Acta Histochemica 119 (2017) 624–631

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.com/locate/acthis

Assessment of bone repair in critical-size defect in the calvarium of rats after the implantation of tricalcium phosphate beta (β-TCP)

Leonardo de Freitas Silva[a,*], Erik Neiva Ribeiro de Carvalho Reis[a], Tânia Aparecida Barbara[b], João Paulo Bonardi[a], Idelmo Rangel Garcia Junior[c], Paulo Sérgio Perri de Carvalho[d], Daniela Ponzoni[e]

[a] DDS, MSc, PhD student on Oral and Maxillofacial Surgery from Araçatuba Dental School – UNESP, São Paulo, Brazil
[b] DDS, MSc student on Dental Implants from São Leopoldo Mandic Dental School, São Paulo, Brazil
[c] Adjunct Professor from Department of Surgery and Integrated clinic, Araçatuba Dental School – UNESP, São Paulo, Brazil
[d] Professor of Oral and Maxillofacial Surgery Department from Bauru Dental School – USP, São Paulo, Brazil
[e] Assistant Doctor Professor from Department of Surgery and Integrated clinic, Araçatuba Dental School – UNESP, São Paulo, Brazil

ARTICLE INFO

Keywords:
Biomaterials
Bone substitutes
Animal experiments

ABSTRACT

Objectives: Evaluating the osteoconductive property of tricalcium phosphate beta (β-TCP) in comparison to that of inorganic bovine bone for repair in a critical-size defect in the rat calvarium.

Materials and

Methods: Critical-size defects of 7 mm were made with a trephine in the calvaria of 48 Wistar rats. The animals were divided into four groups, and the defects in each group were filled with tricalcium phosphate beta (β-TCP), inorganic bovine bone (Bio-Oss), autogenous bone, or left empty. The animals were euthanized at two different time points (30 and 60 days post-operation). All defects were recovered with a absorbable membrane of bovine cortical bone. Histological, histometric, and immunohistochemical (osteocalcin) assessments were carried out at 30 and 60 days post-operation.

Results: At 30 days post-operation, all groups showed areas of bone formation, predominantly when autogenous grafts were used. However, there were no statistically significant differences between the treatment groups ($p > 0.05$). After 60 days, there were similarities in the bone formation patterns between the β-TCP (26.32 ±) and Bio-Oss (17.35 ±) groups ($p = 0.549$). In terms of the immunohistochemical assessment of osteocalcin, the clot group showed light to moderate staining at 30 and 60 days. The autogenous group showed moderate staining at 30 days and moderate to intense staining after 60 days. The Bio-Oss group showed light to moderate staining after 30 days and intense staining at 60 days. The β-TCP group showed moderate staining at 30 and 60 days post-operation.

Conclusion: β-TCP is a good osteoconductive material with similar effects to those of inorganic bovine bone graft and is suitable for utilization in the repair of bone defects.

1. Introduction

Biomaterials have been utilized in bone reconstruction procedures, such as maxillary sinus lifts, alveoli fillings after tooth extraction, and osseointegrated implant installations, in order to eliminate donor site morbidity (Gorla et al., 2015; Ananth et al., 2015; Santos et al., 2013). They can be classified as osteoinductive, osteoconductive, or osteogenic materials.

An osteoinductive material induces undifferentiated mesenchymal stem cells surrounding the tissue to differentiate into osteogenic cells that later form bone (Yuan et al., 2001). Osteoconduction occurs when the graft material permits osteogenic cells from the bone margins to infiltrate the graft material (Jensen et al., 2006). Osteogenic materials contain viable osteoprogenitor cells capable of differentiating into osteoblasts and producing new bone (Nazirkar et al., 2014).

Autogenous bone grafts are still considered as the gold standard in bone reconstruction because of their osteogenic properties (Burchardt 1987). These grafts have viable osteogenic cells, osteoinductive properties owing to the presence of bone morphogenetic proteins (BMPs), and osteoconductive properties as a result of the porous mineralized compound of the bone (Nazirkar et al., 2014).

Several materials such as allografts, xenografts, and alloplastic

* Corresponding author at: José Bonifácio street, number 1193, Vila Mendonça, 16015 – 050 – Araçatuba, São Paulo, Brazil.
E-mail address: leonardofreitas86@gmail.com (L. de Freitas Silva).

http://dx.doi.org/10.1016/j.acthis.2017.07.003
Received 30 March 2017; Received in revised form 23 June 2017; Accepted 13 July 2017

implants have been used as alternatives to autogenous grafts to repair bone defects. Among them, tricalcium phosphate beta (β-TCP) is a synthetic material with a high level of purity (99%), with the pure phase belonging to the apatite family. This type of material is biocompatible and readily available, can be easily sterilized, and has a long lifetime (Le Nihouannen et al., 2007). Inorganic bovine bone is considered the gold standard among xenografts (Manfro et al., 2014), showing similar results to those observed with autogenous bone in several studies (Jensen et al., 2012; McAllister et al., 1999; Schlegel et al., 2003).

