004
	2	0.6	5.4	2.6	2.7	1.7	
	3	0.3	1.6	1.3	1.2	0.5	1 > (2 = 5) > (3 = 4)
	4	0.2	1.6	1.3	0.9	0.6	
	5	1.5	2.8	2.2	2.2	0.5	
Bioceramics	1	0.0	0.0	0.0	0.0	0.0	<0.001
	2	0.0	0.0	0.0	0.0	0.0	
	3	10.3	17.9	13.8	13.9	3.0	3 > 4 > (1 = 2 = 5)
	4	10.3	17.9	13.8	13.9	3.0	
	5	0.0	0.0	0.0	0.0	0.0	
Adipose tissue	1	0.0	14.0	0.1	3.6	6.1	0.001
	2	27.6	53.7	37.9	41.3	11.5	
	3	0.0	6.0	0.7	1.9	2.5	(25) > 4.3e1
	4	0.3	15.3	5.1	5.4	6.0	
	5	15.5	47.2	33.9	29.4	13.8	
Others	1	0.0	14.5	0.0	3.6	6.3	0.063
	2	0.7	12.6	10.3	8.9	4.9	
	3	1.5	10.4	5.6	6.1	3.5	1 = 2 = 3 = 4 = 5
	4	12.0	24.1	18.2	18.0	4.4	
	5	0.0	27.6	2.7	11.7	14.5	

* Treatment groups: 1—sham, 2—no reconstruction, 3—bioceramics, 4—bioceramics + platelet concentrate, 5—platelet concentrate

** SD: standard deviation

freeze-dried bone allografts [21]. The use of PRP in conjunction with bone morphogenetic proteins has also been described with good results [31]. This data suggests that the success of these techniques is directly dependent on the correct choice of the biomaterial to be used and on its capacity to function as a reliable carrier for these osteoinducers. Moreover, individual variations of growth factor concentrations must be considered and evaluated since these variations are also related to species [36, 37].

The liquid or gel presentation form of PRP does not seem to influence the final results. The gel presentation could only contribute to an easier manipulation of the graft [25]. In our research, a liquid platelet concentrate was used because we avoided the addition of bovine thrombin, which is a heterologous substance. Thrombin is one of the most important components of fibrin glue, which can be used as novel scaffolds when mixed with micro and macroporous biphasic calcium phosphate granules [38]. In our study, the

Table 4 Quantification of bonematrix, osteoblasts, osteocytes, blood vessels, bioceramics, adipose tissue and others, at medullar region, 30 days after surgery

Variable	Treatment*	Minimum	Maximum	Median	Mean	SD**	*P* value
Bone matrix	1	0.0	0.0	0.0	0.0	0.0	<0.001
	2	0.0	20.8	7.9	9.3	9.0	
	3	67.6	78.2	68.6	71.5	4.9	3 > 4 > 2 > (5 = 1)
	4	33.2	47.6	44.6	42.1	5.6	
	5	0.0	5.1	0.0	1.0	2.3	
Osteoblasts	1	0.0	0.0	0.0	0.0	0.0	0.002
	2	0.0	1.7	0.0	0.3	0.8	
	3	1.6	9.1	2.6	4.0	3.1	(3 = 4) > (5 = 2 = 1)
	4	0.4	3.1	1.8	1.6	1.1	
	5	0.0	2.5	0.0	0.5	1.1	
Osteocytes	1	0.0	0.0	0.0	0.0	0.0	<0.001
	2	0.0	0.9	0.2	0.4	0.4	
	3	6.1	11.6	8.4	8.8	2.4	(4 = 3) > 2 > (5 = 1)
	4	6.0	11.9	8.7	9.1	2.2	
	5	0.0	1.8	0.0	0.4	0.8	
Blood vessels	1	0.0	5.3	0.4	1.8	2.4	0.026
	2	3.1	12.5	10.4	8.8	3.8	
	3	1.1	3.1	1.4	1.8	0.8	2 > 5,3,1e4
	4	0.0	2.0	1.3	0.9	0.9	5 > (1 = 4)
	5	0.1	11.3	3.8	4.5	4.7	
Bioceramics	1	0.0	0.0	0.0	0.0	0.0	<0.001
	2	0.0	0.0	0.0	0.0	0.0	
	3	5.1	14.3	8.7	9.1	3.4	4 > 3 > (1 = 2 = 5)
	4	6.8	14.5	12.1	11.7	3.0	
	5	0.0	0.0	0.0	0.0	0.0	
Adipose tissue	1	94.8	100.0	99.6	98.2	2.4	<0.001
	2	70.3	95.1	81.1	81.2	11.0	
	3	0.0	0.8	0.0	0.2	0.4	1 > 5 > 2 > 4 > 3
	4	7.1	24.3	12.6	14.5	6.3	
	5	88.7	99.5	93.1	93.6	4.3	
Others	1	0.0	0.0	0.0	0.0	0.0	
	2	0.0	0.0	0.0	0.0	0.0	<0.001
	3	2.5	8.5	3.3	4.7	2.7	
	4	13.5	30.9	19.0	20.2	6.5	4 > 3 > (1 = 2 = 5)
	5	0.0	0.0	0.0	0.0	0.0	

* Treatment groups: 1—sham, 2—no reconstruction, 3—bioceramics, 4—bioceramnics + platelet concentrate, 5—platelet concentrate

** SD: standard deviation

bioceramics granules were easily applied even when used alone.

