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Original Article

Bone repair induced by different bone graft substitutes in critical-sized defects in rat calvaria

Avaliação do reparo ósseo induzido por diferentes substitutos ósseos em modelo de defeito crítico de calvária de ratos

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Resumo

Introdução: A utilização de substitutos ósseos em procedimentos de enxertia de forma alternativa ao uso do osso autógeno tem sido indicada, entretanto a comparação direta entre esses biomateriais tem sido pouco explorada. **Objetivo:** Avaliar o efeito de diferentes biomateriais osteocondutores sobre o reparo de defeitos críticos em calvárias (DCC) de ratos. **Material e método:** Foram utilizados 40 ratos que foram submetidos a confecção de um DCC com 8 mm de diâmetro. Os animais foram aleatoriamente divididos em 5 grupos com 8 animais, de acordo com o tipo de biomaterial utilizado para preencher os DCC: Grupo COA (coágulo); Grupo AUT (osso autógeno); Grupo OBD (osso bovino desproteinizado); Grupo HA/ TCP (cerâmica bifásica composta de hidroxiapatita e β fosfato tricálcio); Grupo TCP (β fosfato tricálcio). Foram executadas análise microtomográfica para avaliação do comprimento linear remanescente (DLL) do DCC e o volume dos tecidos mineralizados (MT) dentro do DCC nos períodos de 3, 7, 15 e 30 dias após cirurgia. Adicionalmente, foi executado análise histométrica para avaliar a composição do tecido ósseo reparado (% Osso e % Biomaterial) no período de 30 dias. **Resultado:** O grupo COA apresentou o menor DLL e MT dentro do DCC e maior % osso do que os outros grupos. O grupo OBD apresentou maior volume de tecidos mineralizados e maior % biomaterial do que o grupo os grupos AUT e TCP. Os grupos OBD e AUT apresentaram maior % osso que o grupo TCP. **Conclusão:** O OBD promoveu melhor padrão de aumento de disponibilidade óssea e qualidade do osso reparado em comparação ao TCP e HA/TCP, porém biologicamente inferior ao grupo AUT.

Descritores: Histologia; microtomografia; regeneração óssea; substitutos ósseos.

Abstract

Introduction: The use of bone substitutes in grafting procedures as an alternative of the use of autogenous bone graft has been indicated, however, the direct comparison between these biomaterials has been little explored. **Objective:** To evaluate the effect of different osteoconductive bone substitutes on the bone repair in critical-sized defects (CSDs) in rat calvaria. **Material and method:** One CSD with an 8 mm diameter was made in each of the 40 rats used in this study. The animals were randomly allocated into 5 groups (n=8), according to the type of bone substitute used to fill the CSD: COA (Coagulum); AUT (autogenous bone); DBB (deproteinized bovine bone graft); HA/TCP (biphasic ceramic composed of hydroxyapatite and β-phosphate tricalcium); and TCP (β-phosphate tricalcium). A microtomographic analysis was performed to evaluate the remaining defect linear length (DLL) of the CSD and the volume of the mineralized tissues (MT) within the CSD at 3, 7, 15 and 30 days after the surgical procedure. In addition, a histometric analysis was performed to evaluate the composition of the repaired bone tissue (% Bone and % Biomaterial) at the 30-day period. **Result:** It was shown that the COA had the lowest DLL and MT within the CSD. In addition, the COA presented the highest % of bone in CSD. The DBB had a higher MT and a higher

% of bone substitute particles in the CSD than the AUT and TCP groups. The DBB and AUT groups presented higher % of bone in the CSD than the TCP group. **Conclusion:** The use of the DBB promoted a better pattern of bone volume gain and formation compared to TCP and HA / TCP but was biologically inferior to the AUT.

Descriptors: Histology; x-ray microtomography; bone regeneration; bone substitutes.

INTRODUCTION

The regeneration of bone defects caused by different aetiological agents remains a major challenge in the medical and dental fields[1-3]. Although autogenous bone graft (AUT) is considered the gold standard for bone availability enhancement techniques[4-7], the use of this graft presents some limitations, such as the donor bed morbidity, the increased surgical time, the limited amount of available graft and the elevated resorption rates, especially when used in particulate form[5].