Several biomaterials have been introduced in the market that aim to repair bone defects and thus, the study of these biomaterials is necessary in order to obtain the best knowledge of their behavior as osteoconductive materials. The goal of this study was to assess the bone repair of critical-size defects in the calvaria of rats after the implantation of β-TCP as an osteoconductive material in comparison to inorganic bovine bone.

2. Materials and methods

2.1. Animals

This study was approved by the Ethics Commission on Use of Animals of Araçatuba Dental School Process 2014-01457. Forty-eight male *Rattus norvegicus* (order Rodentia, lineage Wistar) rats weighing 250–300 g, all with the same age, were obtained from the vivarium of the Araçatuba Dental School − Unesp (Araçatuba, São Paulo, Brazil). The animals were distributed in cages with four animals per cage and fed a standard diet and water *ad libitum*.

The website http:www.lee.dante.br was used to calculate the sample size, which substantiates previous results (Bizenjima et al., 2016). The standard deviation used was 12.5, the difference of means was 20.8, the power of the test was 80%, and $p < 0.05$, with four animals per group. Therefore, forty-eight animals were used for this study.

2.2. Experimental design and surgical procedure

The animals were randomly divided into four groups (six animals per group) for experimental periods of 30 and 60 days. In the autogenous group, the defects were filled with crushed autogenous bone. In the Bio-Oss group, the defects were filled with inorganic bovine bone (Bio-Oss, Geistlich Pharma, Wolhusen, Switzerland) with granules of 0.25–1 mm. Defects in the Clot group and were filled with clot, and those in the β-TCP group were filled with β-TCP (BETAPro®, São Paulo, São Paulo, Brazil) with granules of 50–150 μm. In all groups, the defects were covered with an absorbable membrane of bovine cortical bone (Baumer®, Mogi Mirim, São Paulo, Brazil).

Calvarium defects in the autogenous, Bio-Oss, and β-TCP groups were filled with 100 μg of each respective biomaterial. The biomaterials were implanted without passing the defect borders.

The animals fasted pre-operatively for 8 h. Anesthesia by sedation was performed intramuscularly with 60 mg/kg 1% ketamine (Vetaset®, Fort Dodge, Saúde Animal LTDA, Campinas, São Paulo, Brazil) and 8 mg/kg 2% xylazine (Dopaser®, Laboratório Calier do Brasil Ltda, São Paulo, Brazil). Local infiltration of the calvarium with 0.3 mL/kg 2% mepivacaine hydrochloride with epinephrine (1:100,000, Mepiadre 100®, DFL LTDA, Rio de Janeiro, Brazil) was also performed.

Trichotomy of the calvarium was performed, and the region was disinfected with 10% polyvinylpyrrolidone iodine degermante solution with 1% active iodine (Riodeine®, Rioquímica, São José do Rio Preto, Brazil). Soon after, a triangular incision was made on the sagittal region of the cranium with a lamina number 15, followed by detachment of the periosteum and confection of the bone defects in the center of the calvarium with a 6-mm trephine (Neodent, Curitiba, Paraná, Brazil) mounted at a reducer contra-angle of 20:1 (Kavo® do Brasil, Joinvile, Brazil) and connected to an electric motor with controlled rotation (BLM 600 plus, Driller®, Jaguaré, São Paulo, Brazil) at 1200 rotation per minute, under 50% saline irrigation (Fisiológico®, Laboratórios Biosintética Ltda®, Ribeirão Preto, SP, Brazil). In the autogenous group, the harvested bone was manually crushed and used as the filling material for the cavity. The defects in the other groups were filled with the previously specified materials. All defects were covered with an absorbable membrane. The skin was sutured with mononylon 5-0 sutures (Ethicon-Johnson & Johnson do Brasil, São Paulo, São Paulo, Brazil).

Post-operatively, the animals received one dose of pentabiotic (0.1 mL/kg, Fort Dodge Saúde Animal Ltda, Campinas, São Paulo, Brazil) intramuscularly and three doses of morphine (2.5 mg/kg, Dimorf®, Itapira, São Paulo, Brazil) at intervals of 24 h.

Animals were euthanized with a lethal dose of thiopental (150 mg/kg) plus lidocaine (10 mg/mL intraperitoneally), at post-operative periods of 30 and 60 days. The calvaria were extracted via rectangular cutaneous incision using an oscillating necropsy saw and were immediately submerged in 10% neutral buffered formalin for 48 h prior to paraffin embedding.

2.3. Laboratory processing

The calvaria were removed and fixed with 10% buffered formalin, decalcified with 4.13% ethylenediaminetetraacetic acid (Merck, Darmstadt, Germany), dehydrated using ascending alcohol gradients, and embedded in paraffin. Semi-serial cuts of 5 μm in thickness were prepared and stained with hematoxylin and eosin (Merck & Co., Inc., NJ, EUA) for histological and histometric analyses (laminae pairs), and the others were used for immunohistochemical analysis (Fig. 1).