In this study, the use of a platelet concentrate, even in conjunction with the bioceramics, led to higher percentages of adipose tissue. This suggests that it influences the augmentation and maintenance of adipose tissue and not bone tissue. This data is in agreement with a previous publication which showed the capacity of autologous platelet-rich plasma to support the refined adipose tissue graft obtained after the centrifugation of lipoaspirate cells, preserving its volume for one year [27]. Both studies open new perspectives to the use of platelet concentrates in plastic surgeries.

Another crucial factor in bone formation is vascularization, which plays a major role in regenerative medicine [36, 39]. The new angiogenesis which occurs inside three-dimensional scaffolds guarantees the functionality of the implanted biomaterial [39]. The formation rate and direction of the new blood vessels are controlled not only by

Table 5 Quantification of bonematrix, osteoblasts, osteocytes, blood vessels, bioceramics, adipose tissue and others, at cortical region, 90 days after surgery

Variable	Treatment*	Minimum	Maximum	Median	Mean	SD**	*P* value
Bone matrix	1	74.0	80.3	70.8	76.9	2.9	0.001
	2	7.6	7.6	7.6	7.6	0.0	
	3	43.7	56.2	50.0	49.3	4.8	1 > 5 > (3 = 4) > 2
	4	8.0	58.9	48.8	42.5	19.8	
	5	34.5	71.8	67.3	59.2	16.4	
Osteoblasts	1	0.1	3.4	1.6	1.5	1.4	0.013
	2	0.0	0.0	0.0	0.0	0.0	
	3	0.2	5.0	2.3	2.6	2.0	(4 = 3 = 5 = 1) > 2
	4	1.6	5.6	2.0	2.7	1.7	
	5	0.9	2.1	1.9	1.7	0.5	
Osteocytes	1	9.1	19.2	10.8	12.5	4.1	0.001
	2	0.4	0.4	0.4	0.4	0.0	
	3	5.3	9.3	7.6	7.6	1.5	5 > (4 = 1) > 3 > 2
	4	11.5	13.0	12.2	12.2	0.6	
	5	9.6	18.8	18.2	15.7	2.1	
Blood vessels	1	2.8	6.7	4.3	4.6	1.6	0.007
	2	2.0	2.0	2.0	2.0	0.0	
	3	1.6	4.7	2.5	2.7	1.3	1 > (2 = 3) > (4 = 5)
	4	0.2	2.2	1.5	1.4	0.7	
	5	0.2	3.3	0.9	1.3	1.2	
Bioceramics	1	0.0	0.0	0.0	0.0	0.0	<0.001
	2	0.0	0.0	0.0	0.0	0.0	
	3	9.5	20.8	18.8	16.4	4.7	3 > 4 > (1 = 2 = 5)
	4	4.1	19.5	4.8	9.1	6.7	
	5	0.0	0.0	0.0	0.0	0.0	
Adipose tissue	1	0.0	14.0	2.4	3.3	4.1	0.006
	2	84.9	53.7	84.9	84.9	0.0	
	3	1.5	6.0	5.6	12.9	14.0	2 > 5,4,3e1
	4	1.7	15.3	6.4	9.4	6.9	
	5	2.2	47.2	10.3	20.0	21.3	
Others	1	0.0	5.4	0.0	1.2	2.4	0.026
	2	5.1	5.1	5.1	5.1	0.0	
	3	0.0	14.6	11.4	8.5	6.6	(4 = 3 = 2) > (5 = 1)
	4	2.6	22.4	14.5	12.8	7.3	
	5	0.0	4.8	2.4	2.2	2.0	

* Treatment groups: 1—sham, 2—no reconstruction, 3—bioceramics, 4—bioceramics + platelet concentrate, 5—platelet concentrate

** SD: standard deviation

temporal and spatial gradients of both diffusible and matricellular signals [39] but also by the biomaterial's properties, such as the porosity and chemical composition. In this experiment, the quantity of blood vessels was increased when the platelet concentrate was used alone (without bioceramics), as well as the number of osteocytes, indicating that the platelet concentrate did not contribute to the volume of the new bone tissue but had an impact on its maturation. On the other hand, the number of blood vessels observed in the bioceramics group was sufficient to guarantee the viability of the cells that were observed around and inside the granules.

