Efecto de la matriz de beta-fosfato tricálcico con fibronectina en la reparación de defectos óseos críticos: estudio experimental del potencial de regeneración ósea y su aplicabilidad clínica

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Received: 31 January 2018 | Revised: 23 August 2018 | Accepted: 26 August 2018

DOI: 10.1111/clr.13370

ORIGINAL RESEARCH

WILEY CLINICAL ORAL IMPLANTS RESEARCH

Guided bone regeneration using beta-tricalcium phosphate with and without fibronectin—An experimental study in rats

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Funding information
This study was performed by the research group "Odontological and Maxillofacial Pathology and Therapeutics" of the Biomedical Research Institute of Bellvitge (IDIBELL) of Barcelona, Spain. Llorenç Badiella-Busquets was recipient of MTM2015-69493-R grant from the Spanish Ministry of Economy, Industry and Competitiveness. The study forms part of the interimplant project ("Biomateriales Avanzados para una Nueva Generación de Implantes") under the leadership of the Biotechnology Institute and has been one of the 16 projects approved by the CDTI (Centre for Industrial Technological Development) in the third announcement of the CENIT program supported by the initiative INGENIO 2010 of the Spanish Government.

Abstract

Objective: This histomorphometric study compared bone regeneration potential of beta-tricalcium phosphate with fibronectin (β-TCP-Fn) in critical-sized calvarial defects (CSDs) in rats to assess whether fibronectin (Fn) improved new bone formation.

Material and methods: Critical-sized calvarial defects were created in 30 adult male Sprague Dawley rats, which were divided into four groups according to the time of euthanasia (6 or 8 weeks of healing) and type of filling (β-TCP-Fn/6 weeks, β-TCP/6 weeks, β-TCP-Fn/8 weeks and β-TCP/8 weeks). The primary variables related to new bone formation were augmented area (AA) and gained tissue (GT; sum of mineralized bone matrix [MBM] and bone substitute [BS]). Secondary variables were the diameter of the defect, MBM, non-mineralized tissue (NMT) and BS.

Results: A total of 29 rats and 58 histological samples were evaluated, 28 (48.3%) samples obtained at 6 weeks and 30 (51.7%) at 8 weeks, homogeneously distributed between right and left sides. Thirteen (22.4%) were treated with β-TCP-Fn, 16 (27.6%) with β-TCP and 29 (50%) were controls. At 8 weeks, histomorphometric analysis showed significant differences in AA using β-TCP and β-TCP-Fn versus controls ($p = 0.001$ and $p = 0.005$, respectively). Bone turnover expressed as % within the target area was slightly higher but not statistically significant in the β-TCP-Fn than in β-TCP (MBM) at 6 weeks versus 8 weeks ($p = 0.067$ and $p = 0.335$, respectively). Finally, the total GT area in mm^2 was higher using β-TCP-Fn as compared to β-TCP ($p = 0.044$).

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Conclusions: β-TCP-Fn was slightly but non-significantly more effective than β-TCP without Fn for improving the volume of regenerated bone in CSDs of rats, possibly allowing a more efficient bone remodelling process. This effect however should continue being investigated.

KEYWORDS
animal experiments, beta-tricalcium phosphate, bone regeneration, experimental study, fibronectin, histomorphometry, rats

1 | INTRODUCTION

Bone grafts have been used for decades to replace lost bone due to different causes. It is estimated that more than 2.2 millions of reparative surgical interventions with grafts are performed yearly, with either autograft or allograft tissues used in 90% of procedures (Greenwald et al., 2001). However, limitations of autogenous grafts include morbidity, limited amount of tissue graft available from the donor site and the possibility of infection transmission in non-lyophilized allografts (Bigham-Sadegh & Oryan, 2015; Clokie, Moghadam, Jackson, & Sandor, 2002; Wang et al., 2013). Although allogenic demineralized bone matrix (Haddad, Peel, Clokie, & Sándor, 2006) and deproteinized bovine bone matrix (Huh et al., 2015) have been used as alternatives to autogenous bone, alloplastic materials such as hydroxyapatite and beta-tricalcium phosphate (β-TCP) can be used instead of bone grafts due to its excellent biocompatibility and osteoconductivity. The physical properties and bone regeneration effects of hydroxyapatite and β-TCP have been examined in different experimental studies (Calvo-Guirado et al., 2012; Homaeigohar et al., 2005; Lee, Pai, Chang, & Kim, 2016; Rojbani, Nyan, Ohya, & Kasugai, 2011; Suenaga, Furukawa, Suzuki, Takato, & Ushida, 2015). Other grafting materials, such as calcium silicate bioactive ceramics (Xu et al., 2008), the high-density polyethylene (Homaeigohar et al., 2005; Homaeigohar, Shokrgozar, Khavandi, & Sadi, 2008) and calcium phosphate crystals (Cai et al., 2009), have also been investigated.