2.4. Histometric analysis

Blind histometric and immunohistochemical analyses were performed. Images were obtained using an optical microscope (LeicaR® DMLB, Heerbrugg, Switzerland) linked to a capture camera (LeicaR® DC 300F Microsystems Ltd., Heerbrugg, Switzerland) and connected to a microcomputer with the Axio Vision 4.8 software (Carl Zeiss, Oberkochen, Germany). The digitalized images were recorded in JPEG format. The histometric evaluation was performed using ImageJ 150e software (National Institutes of Health, Maryland, USA) at a magnification of 63 × . The bone defect was determined by measuring the area between the osteotomy margins and is expressed as the total percentage of the defect. The area of bone formation was calculated and expressed as a percentage inside the total area of the defect created in the calvarium, as described by Luvizuto et al. in 2012. The obtained values were statistically analyzed by multiple comparison tests, two-factor ANOVA, and Tukey post-hoc testing (differences were considered significant at $p < 0.05$) with the statistics software SigmaPlotTM 12.3 (SigmaPlot Exakt Graphs and Data Analysis, San Jose, CA, USA).

2.5. Immunohistochemistry

Primary antibodies against osteocalcin (OC, goat anti-oc, SC18319, Santa Cruz Biotechnology, CA, USA) were used. Samples were treated with immunoperoxidase and chromogen 3,3′-diaminobenzidina (Sigma, St. Louis, MO, USA) and counter-stained with hematoxylin de Harris. Control samples were not treated with primary antibodies (negative control), and incubation with normal donkey serum (5%) was performed to prevent non-specific binding of the antibody. The slides were analyzed using an optical microscope (LeicaR® DMLB, Heerbrugg, Switzerland) linked to a capture camera (Leica® DFC 300FX, Leica Microsystems, Heerbrugg, Switzerland). Ordinal qualitative analysis was performed, and the scores were based on the area of positive immunostaining. The scores were based on staining of cells in the predetermined areas, which was indicative of dynamic bone tissue. To simplify the comparison among the different groups and periods, the immunohistochemistry scores were converted into frequencies of

Fig. 1. Flow chart.

Table 1
Percentage of new bone formation area according to the groups and euthanasia periods.

Group	Period (Days)	Percentage of bone formation	Number of particles (Median)	P value*
Clot	30	22.012 ± 7.25		0,554
Autogenous	30	30.926 ± 9.02		
Bio-Oss	30	27.54 ± 14.00	34.33	0,823
β-TCP	30	21.722 ± 8.24	42.16	
Clot	60	24.69 ± 8.43	–	< 0,001
Autogenous	60	54.224 ± 10.16	–	
Bio-Oss	60	17.352 ± 8.03	39.4	0,549
β-TCP	60	26.322 ± 9.40	54.33	

* $p < 0.05$ was considered a statistically significant difference.

percentage for the period of evaluation (Pedrosa et al., 2009; Manrique et al., 2015). The histological scoring was light, moderate, or intense, based on the stained area (Table 1).

3. Results

3.1. Histological analysis

3.1.1. 30-day

In the 30-day Clot group, the osteotomy line and new bone formation occurring near the margins of the bone defect were observed (Fig. 2A). At a magnification of 250 × , it was possible to observe new bone formation next to the stump (Fig. 3A). In most of the samples, total closing of the defect was not observed. The center of the bone defect was filled mostly with newly formed bone with a small central area of fibrous tissue rich in fibroblasts and capillaries (Fig. 2A).

In the autogenous group, new bone formation was observed in the regions near the stumps and in the defect center, and in some samples the defects were completely closed (Fig. 2B). At 250 × magnification, particles of autogenous bone (*) incorporated into the newly formatted bone tissue were observed (Fig. 3C).

In the Bio-Oss group, the central region of the wound was filled with particles of the material, which were characterized by negative areas with polyhedral forms, varied sizes, and contours forming right angles with the membrane covering the defect (Figs 2C and 3E). New bone formation was observed from the stump to the defect center (Fig. 2C). In the central part, complete closing of the critical defect was observed. In some samples, particles of the material were surrounded by new bone formation or by fibrous connective tissue (Figs. 3E and 2C), indicating osteoconduction.

In the β-TCP group, the presence of particles of the implant material was observed inside the bone defect covered by the membrane. New bone formation was also observed from the stump to the wound center (Figs 2D and 3G). In the center of the defect, connective tissue rich in fibroblasts and intensive capillary proliferation were observed among the particles, and in some areas, there were regions of new bone formation next to the particles (Fig. 2D).