These limitations have indicated the necessity for the use of other types of bone substitutes that allow an adequate process of bone tissue formation with reduction of morbidity to the host[8,9]. Among the bone substitute alternatives to autogenous bone graft, osteoconductive biomaterials, such as deproteinized bovine bone (DBB) and biphasic ceramics, which are composed of hydroxyapatite and beta-phosphate tricalcium (HA/TCP), have highly diffused clinical application[8-10]. However, the low rate of reabsorption of hydroxyapatite present in both biomaterials implies its complete replacement by bone tissue, and the remnants of these bone substitutes have been observed in the recipient site until the implant placement[9,10]. In addition, these remaining particles do not participate in the direct osseointegration process of the implants due to the lack of vitality of this tissue[11].

Another biomaterial with osteoconductive properties in widespread use as a bone substitute is β-phosphate tricalcium (TCP). TCP has presented clinical outcomes in maxillary sinus elevation similar to DBB[12,13] and AUT[6]. In contrast to other osteoconductive bone substitutes, TCP has been related with higher resorption rates, which could indicate that this bone substitute can induce a greater amount of new bone in the grafted area[7].

The direct comparison between the different osteoconductive bone substitutes has been little explored. A preclinical study compared the potential for the bone repair of TCP with DBB, AUT, and coagulum (COA) in critical-sized defects (CSDs) in rat calvaria[7]. In this study, TCP induced the bone repair with a similar biological characteristic as the areas grafted with DBB, however, with lower bone formation than AUT. Nonetheless, the evaluation periods in this study were over 30 days and volumetric alteration of the tissue repaired in earlier periods was not evaluated. In addition, the direct comparison of the main osteoconductive bone substitutes (DBB, HA / TCP and TCP) with autogenous bone was not previously performed longitudinally in the same animal.

Thus, the aim of this study was to evaluate the influence of different bone graft substitutes (DBB, HA / TCP and TCP) in CSD repair by means of microtomographic analysis (Analysis of remaining defect linear length and volume of mineralized tissue in early stages of bone healing) and histological analysis (Analysis of the composition of the grafted tissue). The null hypothesis of this study is that the different osteoconductive bone substitutes will not present differences in the CSD repair.

MATERIAL AND METHOD

The study was approved by the Ethics Committee for Animal Use of our institution (19/2014) and was conducted according to international guiding principles for biomedical research involving animals and the ARRIVE guidelines. In the present study, 40 adult male rats (Rattus Norvegicus, albinus var., Holtzman) with body masses ranging from 300-350 grams were used.

The animals were kept in an environment with controlled light and temperature, fed with solid feed and had access to water *ad libitum* before and during the experimental period.

Groups

The 40 animals were randomly assigned, by lot after the CSD confection, to 5 groups of 8 animals each: COA group - The CDS was filled with coagulum; AUT – The CSD was filled with autogenous bone graft obtained during the confection of the CSD; DBB group – The CSD was filled with deproteinized bovine bone graft (Bio-Oss®, Geistlich AG, Wolhusen, Switzerland); HA / TCP group - The CSD was filled with biphasic ceramic composed of hydroxyapatite and β-tricalcium phosphate (Straumann® Bone Ceramic, Straumann AG, Basel, Switzerland); and TCP group – The CSD was filled with β tricalcium phosphate (Beta Pro®, Procell, São Carlos, Brazil).

For the calculation of the sample size, bone formation data on CSD that were filled with DBB and TCP and were evaluated by histometric analysis by Silva et al.[7] were used. The minimum difference in means between the groups where significant differences were found was 27.9% with a standard deviation of 9.0%. Thus, when establishing power β of 0.90 and error α of 0.05, a minimum number of 6 animals per group was determined. We used two animals more per group in order to prevent the loss of the statistical power due to the loss of animals during the experimental period.

Surgical Procedure

The animals were anaesthetized by a combination of ketamine and xylazine at a ratio of 0.08 ml/kg of body weight of ketamine (Ketamine Chlorhydrate, Francotar 10%, Virbac do Brasil Ind. Com. Ltda, São Paulo, Brazil) associated with 0.04 ml/kg of body weight of xylazine (Xylazine Hydrochloride - Virbaxyl 2% - Virbac do Brasil Ind. E Com. Ltda., São Paulo, Brazil) and were subsequently submitted to trichotomy in the region of calvaria. The antisepsis of the surgical field was then performed with povidone-iodine solution.