In this process, the existence of interconnection between different pore sizes plays an important role in bone ingrowth, since it allows nutrient and waste exchange from the perimeter of the matrix to its center, consequently proving a viable microenvironment for the tissue that is being formed [6, 14, 15, 17, 40].

Characteristics such as the geometry of the biomaterial, its chemical composition, superficial topography, porosity,

Table 6 Quantification of bonematrix, osteoblasts, osteocytes, blood vessels, bioceramics, adipose tissue and others, at medullar region, 90 days after surgery

Variable	Treatment*	Minimum	Maximum	Median	Mean	SD**	*P* value
Bone matrix	1	0.0	0.0	0.0	0.0	0.0	<0.001
	2	0.0	0.0	0.0	0.0	0.0	
	3	40.0	57.7	49.3	48.0	6.8	3 > 4 > (1 = 2 = 5)
	4	0.0	21.8	7.8	10.4	8.2	
	5	0.0	0.0	0.0	0.0	0.0	
Osteoblasts	1	0.0	0.0	0.0	0.0	0.0	0.001
	2	0.0	0.0	0.0	0.0	0.0	
	3	0.3	4.3	3.1	2.7	1.6	3 > 4 > (1 = 2 = 5)
	4	0.0	3.2	0.0	0.7	1.4	
	5	0.0	0.0	0.0	0.0	0.0	
Osteocytes	1	0.0	0.0	0.0	0.0	0.0	<0.001
	2	0.0	0.0	0.0	0.0	0.0	
	3	4.9	10.2	8.4	7.7	2.1	3 > 4 > (1 = 2 = 5)
	4	0.0	8.1	2.9	3.5	2.9	
	5	0.0	0.0	0.0	0.0	0.0	
Blood vessels	1	0.0	3.5	0.0	1.2	1.7	0.007
	2	1.3	1.3	1.3	1.3	0.0	
	3	1.2	7.8	2.0	3.4	2.8	5 > 3 > (2 = 1 = 4)
	4	0.0	2.7	0.0	0.5	1.2	
	5	3.6	7.0	5.4	5.4	1.3	
Bioceramics	1	0.0	0.0	0.0	0.0	0.0	<0.001
	2	0.0	0.0	0.0	0.0	0.0	
	3	8.9	26.6	16.9	15.2	6.5	3 > 4 > (1 = 2 = 5)
	4	0.0	13.9	6.1	5.7	5.0	
	5	0.0	0.0	0.0	0.0	0.0	
Adipose tissue	1	96.5	100.0	100.0	98.8	1.7	0.001
	2	98.7	98.7	98.8	98.8	0.0	
	3	0.4	39.8	3.1	12.4	16.6	(1 = 2) > 5.4e3
	4	35.5	100.0	75.0	70.1	23.3	5 > 3
	5	93.0	96.4	94.6	94.7	1.3	
Others	1	0.0	0.0	0.0	0.0	0.0	0.003
	2	0.0	0.0	0.0	0.0	0.0	
	3	0.0	18.2	7.6	8.9	6.8	(4 = 3) > 1 = 2 = 5
	4	0.0	17.5	8.6	8.7	6.2	
	5	0.0	0.0	0.0	0.0	0.0	

* Treatment groups: 1—sham, 2—no reconstruction, 3—bioceramics, 4—bioceramics + platelet concentrate, 5—platelet concentrate

** SD: standard deviation

and granulometry have crucial importance to its biological response and are directly related to the intrinsic osteoinduction concept described by some authors, who have found bone formation when calcium phosphate ceramics were implanted intramuscularly and subcutaneously in different animal models without the addition of osteogenetic proteins [2, 4, 12–14, 16, 19, 41, 42].

The fact that in this study the bioceramics was not applied in an ectopic site does not allow us to conclude that it is an intrinsic osteoinducer. However, further studies have to be developed in order to determine whether the bone formation observed inside the adipose tissue was due to the osteoconductivity of the bioceramics or to the presence of molecular signals that had induced cell differentiation. Moreover, this explains the excellent results when bioceramics are clinically applied in older patients, where the red bone marrow is gradually replaced by yellow bone marrow.

5 Conclusion

Our data provides additional evidence and consolidates previous successful clinical reports that showed efficient bone formation with the use of biphasic bioceramics composed of 65% HA and 35% β-TCP. Its application alone led to major bone mass gain, corresponding to a minimum of 4-fold the volume of applied bioceramics, when compared to other protocols used in the present study. The use of a platelet concentrate requires further investigation and knowledge of its behavior in different microenvironments is still necessary. Our results demonstrate that platelet concentrates can contribute to the augmentation and maintenance of the adipose tissue thus representing a new field for future applications in plastic surgery.

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