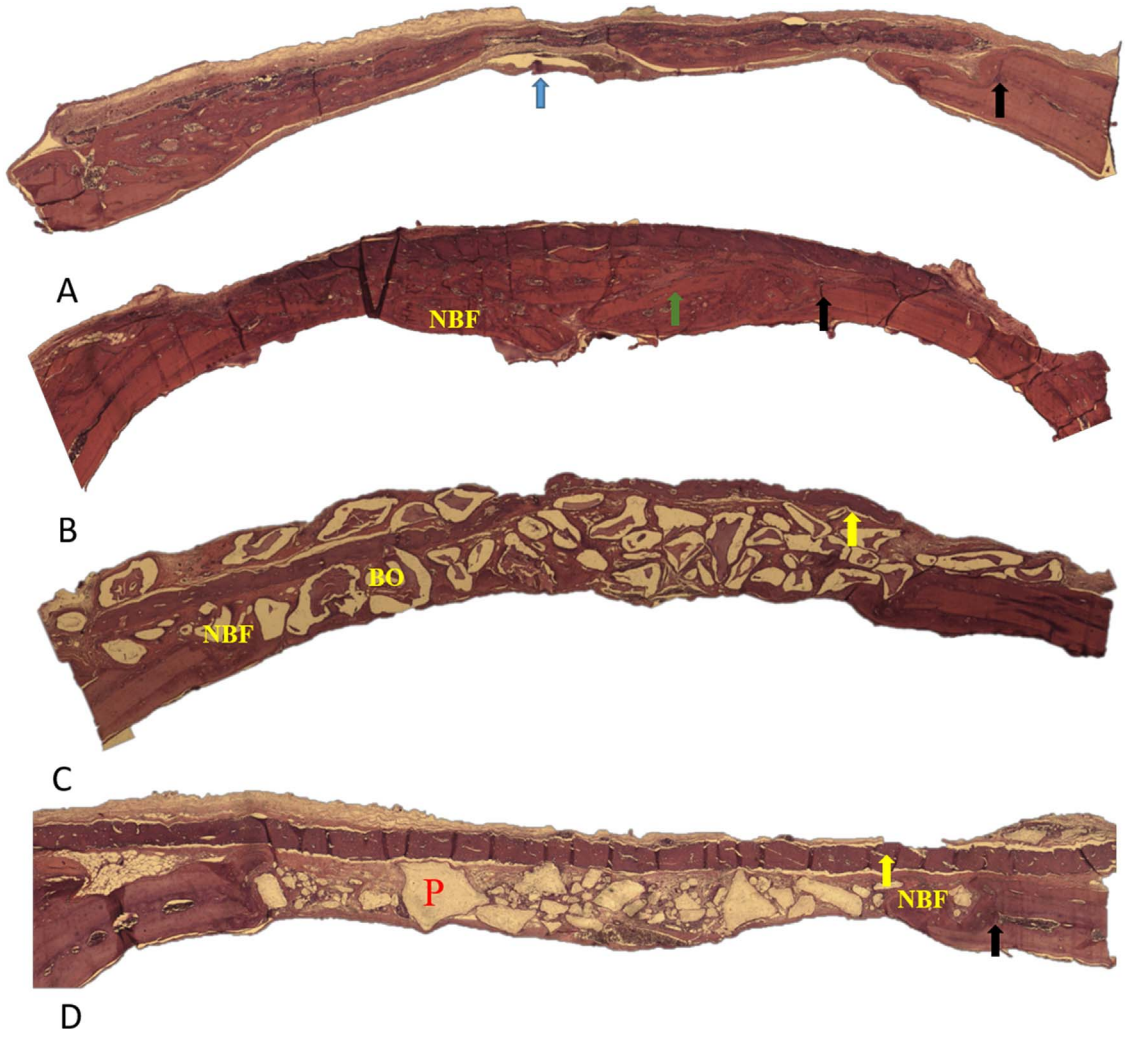

Fig. 2. Bone defects filled with biomaterial, 30-day. (A) Clot group, osteotomy line (black arrow), center of defect with no new bone formation (blue arrow). (B) Autogenous group, center of defect, new bone formation (NBF) filling the entire cavity, presence of particles of autogenous graft (green arrow). (C) Bio-Oss group, particles of the biomaterial (BO), membrane (yellow arrow), new bone formation (NBF). (D) β-TCP group, particles of the biomaterial (P), osteotomy region (black arrow), new bone formation (NBF), membrane (yellow arrow) (HE, increase of 63 ×). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.1.2. 60-day

In the 60-day Clot group, the majority of the defects showed new bone formation occluding the whole extension (Fig. 3B). Next to the stumps, new bone formation with a normal aspect and rounded borders was observed (Fig. 4A). In the autogenous group, most of the defects were closed with new bone incorporated into particles of autogenous bone (Figs. 3D and 4B). As in the 30-day group, the central region of the wound remained filled with several particles of the material, similar to the microscopic pattern. There was an absence of foreign body type granuloma (Figs 3F and 4C). In the β-TCP group, particles of the implant material in the defect were surrounded by fibrous connective tissue or new bone, with the largest amount of bone tissue observed among the particles (Figs. 3H and 4D).

3.2. Histometric analysis

Fig. 5 and Table 1 show the new bone formation, the percentage and number of particles in each studied group at each post-operative period. At 30 days, all groups presented areas of new bone formation, and the largest area of formation was observed in the autogenous group ($p < 0.05$). However, there were no statistically significant differences between the other groups ($p > 0.05$).