In recent years, extensive experimental research has focused on different approaches to accelerate bone regeneration (Gomes & Fernandes, 2011; Li et al., 2011; Luvizuto et al., 2011; Rodriguez et al., 2011). Tissue engineering using combinations of cells, scaffolds and bioactive factors is novel possibilities as bone grafts for skeletal defects (Annibali et al., 2014, 2013 ; Ball, O'Connor, & Pandit, 2009; Fernández et al., 2012). Fibronectin is a glycoprotein of the extracellular matrix that promotes cell adhesion, differentiation and expansion, and has been investigated in combination with regeneration biomaterials for improving proliferation and differentiation of osteoblasts cultivated on composite scaffolds (Ball et al., 2009; Fernández et al., 2012). Moreover, it has been shown that anodized titanium implants treated with fibroblast growth factor-Fn fusion protein enhanced osseointegration (Park et al., 2005). In another study, Fn accelerated osteoblast differentiation in osteoprogenitor cells cultured on rigorously cleaned titanium alloy implant material (Rapuano, Hackshaw, Schniepp, & MacDonald, 2012). On nanoporous titanium surfaces, Fn and vitronectin promoted the attachment and proliferation of human foetal osteoblast cell lines (Rivera-Chacon et al., 2013).

In previous studies of our group, the bone regeneration potential of β-TCP-Fn with autologous adipose-derived stem cells (β-TCP-Fn-ADSCs) was examined in critical-sized defects (CSDs) of alveolar ridges in a dog model (Alvira-González et al., 2016) and in dehiscence-type defects associated with dental implants (Sánchez-Garcés et al., 2017). The use of ADSCs does not seem to improve the area of bone regeneration and bone-implant contact (BIC) and did not entail an advantage as compared with other biomaterials. However, the use of β-TCP-Fn was superior to β-TCP alone.

Native bovine resorbable collagen barrier membranes for guided bone regeneration procedures have demonstrated a positive soft tissue exclusion effect (Donos, Dereka, & Mardas, 2015). Nevertheless, they do not maintain the required preformed space when there is a lack of grafted material beneath those membranes during the bone formation period (Kostopoulos & Karring, 1994). This process depends also on the resorption ratio of the membrane and its time/effectiveness as a tissue barrier (Moses et al., 2008). Donos et al. (2004) in an experimental rat model study concluded that all the calvarial CSDs covered by collagen membranes healed completely at 4 months without any filling materials, although most of them exhibited a concave shape and less vertical gain because of the membrane collapse.

This histomorphometric study was designed to assess bone regeneration potential of β-TCP without and with Fn (β-TCP-Fn) by comparing them with a control in calvarial CSDs from an experimental rat model, when all defects are covered by a native collagen barrier membrane. The primary objective of the study was to assess new bone formation regarding the following histomorphometric variables (Figure 1): (a) augmented area (AA) defined as total regenerated area within the target area composed by mineralized bone matrix, non-mineralized tissue and bone substitute; and (b) gained tissue (GT) defined as mineralized bone matrix plus bone substitute. Secondary objectives included the diameter of the defect, mineralized bone matrix, non-mineralized tissue area and residual bone substitute. To support the effect of the addition of Fn on β-TPC grafting, all variables were calculated in mm^2 and as percentages in the target area. It was hypothesized that β-TCP-Fn would improve bone formation as compared with β-TCP alone in this experimental rat model.