The surgical access to the calvaria was obtained through a cutaneous and muscular bicoronal incision, which allowed the exposure of the bone tissue. Then, the tissues were divulsed until the exposure of the periosteum, which was incised and detached, so that the exposure of the bone tissue was obtained.

A circular bone defect was made immediately after the apex of the coronal suture of the animal using a trephine milling drill with an external diameter of 8 mm (3i – 3i implantes do Brasil, São Paulo, Brazil). Then, the CSD presented with an 8 mm diameter and approximately 1.5 mm thickness, given by the total removal of the bone tissue. The CSDs were performed with the aid of a contra-angle (Anthogyr, Injecta, Diadema, Brazil) with a reduction of 16: 1 coupled with an engine speed of 1500 rpm (BML 600 Plus Driller, CK Driller, Cacapicuíba, Brazil), under constant irrigation with saline solution. All the CSD were made by the same surgeon (MATA).

Each biomaterial was implanted in the CSD until it was completely filled without extravasation. Then, all defects were covered by collagen membrane (Genius-Baumer, Mogi-Mirim, Brazil) and sutured with 4.0 silk suture thread (Ethicon, Johnson & Johnson, São José dos Campos, Brazil). The animals were treated in the postoperative period with a single multibiotic (Pentabiotico veterinario veterinária, Zoetis Dodge, São Paulo, Brazil, Subcutaneous: 0.03 ml / kg) and a ketoprofen dose (Ketoflex; Mundo Animal, São Paulo, Brazil, Intramuscular: 0.03 ml / kg).

Micro CT Analysis

The animals were anaesthetized to be submitted to scanning at 3, 7, 15 and 30 days after the surgical procedure using a Micro CT scanner (SkyScan, Kontich, Belgium). The parameters used for scanning were as follows: Pixel of the camera: 12.45; Voltage: 65 Kv; Amperage: 385 μA; and Filter: Aluminium 1 mm. The generated images were later reconstructed, spatially reoriented and analysed by specific software (NRecon / DataViewer / CTan, Skyscan, Aartselaar, Belgium). Two analyses were performed: 1) analysis of the remaining linear length (RLL) of the CSD that was measured in the larger portion of the CSD in the sagittal and coronal planes, and 2) analysis of the volume of the mineralized tissues (MT) within the CSD, which was performed within a circular region of interest (ROI), 8 mm in diameter with a thickness of 30 axial cuts with 18 μm thickness. The bone tissue associated with the bone substitutes was detected by applying a threshold within the range of 55-250 in the greyscale within the ROI. The micro CT analysis was performed by a blinded, trained and calibrated evaluator (GJO).

Surgical Samples Harvesting and Histological / Histometric Analysis

After the micro CT scan was performed at the 30-day period, the animals were euthanized by anaesthetic overdose. Afterwards, the regions of calvaria related to the defects were removed and fixed in 4% paraformaldehyde for 48 hours and decalcified in 7% EDTA for 3 months for later execution of laboratory processing for inclusion of the samples in paraffin and for obtaining histological sections of 5 μm thick that were stained using the haematoxylin and eosin technique.

Subsequently, three equidistant histological sections (50 μm apart) of each animal were photographed using a camera (Leica Microsystems DFC-300-FX; Leica Reichert & Jung Products, Wetzlar, Germany), which was coupled under a microscope optical (DM2500, Leica Reichert & Jung products, Wetzlar, Germany) at a 25x magnification.

In the histometric analysis, the original CSD was delimited based on the reversion lines that marked the original defect, and later, the different components within the total area of the original defect (newly formed bone and bone graft remnants) were measured by software image analyser (ImageJ, Jandel Scientific, San Rafael, CA, USA), and these values were given as a percentage, relative to the total area. Histometric analysis were performed by blinded and trained evaluator for these analyses (MATA).

Statistical Analysis

GraphPad Prism 6 software (San Diego, CA, USA) was used to perform the statistical analysis. The histological and microtomographic data had the normal distribution confirmed by the Shapiro-Wilk normality test. The evaluation comparing the different groups was carried out using the parametric tests of one-way ANOVA complemented by Tukey's test. The evaluation within each group comparing different experimental periods was performed using the parametric tests of the repeated measures ANOVA test complemented by Tukey's test. All statistical tests in this study were applied at a significance level of 5%.