At 60 days, a tendency of major new bone formation was observed in comparison to the 30-day period, but there were no statistical differences between the β-TCP and Bio-Oss groups ($p = 0.549$). The largest percentage of new bone formation was observed in the autogenous group ($p < 0.05$). Some defects filled with β-TCP, inorganic bovine bone, and autogenous grafts showed complete healing.

3.3. Immunohistochemical analysis

The presence of osteocalcin was assessed on the tissue and surrounding the particles of the biomaterial. At 30 days post-operation, the β-TCP and autogenous groups exhibited moderate staining, the Bio-Oss group exhibited light to moderate staining, and the Clot group exhibited light staining. After the 60-day period, the autogenous group showed moderate to intensive staining, the Bio-Oss group showed moderate staining, the β-TCP group showed light to moderate staining, and the Clot group showed light staining (Fig. 6).

4. Discussion

The purpose of this study was to assess the osteoconductive properties of β-TCP in comparison with those of Bio-Oss, autogenous bone, and clot. The null hypothesis was that the β-TCP biomaterial would exhibit behaviors similar to those of Bio-Oss, and this hypothesis was validated in this study with the observed results.

The rat calvarium is similar to the human mandible, and both

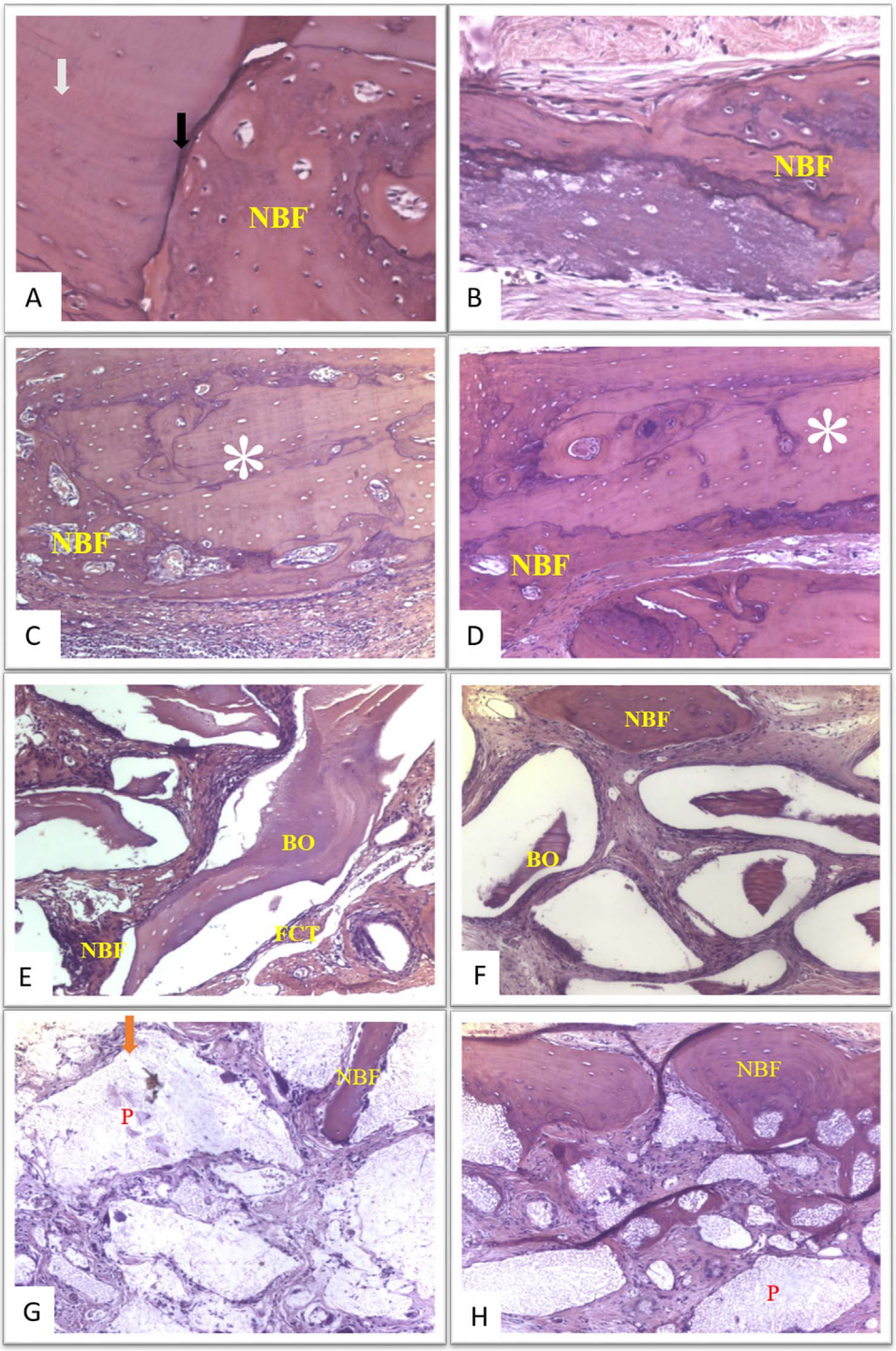

Fig. 3. (A) Clot group, 30-day, left stump (gray arrow), osteotomy line (black arrow), new bone formation (NBF) from the stump. (B) Clot group, 60-day, center of defect, new bone formation (NBF). Autogenous group, center of defect, detail of the particle of autogenous bone (*) incorporated in new bone tissue (NBF) at (C) 30 days and (D) 60 days. Bio-Oss group, center of defect, particle of Bio-Oss (BO) surrounded by fibrous connective tissue (FCT) and new bone formation (NBF) at (E) 30 days and (F) 60 days. β-TCP group, particles of biomaterial (P), new bone formation (NBF), fibrous connective tissue (arrow) at (G) 30 days and (H) 60 days (HE, increase of 250 ×).

