Demineralized bone matrix and calcium-phosphate cement in bone regeneration in rats[1]

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Abstract

Purpose: To compare bone regeneration in critical-sized defects in rat calvarium using demineralized bone matrix and calcium phosphate cement.
Methods: Thirty Wistar rats were divided into 3 groups of 10 animals each. Two defects of 5-mm were made in the parietal bones of each animal. Group I had calcium phosphate cement placed in the experimental defect, Group II had filled with demineralized bone matrix and Group III had with the combination of the matrix and cement in equal parts. All animals had one defect left unfilled to serve as controls. Five animals in each group were sacrificed at 4 and 8 weeks. Histomorphometric analysis was used to quantify the amount of new bone within the defects.
Results: The results showed that demineralized bone matrix-treated defects had significantly more new bone at 4 weeks compared to calcium phosphate cement-treated defects (p=0.03) and also had significantly more new bone at 8 weeks compared to unfilled defects (p=0.04).
Conclusions: The demineralized bone matrix was superior to calcium phosphate cement in bone regeneration. It seems that calcium phosphate cement acted by inhibiting the osteogenesis when associated with a demineralized bone matrix and this combination should not be recommended.
Key words: Bone Matrix. Bone Regeneration. Rats.

■ Introduction

The restoration of bone defects in the maxillofacial skeleton remains one of the most challenging problems faced by oral and maxillofacial surgeons[1]. Trauma, osteotomy sites, oncologic resections, infections, or developmental anomalies are clinical situations in which defects need to be reconstructed. In adults, only minor bone defects are capable of healing spontaneously[2,3]. The reason for this impaired defect healing seems to be related to the fast ingrowth of soft tissue, thus blocking bone formation at the edges of the defect and thereby preventing bone healing[4].

Currently, autogenous bone grafts are the preferred material for craniofacial reconstruction[5,6]. Despite being highly effective, there are several problems associated with their use. Clinically, autogenous bone grafts may increase operative time and morbidity, they are often mechanically unstable during surgery, may be insufficient for reconstruction of large defects, and undergo significant resorption, thus requiring further bone grafting procedures[7]. Recently, to minimize these problems, interest has been shown in the use of allogenic and alloplastic bone substitutes. The ideal graft should be osteoinductive to stimulate osteogenesis and osteoconductive to provide a scaffold for establishing optimal conditions for ingrowth of blood vessels and cells with osteogenic potential[5,6,8].

The demineralized allograft may be used as a matrix, especially since it is immunologically harmless, stimulates osteogenesis and is incorporated into the new bone formed[6]. Calcium phosphate cement has been used as a bone substitute for reconstruction of bone loss[8]. It bonds chemically to the bone, restoring the contour and increasing the biomechanical property; in addition it can promote osteoinduction when in direct contact with the bone[9,10].

The aim of this study was to compare bone regeneration in critical-sized defects in the calvarium of rats using demineralized bone matrix and calcium phosphate cement in the expectation that the use of the matrix and cement together would increase the osteoinductive and osteoconductive capacity of the bone defect.

■ Methods

This research was approved by the Ethics Committee, Universidade de Pernambuco.

Thirty male Wistar rats were used for the experiment. The rats were divided into 3 groups of 10 animals each, according to the type of filling. Two calvarial defects (experimental and control) of 5-mm were made in the parietal bones of each animal. Group I had calcium phosphate cement (Bone Source®, Leibinger, Dallas, TX, USA) placed in one defect, Group II had one defect filled with demineralized bone matrix (Gen-ox-org®, Baumer, Mogi-Mirim, Brazil), and Group III had one defect filled with a combination of the matrix and cement in equal parts. All the animals had the other defect left unfilled to serve as controls.

The time of evaluation for all groups was at 4 and 8 weeks after the surgical procedure. Thus, five animals in each group were sacrificed at these times.

Thirty animals were submitted to surgery under general anesthesia, which was induced by muscular injection of ketamine and xylaxine diluted 1:1 in a dose of 0.1 ml per 100 g of weight. A sagittal incision of approximately 10 mm was made from the fronto-nasal region to the occipital protuberance. Blunt dissection was created and the calvarium was exposed.