RESULT

All animals survived the surgical procedures and remained healthy throughout the experimental period. To evaluate the calibrations of the examiners, 10 microtomographic scans and 30 histological slides were measured in duplicate with an interval of one week. Pearson's correlation showed an index r> 0.89 for the examiners in all evaluated parameters.

Micro CT Analysis

All the groups presented a progressive reduction of the RLL in longer periods of evaluation. It was also shown that the COA group (6.34 ± 1.49 mm) had a lower RLL compared to the DBB (7.64 ± 0.41 mm), TCP / HA (7.55 ± 0.39 mm), and TCP (7.59 ± 0.33 mm) groups at the 3-day period ($p < 0.05$). In addition, the COA group presented a lower RLL compared to the DBB group (5.80 ± 1.24 mm vs. 7.01 ± 0.38 mm) at the 30-day period ($p < 0.05$) (Table 1).

Table 1. The mean and standard deviation of RLL data (mm) in all groups and evaluation periods

Group/Period	3 days	7 days	15 days	30 days
COA	6.34 ± 1.49*^{B}	6.41 ± 0.63^{B}	6.48 ± 0.93^{B}	5.80 ± 1.24$^{\#A}$
AUT	7.37 ± 0.67^{B}	7.17 ± 0.83^{AB}	6.65 ± 0.72^{A}	6.27 ± 0.62^{A}
DBB	7.64 ± 0.41^{B}	7.31 ± 0.86^{AB}	7.29 ± 0.36^{AB}	7.01 ± 0.38^{A}
TCP/HA	7.55 ± 0.39^{B}	6.85 ± 0.45^{A}	6.82 ± 0.51^{A}	6.53 ± 0.53^{A}
TCP	7.59 ± 0.33^{B}	6.94 ± 0.51^{AB}	6.84 ± 0.70^{A}	6.85 ± 0.71^{A}

*Shorter RLL compared with the DBB, TCP/HA, and TCP groups at the 3-day period; #Shorter RLL than the DBB group at the 30-day period. One-way ANOVA complemented by Tukey's test ($p<0.05$). Different letters represent intragroup statistical differences – Repeated Measurements ANOVA complemented by Tukey's test.

Regarding the volume of MT, it was verified that the COA group always presented lower values of this parameter (5.17 - 28.04%) in relation to all other groups (> 42.77%) in all the evaluation periods ($p < 0.05$). The DBB group (65.66 ± 3.95%) had a higher volume of MT than the AUT group (42.77 ± 6.12%) at the 7-day period ($p < 0.05$). In addition, the DBB group (59.05 ± 6.13%) had a higher volume of MT than the AUT (44.93 ± 5.72%) and TCP groups (45.16 ± 5.03%) at the 15-day period ($p < 0.05$) (Table 2). Comparing the different experimental periods, it was noted a progressive reduction in the volume of the MT in all the groups, except in the COA group that presented a progressive improvement in this parameter in longer periods of evaluation. Figure 1 shows the representative images of microtomographic analysis of all the groups.

Table 2. The mean and standard deviation of MT volume data (%) in all groups and evaluation periods

Group/Period	3 days	7 days	15 days	30 days
COA	5.12 ± 2.48*^{C}	18.92 ± 3.58*^{B}	28.04 ± 3.90*^{A}	27.54 ± 4.66*^{A}
AUT	63.62 ± 3.86^{A}	42.77 ± 6.12$^{\#B}$	44.93 ± 5.72$^{\delta B}$	46.82 ± 4.21^{B}
DBB	72.14 ± 4.72^{A}	65.66 ± 3.95^{A}	59.05 ± 6.13^{B}	51.43 ± 3.85^{B}
TCP/HA	68.37 ± 3.90^{A}	54.48 ± 4.33^{B}	47.09 ± 7.52^{B}	44.12 ± 2.97^{C}
TCP	66.80 ± 7.31^{A}	50.71 ± 3.61^{B}	45.16 ± 5.03$^{\delta B}$	43.81 ± 4.00^{B}

*Lower MT volume than all the other groups in all the experimental periods; #Lower volume of MT than the DBB group at the 7-day period; δLower MT volume than the DBB group at the 15-day period. One-way ANOVA complemented by Tukey's test ($p<0.05$). Different letters represent intragroup statistical differences – Repeated Measurements ANOVA complemented by Tukey's test.