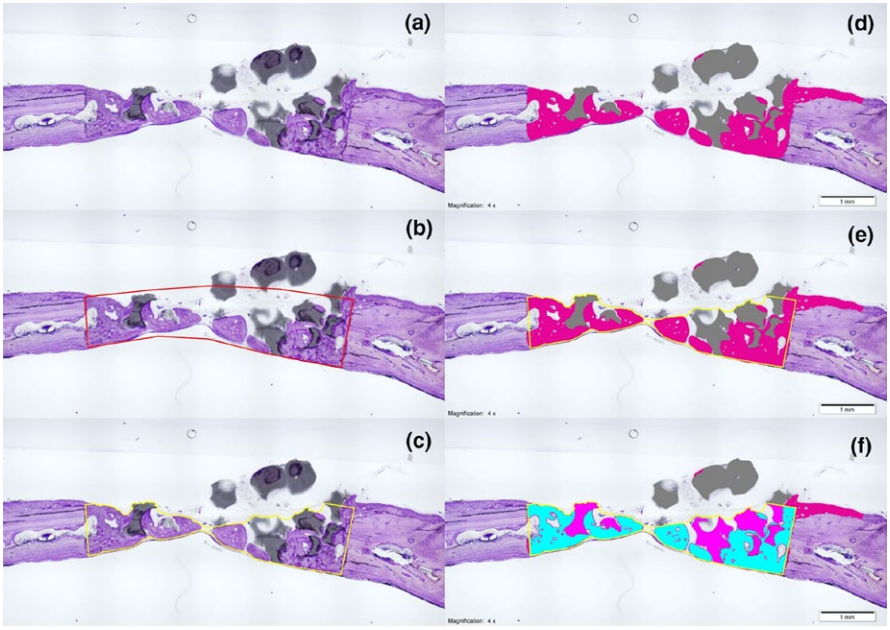

FIGURE 1 Histomorphometric analysis. Definition of the regions of interest (ROI) for the digital tissue differentiation procedure. Original image: (a). Defect area region (DA) was defined as the area occupied by the bone extracted during the surgery. The interface between the new and pristine bone was first detected, outlined and finally linked following the curvature of the skull with straight lines (red polygon). The increase in the cortical thickness caused by periosteal reaction was avoided (b). Augmented area region (AA) was outlined (yellow polygon) following the surface of the DA occupied by mineralized bone matrix (MBM) and bone substitute (BS) (c). Using a digital pen, the proportions occupied by new MBM (rhodamine red) and BS were coloured (d) and the AA overlapped (e). The image analysis software calculated the area of MBM and BS within the AA (f). Levai-Laczkó ×40

2 | MATERIAL AND METHODS

2.1 | Material

Beta-tricalcium phosphate (β-TCP) used was 99% pure (KeraOs®, Keramat, A Coruña, Spain), in size particles between 0.25 and 1 mm, alone or coated with Fn. Twenty-four hours before to surgery, 500 µl of Fn solution (10 µg in Dulbecco's modified Eagle medium [DMEM] 1 g/L) was added per gram of bone graft and incubated for 24 hr at 37°C. The coating solution was then eliminated, and the grafts were washed with Dulbecco's phosphate-buffered saline (DPBS) and stored in multi-dose blisters, in sterile conditions, ready to be refrigerated, transported and used as previously reported (Alvira-González et al., 2016; Sánchez-Garcés et al., 2017).

2.2 | Study design

The study was approved by the Ethics Committee on Animal Research (CEEA 346–12) of the University of Barcelona, Barcelona (Spain), and it is in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and with local laws and regulations.

Thirty Sprague Dawley rats, allocated at random with data collected at 6 and 8 weeks were included in a prospective controlled study. Animals were ex-reproductive adult males (14 weeks' old weighing between 250 and 300 g) chosen to minimize the effect of the spontaneous regeneration occurring in young animals (Vajgel et al., 2014). All were kept in the same stall in standard conditions of 12 hr cycles between day and night, 22 ± 2°C and 50 ± 10% of relative humidity. Each animal was identified with a number in the tail. The calvarial CSDs used in this study had a standard model, economic and adequate, being able to evaluate properly the bone formation in bone skull defects (Vajgel et al., 2014).

2.3 | Surgical protocol

Anaesthesia was induced with 5% isoflurane (Forane®, Abbot Laboratories, Madrid, Spain) 5 L/min in an anaesthetic chamber. Buprenorphine (0.05 mg/kg; Buprex®, RB Pharmaceuticals Ltd., Slough, Berkshire, UK) was injected subcutaneously. A single dose of 5 mg/ml of enrofloxacin (Baytril®, Bayer Hispania, Sant Joan Despí, Barcelona, Spain) as antibiotic prophylaxis was given. Animals were then connected to a breather for anaesthetic maintenance (New Generation Black Mk-TCIII, NSS, England) with a flux of 0.7–0.8 L/min of oxygen and vaporized isoflurane 2%–2.5% at 3½.

The surgical area was shaved and disinfected using 10% topic povidone iodine (Iodina®, Laboratorios Reig Jofré, Sant Joan Despí, Barcelona, Spain) and surgically draped. The incision area was infiltrated with 1 cc of articaine 4% with epinephrine 1:100,000 (Ultracain®, Laboratorios Normon, Tres Cantos, Madrid, Spain) subcutaneously. The cranial cutaneous incision was done in an antero-posterior direction following the sagittal suture, approximately 2 cm long going from the occipital until the frontal bone. The