A critical defect of 5 mm on both sides of the median suture was performed using a trephine, always taking care to avoid injuring the dura mater. One osteotomy was made more anteriorly than the other in order to avoid dislocation of the material from one cavity to another. The procedure was concluded with suture in layers using nylon.

After sacrifice, the specimens were fixed with 10% formaldehyde and decalcified in a solution of 0.5% nitric acid for about 15 days. Semi-serial sections with a thickness of 5µm were cut in the coronal plane, using the greatest diameter through the defect, and stained with haematoxylin and eosin.

The slides were submitted to histological study in order to observe the bone regeneration in a descriptive manner using scores (Chart 1). The overall area of the defect was considered for this classification, using slides until the middle of the defect was reached. The type of tissue reaction found in the bone defect was also observed, such as acute or chronic inflammation and foreign body reaction. The presence or absence of the material inserted was observed as well, the purpose being to detect its presence or absence rather than quantify it, which was done by histomorphometric analysis.

Histomorphometric analysis was used to quantify the amount of new bone within the defects using the Motic Images Advanced 3.0 Program (Micro-Optic Industrial Group CO. Ltda, Guiyang, China).

Chart 1 - Histopathological scores.

1st Category: type of tissue found in the bone defect
1 – presence of fibrous connective tissue
2 – presence of immature bone tissue
3 – presence of mature bone tissue

■ Statistics

A descriptive statistical analysis calculated mean, median and standard deviation of control and test groups. Intragroup evaluation was carried out using the Wilcoxon test. The comparative evaluation between groups in the test cavities was performed using the paired comparison of Kruskal-Wallis. A Statistically significant difference was considered at the 5.0% level.

■ Results

There was bone formation (scores 2 and 3) in the experimental group (calcium phosphate cement) in almost all animals for both times of evaluation. There was foreign body reaction in almost all animals in the experimental group at 4 weeks of evaluation (n=4), and no tissue reaction was observed, except in one animal, in the experimental group at 8 weeks (n=4). The presence of the material inserted was seen in almost all animals in the experimental group for both times of evaluation (n=5 at 4 weeks; n=3 at 8 weeks) (Figures 1 and 2).

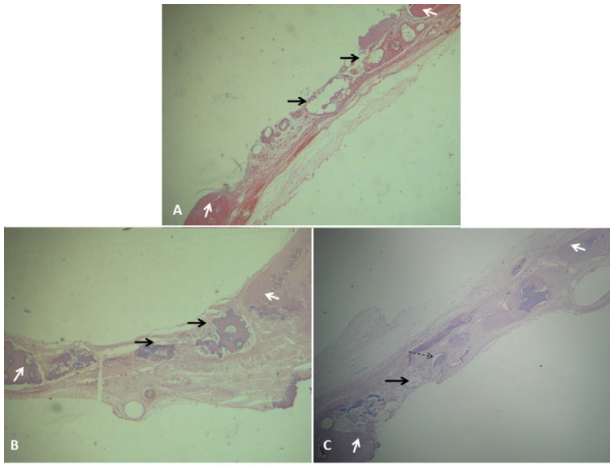

Figure 1 – Evaluation within 4 weeks (A-C). **A.** Group I (calcium phosphate cement): bone defect extremities (*white arrows*) and empty spaces (*black arrows*) leaved by the material used during the decalcification process. **B.** Group II (demineralized bone matrix): particles of the material used (*black arrows*) in the center of the bone defect. **C.** Group III (matrix and cement): osteoid areas (*black arrow*) close to the inserted material (*traced black arrow*) (H&E, x10).