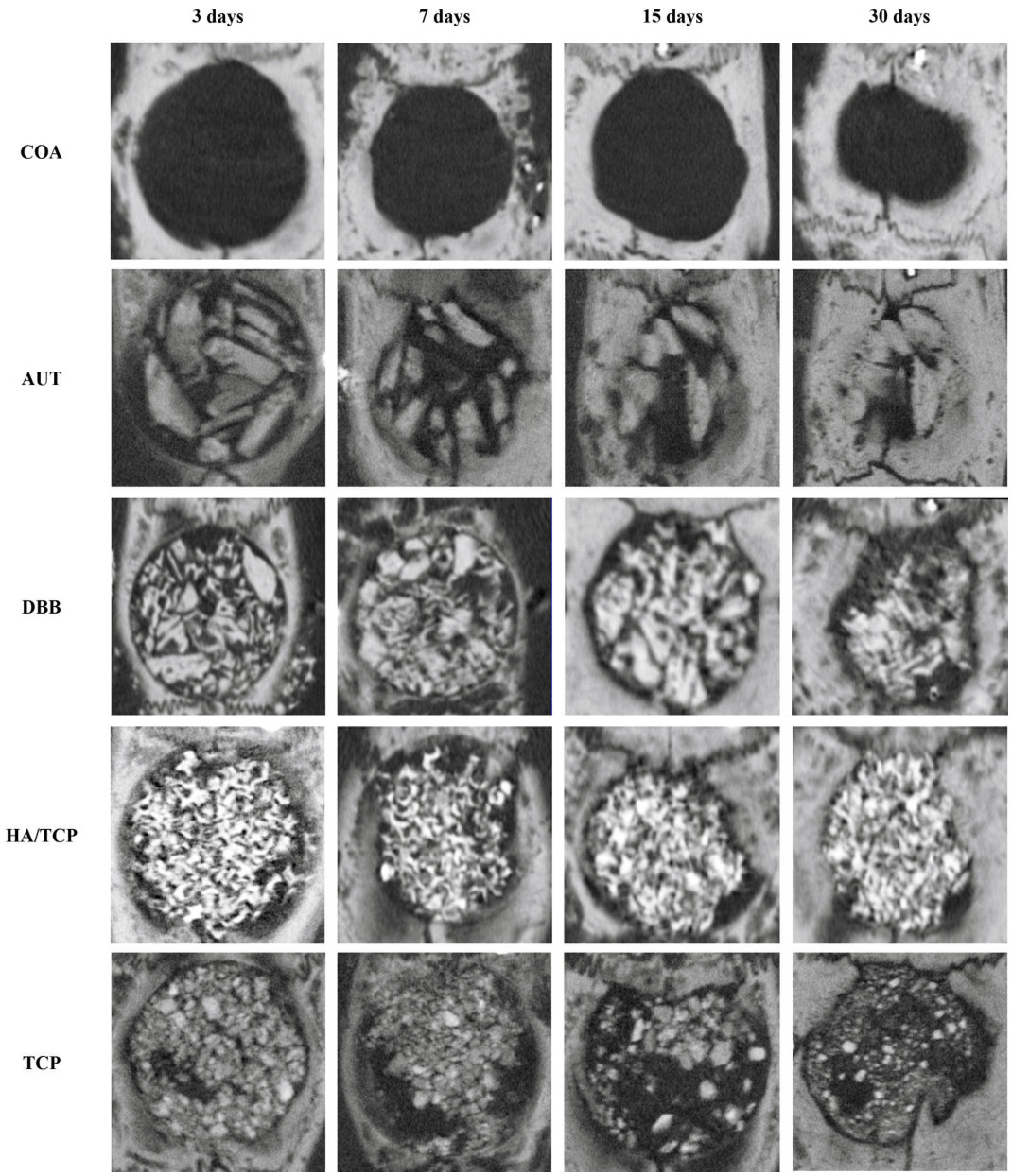

Figure 1. Representative Micro CT scanned images. It is possible to observe that the groups where the CSD were filled with biomaterials presented more MT than the COA group; however, this subgroup presents a greater closure of the CSD in relation to the other groups, with the exception of the AUT group.