develop through intramembranous bone formation and show limited regenerative potential (Yun et al., 2010). A critical-size defect is defined as the smallest intraosseous wound in a particular bone and animal species that will not heal spontaneously during the lifetime of the animal (Schmitz and Hollinger 1986). Thus, in the present study, the Clot group was used as a negative control with a defect of 7 mm, as utilized in previous studies (Schmitz and Hollinger 1986; Schmitz et al., 1990; Vajgel et al., 2014). Autogenous grafts and Bio-Oss were used because they are considered as the gold standards among biomaterials and xenografts, respectively (Burchardt 1987; Manfro et al., 2014).

In the currently study, the autogenous graft induced the highest rate of new bone formation. However, in the β-TCP and Bio-Oss groups, the differences in new bone formation were not statistically significant. This suggests that β-TCP, as assessed in this experimental model (rat calvarium), is a good bone substitute for the reconstruction of critical-size defects.

According to Ueno et al., in 7-mm defects in the rat calvarium, β-TCP in combination with autologous tibia periosteum resulted in a higher percentage of new bone formation than did β-TCP alone (50.3 ± 2.5 *vs* 23.8 ± 1.5) (Ueno et al., 2007). Yun et al. carried out a study with a critical-size defects of 8 mm in rat calvaria, and they observed that the subcutaneous use of parathyroid hormone resulted in greater new bone formation than did that of β-TCP alone after four weeks (Yun et al., 2010). The current study results correspond to those