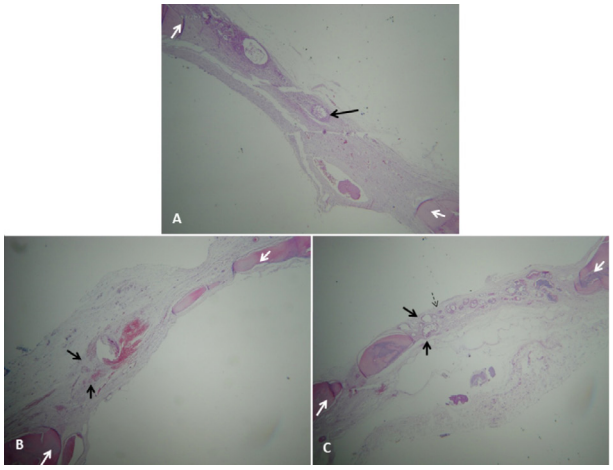

Figure 2 - Evaluation within 8 weeks (A-C). **A**. Group I (calcium phosphate cement): deposition of new bone around the place leaved by the particles of the material used during the decalcification process. **B**. Group II (demineralized bone matrix): material used (*black arrows*) being resorbed. **C.** Group III (matrix and cement): empty spaces (*black arrows*) leaved by the material used during the decalcification process and material used (*traced black arrow*) being resorbed (H&E, x10) (*White arrows* indicate the bone defect extremities in all figures).

There was bone formation (scores 2 and 3) in the experimental group (demineralized bone matrix) in almost all animals for both times of evaluation. There was foreign body reaction in half the animals in the experimental group at 4 weeks of evaluation (n=3). The presence of the material inserted was seen in all animals in the experimental group at 4 weeks of evaluation (n=5), and by 8 weeks the material had been absorbed in the experimental group. The presence of the material inserted was also observed in one animal in the control group at 4 weeks of evaluation (Figures 1 and 2).

There was bone formation (scores 2 and 3) in the experimental group (matrix and cement) in almost all animals for both times of evaluation. There was foreign body reaction in about half the animals in the experimental group for both times of evaluation (n=3 at 4 weeks; n=2 at 8 weeks). The material inserted was seen in all animals in the experimental group for both times of evaluation (n=5 at 4 and 8 weeks). The presence of the material inserted was also observed in two animals in the control group at 8 weeks of evaluation (Figures 1 and 2).

Table 1 shows the mean, median and standard deviation of the hismorphometric bone gain according to group and time of evaluation for experimental and control defects (Figures 1 and 2).

Table 1 – Mean, median and standard deviation of the quantity of bone tissue formed according to the group and time of evaluation.

Group	Statistics	Time of evaluation	
		4 weeks	8 weeks
	(mm^2)		
• **Group I** (calcium-phosphate cement)			
Control	Mean	0.0000	0.0000
	Median	0.0000	0.0000
	Standard deviation	-	-
Test	Mean	0.0000 [(A)]	0.0134
	Median	0.0000	0.0000
	Standard deviation	-	0.0280
Value of p		P[(1)] = 1.000	p[(1)] = 0.180
• **Group II** (demineralized bone matrix)			
Control	Mean	0.0000	0.0000
	Median	0.0000	0.0000
	Standard deviation	-	-
Test	Mean	0.1335 [(B)]	0.0825
	Median	0.1437	0.0642
	Standard deviation	0.0885	0.0725
Value of p		P[(1)] = 0.068	p[(1)] = 0.043*
• **Group III** (matrix and cement)			
Control	Mean	0.0147	0.0000
	Median	0.0000	0.0000
	Standard deviation	0.0202	-
Test	Mean	0.0745 [(AB)]	0.0707
	Median	0.0598	0.0290
	Standard deviation	0.0734	0.1079
Value of p		P[(1)] = 0.068	p[(1)] = 0.109
Value of p		p[(2)] = 0.030*	p[(2)] = 0.112

(*) – Statistically significant difference at the 5.0% level.
(1) – By means of Wilcoxon´s signed posts test.
(2) – By means of the Kruskal-Wallis test for comparison between groups in the test cavities.
Note: If all the letters in parentheses are different, there is a significant difference between the means of the corresponding groups.

Intragroup evaluation was carried out using the Wilcoxon test, which showed that only in Group II (demineralized bone matrix) was there a significant difference at 8 weeks of evaluation (p=0.04). This indicates that there was more new bone formation in the experimental defects when compared to the control ones (Table 1).