Histometry

The histometric analysis was performed only at the 30-day experimental period. It was verified that the COA group had a greater amount of newly formed bone than all the other groups (17.42 ± 0.68%). In addition, the DBB (8.39 ± 0.71%) and AUT (7.96 ± 0.79%) groups had more newly formed bone than the TCP group (6.32 ± 0.80%). Furthermore, the DBB group (24.21 ± 1.44%) had a greater bone graft remnants than the AUT (17.48 ± 0.72%) and TCP groups (17.17 ± 0.74%). The TCP / HA group did not present significant differences in relation to the other groups of biomaterials in the amount of newly formed bone (7.22 ± 0.76%), as well as the amount of bone graft remnants (22.78 ± 0.90%) (Table 3). Figure 2 shows the representative histological images of each group.

Table 3. The mean and standard deviation of the data of the composition of the repaired tissue within the CSD (% newly formed bone and % bone graft remnants) determined by histometric analysis

Group/Tissue	% Newly formed bone	% Bone graft remnants
COA	15.95 ± 0.91[a]	-
AUT	10.74 ± 0.81[b]	17.48 ± 0.72[b]
DBB	8.39 ± 0.71[c]	24.21 ± 1.44[a]
TCP/HA	7.22 ± 0.76[c,d]	22.78 ± 0.90[a]
TCP	6.32 ± 0.80[d]	17.17 ± 0.74[b]

Different letters represent different levels of significant differences between groups - One-way ANOVA complemented by Tukey's test ($p < 0.05$).

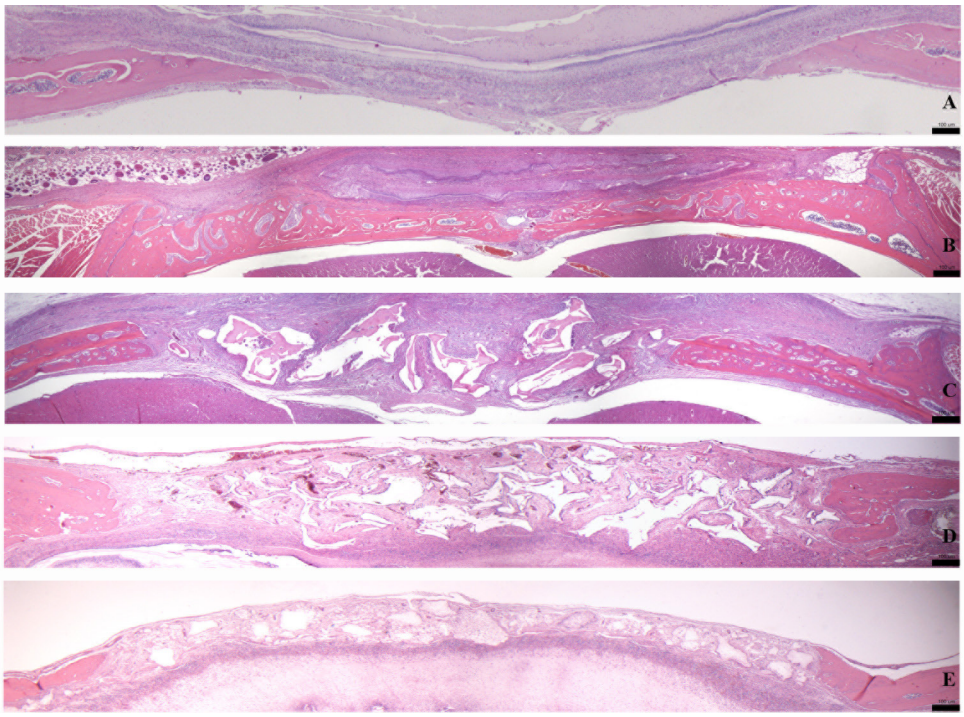

Figure 2. Representative histological images of all groups. A) COA; B)AUT; C)DBB; D)HA/TCP; E)TCP. Despite the lower volumetric gain, the COA group presents greater newly formed bone than the DBB, HA/TCP and TCP groups. The AUT group presented the best association of bone formation and bone volume augmentation. In all groups, the bone formation began at the edges and moved towards the centre of the CSD (Original magnification -25X- HE staining).

DISCUSSION

This study demonstrated that the different osteoconductive bone substitutes tested in this study (DBB, HA / TCP, and TCP) promoted a volumetric increase of MT within the CSD that was associated with the formation of bone tissue and the presence of remnants of biomaterial particles that were in contact with the neoformed bone, mainly at the edges of the CSD, which confirms the osteoconductive properties of these bone substitutes. However, bone formation in these groups was lower than that observed in the COA and AUT groups.