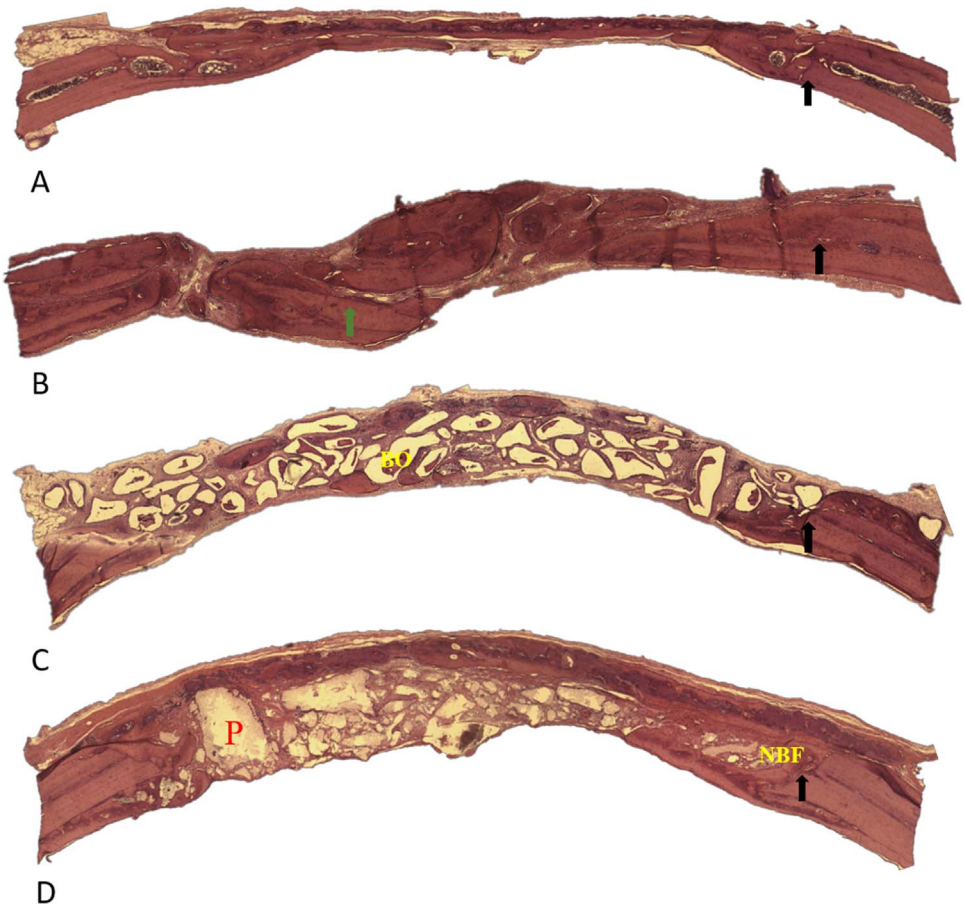

Fig. 4. Bone defects filled with biomaterial at 60-day period. (A) Clot group, newly formed bone tissue filling the defect with normal aspect and rounded borders. (B) Autogenous group, newly formed bone tissue filling entire cavity, graft particles (green arrow), osteotomy line (black arrow). (C) Bio-Oss group, biomaterial particles (BO), new bone formation among the particles in. (D) β-TCP group, line of osteotomy (black arrow), new bone formation (NBF), biomaterial particles (P) (HE, increase of 63 ×). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

found by Ueno et al., with 21.72% new bone formation after the 30-day period in defects filled with β-TCP (Ueno et al., 2007). However, in this study, a bovine cortical membrane was used in all defects, which differs from previous approaches (Yun et al., 2010; Schmitz and Hollinger 1986). Ueno et al. did not use any kind of membrane in one study group and Yun et al. used a tetrafluoroethylene membrane for all defects (Ueno et al., 2007; Yun et al., 2010).

After the 60-day period, the β-TCP group presented similar results to those found by Yun et al. (2010). The mean new bone formation was 26.32% compared with 21.3 ± 4.4% observed by Yun et al. However, the methodology of Yun et al. differs with regards to the defect size (8 mm) and the use of tetrafluoroethylene membrane (Yun et al., 2010).

Bizenjima et al. observed that after a 4-week period, defects of 5 mm in the rat calvarium filled by β-TCP exhibited new bone formation inside the defects and islands of immature bone, with no signs of inflammatory infiltrates or foreign body type reactions in most of the samples (Bizenjima et al., 2016). In previous studies (Luvizuto et al., 2012; Bizenjima et al., 2016), after a 60-day period, the presence of biomaterial with limited resorption and new bone formation was observed.

A critical-size defect of 6 mm in a rat calvarium filled with Bio-Oss showed 25.26% new bone formation after four weeks (Khoshzaban et al., 2011). Park et al. and Mokbel et al. observed new bone formation rates of 6.4% ± 4.3 and 23.3%, respectively, for 8-mm rat calvarial defects filled with Bio-Oss at six and eight weeks (Park et al., 2009; Mookbel et al., 2008). Despite that the percentage of new bone formation in the Bio-Oss group at 60 days was inferior to that observed at 30 days, the results are in accordance with previous findings after experimental periods of four and eight weeks, and there was no statistical difference between the results after 30 and 60 days. Additionally, the

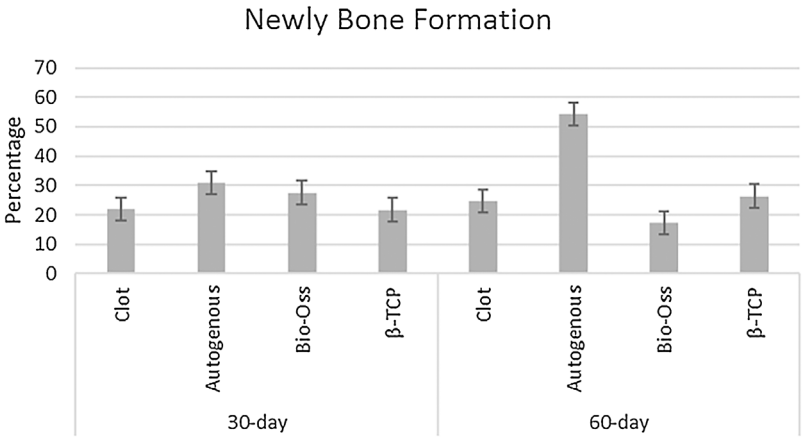

Fig. 5. Representative graphic of the percentage and standard deviations of the experimental groups (Clot, Autogenous, Bio-Oss, and β-TCP) for the area of new bone formation, 30 and 60 days post-operation.