The comparative evaluation of the groups was performed using the paired comparison of Kruskal-Wallis, which showed that only at 4 weeks of evaluation was there a significant difference (p=0.03). This indicates that there was more new bone formation in Group II (demineralized bone matrix) when compared to the other groups, as shown in Table 1.

■ Discussion

The regeneration capacity of bone presents limitations and in many cases the reconstruction of the defects is necessary, as in traumas, oncologic resections, infections, or prior to the placement of dental implants[5,11,12]. The reconstructions may be done with autogenous bone and synthetic or natural material[13-16].

Autogenous bone is regarded as the material of choice for bone reconstructions, although it may present some disadvantages such as greater surgical morbidity due to the need for a second surgical site[17]. In addition, many factors may change the initial result with bone grafts, particularly as a result of postoperative bone resorption[18].

Several materials have been studied and used as bone substitutes in order to avoid the use of autogenous bone[19]. Among these, demineralized bone matrix[7,13,18] and calcium phosphate cement[2,14,20,21] deserve special mention. The present study used bovine demineralized bone matrix (Gen-ox-org®, Baumer) based on Torricelli *et al.*[25], who showed that xenogenous materials may be more osteoinductive than human ones.

A demineralized bone matrix has been shown to be inert from the immunologic point of view, stimulates osteogenesis, is incorporated into the body and replaced with new bone from the hostage[6,7]. In this study, the matrix proved to be biocompatible, because even though there was an inflammatory reaction and the presence of giant cells at 4 weeks of evaluation, at 8 weeks this reaction was mild and there were no giant cells at all.

In relation to the calcium phosphate cement, in this study, at 4 and 8 weeks the presence of giant cells was observed near the particles of the material. It is important to note that during the process of histological decalcification the material is lost, leaving a gap corresponding to the material. This is in agreement with the studies of Indovina Jr. and Block[22], and Moghadam *et al.*[12], who observed an inflammatory reaction and giant cells around the spaces corresponding to the particles of the material at 8 and 12 weeks of evaluation.

In this study, when the matrix was associated with the cement, for both times of evaluation, there were giant cells. When the matrix was used alone, these cells were only observed at 4 weeks of evaluation. This suggests that the presence of calcium phosphate cement could be the determining factor for the maintenance of the inflammatory reaction and the presence of giant cells.

According to Moghadan *et al.*[12], the bleeding during or after the surgical procedure may account for the inappropriate hardening of the cement and for the presence of an inflammatory reaction.

The presence of giant cells noted in this study may be related to the resorption of the particles of the material. These cells may originate from the clastic cells, which are responsible for resorption and remodeling the area. These cells were seen in all experimental defects at 4 weeks of evaluation. In the matrix group, these cells were no longer seen at 8 weeks. This could be due to the greater velocity of resorption of the particles of the matrix when compared to the cement, which is in agreement with Clokie *et al.*[20], and Moghadan *et al.*[12].

The new bone formation in the experimental groups of this study was greater in the experimental defects than in the control ones (filled with blood clots) with a significant statistical difference at 8 weeks of evaluation in group II (bone matrix) (p= 0.043). This is in agreement with Laureano Filho[19] and Torriceli *et al*.[22], who found greater bone formation in the defects that were filled with demineralized bone matrix than in the one filled only with blood clots.

The ossification observed in this study was intramembranous, although in some studies it was endochondral or intramembranous[18,23].

When the cement was placed with a demineralized bone matrix, the mean of new bone formation was less than when the matrix was used alone, and greater when compared to the use of cement alone, although there was no significant difference. These findings may suggest that the cement was an osteogenesis inhibitor, because it acts as a hydroxyapatite bloc even though it has micropores. The development of calcium phosphate cements with larger pores may increase bone formation. Moreover, the addition of osteoinductive factors, such as morphogenetic proteins, may increase the resorption rate and at the same time stimulate bone formation[11,12].

■ Conclusions

The demineralized bone matrix was superior to calcium phosphate cement in bone regeneration. It seems that calcium phosphate cement acted by inhibiting the osteogenesis when associated with a demineralized bone matrix and this combination should not be recommended

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