The COA group presented a greater closure of the CSD in comparison to the groups where the osteoconductive bone substitutes were used to fill the CSD already at the 3-day period, and this finding was associated with the greater bone formation also observed in the COA groups. Preclinical studies have shown that filling of post-extraction sockets with biomaterials reduces the formation of bone tissue compared to the post-extraction sockets that were kept without biomaterials during the healing period[14-16]. This phenomenon is due to the reduction of space for

bone formation since it is occupied by the remnants of biomaterials[16,17], an event confirmed by this study. In addition, the coagulum presents growth factors that accelerate bone regeneration[18,19]. However, it is worth mentioning that the COA group had a lower volume of MT, which demonstrates that the use of biomaterials is important to obtain sufficient repair tissue availability to maintain or to improve the bone morphology[14,20].

The CSD filled with AUT presented similar closure to the COA group and higher bone formation than the CSD filled with HA / TCP and TCP, as well as fewer biomaterial remnants than the CSD filled with DBB. These data, together, show that the area repaired by the use of AUT presented the best biological characteristics among the groups evaluated, confirming its position as a gold standard bone substitute because it is the only one that presents the biological properties of bone formation such as osteoinduction, osteogenesis and osteoconduction[4,7].

Among the bone substitutes tested in this study, the DBB group presented a greater amount of MT volume than the AUT and TCP groups, and this finding was related to the greater amount of DBB particle remnants. This fact confirms the findings of other studies that showed that the bovine hydroxyapatite component is less prone to resorption than the other bone substitutes[21]. The amount of biomaterial particles and bone formation in the HA / TCP groups was similar to the DBB group, although it did not differ from the AUT and TCP groups in terms of volume of MT and the amount of biomaterial remaining. The mechanism of action of HA / TCP is based on the reabsorption of the TCP component that would promote a differentiation of mesenchymal cells in osteoblasts and the maintenance of the HA component that would serve as a framework for bone formation in close contact with this component[8,20,22]. The association of TCP, which is more resorbable with HA, which is poorly resorbable, may be the justification for the biphasic ceramics of HA / TCP to have not differed from the other osteoconductive biomaterials tested in this study.

On the other hand, the TCP group presented lower MT volumes than the DBB group at the 15-day period, a lower amount of bone substitute remaining than the DBB and HA / TCP groups, and a lower amount of neoformed bone than the DBB and AUT groups. This finding is in agreement with previous studies that demonstrated that TCP presents higher rates of resorption than HA[22,23]. As TCP maintains less space than HA, it is likely to make it less competent in terms of volumetric maintenance of the grafted area. The highest resorption rate relative to DBB[23,24], and the lower biological properties of bone formation in relation to autogenous bone graft[7,25], may justify the lower bone formation shown in the TCP group.

The results of this study should be interpreted with caution because the superiority of one biomaterial in relation to another does not mean that clinically this difference will have some relevance in such a way that it can differentiate rates of survival and success of implants in areas grafted with these biomaterials. Indeed, implants placed in areas grafted with DBB, HA/TCP and TCP present high levels of survival and success[6,26,27]. Then, it may be possible that implants can be placed earlier in grafted areas with autogenous bone, DBB or HA / TCP compared to areas grafted with TCP, however this hypothesis must be test in other studies.

This study presents limitations that must be taken into account when interpreting our results. The outcomes of preclinical studies will not necessarily present the same pattern in humans, so the clinical inferences based the data of this study is limited. In addition, the type of defect evaluated was of the critical and the response in non-critical defects such as in maxillary sinus and post-extraction alveolar sockets presents different biological behaviour which may alter the healing promoted by the different bone substitutes evaluated in this study. Despite these limitations, it is worth mentioning that the CSD have been a widely used model for evaluation of bone healing in grafted areas. The 8-mm CSD used in this study has limited regenerative potential with good predictability of absence of complete defect closure that is characteristic of critical defects. So, this model represents a challenging condition to evaluate the regenerative potential of the different bone substitutes[28].

It can be concluded that DBB promoted a better pattern of volume of MT and quantity of newly formed bone compared to TCP and HA / TCP but with less bone formation and a greater remnant of particles than the AUT group. Due of it, the null hypothesis of this study was rejected.

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CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

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