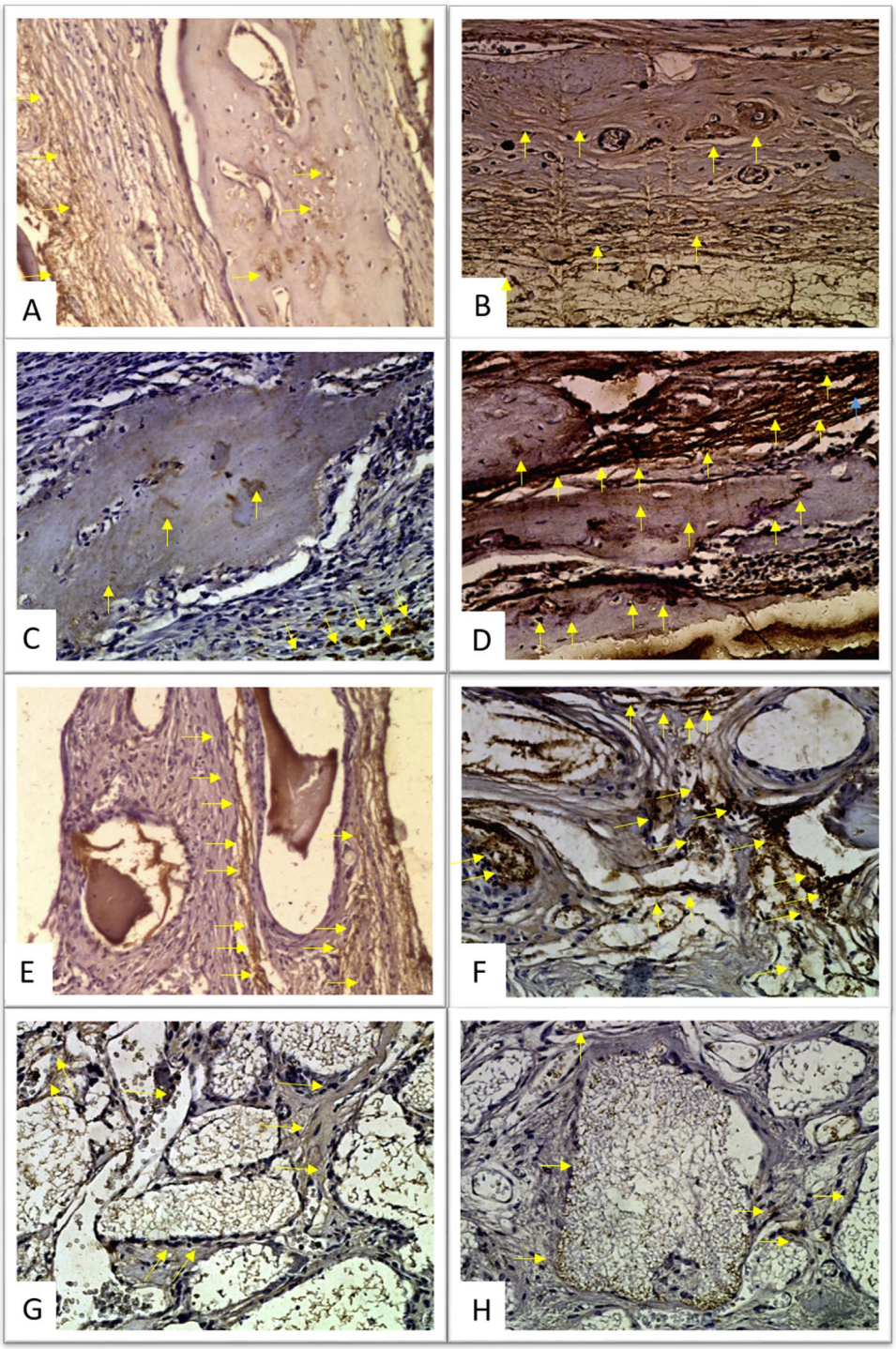

Fig. 6. (A) Clot group, 30-day, light staining. (B) Clot group, 60-day, light staining. (C) Autogenous group, 30-day, moderate staining. (D) Autogenous group, 60-day, moderate to intensive staining. (E) Bio-Oss group, 30-day, light to moderate staining. (F) Bios-Oss group, 60-day, moderate staining. (G) β-TCP group, 30-day, moderate staining. (H) β-TCP group, 60-day, light to moderate staining (immunostaining against osteocalcin, yellow arrows, increase of 250 ×). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

evidence of osteocalcin from the immunohistochemical analysis suggests a greater mineralization level for the 60-day period than for the 30-day period in the Bio-Oss group.

Osteocalcin is considered to be a marker of differentiation into the osteoblastic phenotype because of its expression in cells of the osteoblastic lineage both *in vivo* and *in vitro* (Han et al., 2008). Despite that its role in bone remodeling are not completely understood, osteocalcin has been shown to be involved in the activation of osteoclastic bone resorption and has effects on both osteoclasts and osteoblasts (Han et al., 2008). In this study, the β-TCP group showed moderate osteocalcin staining after the 30-day period and light to moderate staining after the 60-day period. Similar metabolic activities were observed in the autogenous and Bio-Oss groups. However, at 60 days, this activity was less intense than that in the other groups, except for the Clot group, which presented light staining.

The main limitation of this study was the utilization of animals to test the biomaterials. Although the rat calvarium is similar to the human mandible, it does not guarantee that the behavior of the tested biomaterials would be the same in humans. Thus, it is important to carry out clinical trials to gain a better understanding of the behavior of β-TCP.

5. Conclusions

The findings of this study suggest that β-TCP is a good osteoconductive material, is biocompatible, has similar effects to those of inorganic bovine bone, and is suitable for utilization in the repair of bone defects.

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