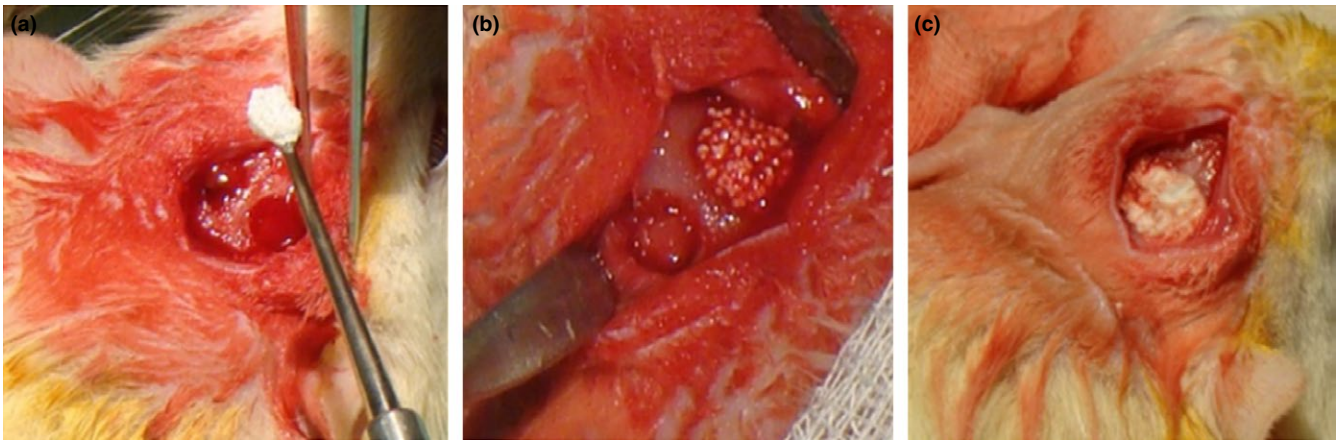

FIGURE 2 Pictures illustrating the clinical procedures. (a) Insertion of the graft material into one of the bilateral bicortical parietal critical-sized calvarial defects (CSDs); (b) test defect grafted and empty control; (c) placement of the collagen membrane covering both CSDs

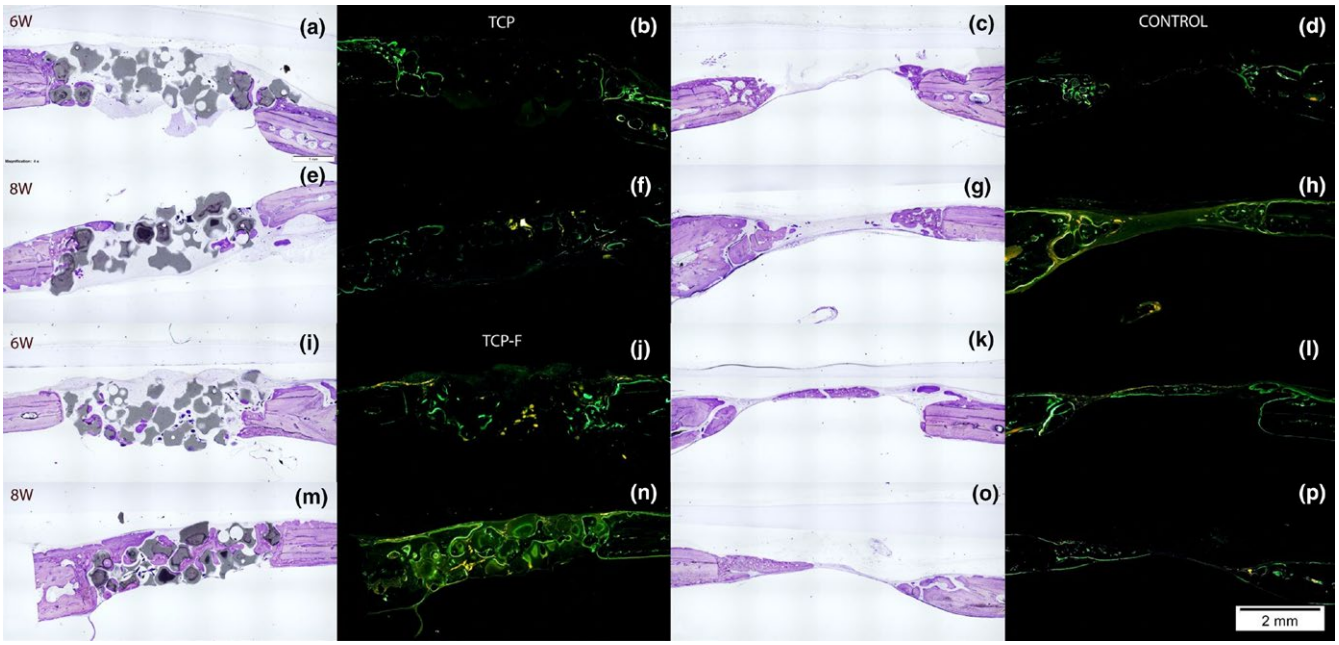

FIGURE 3 Representative images of the defects filled with TCP or TCP-Fn (left side) and control (right side) at 6 and 8 weeks (6 and 8 weeks, respectively). One week before sacrificing the animals, they received a dose of fluorochrome (calcein green) subcutaneously (images b, d, f, h, j, l, n, p). It can be observed differences between the control and test sites concerning the mineralized bone and augmented area within the defects. Levai-Laczkó (images a, c, e, g, i, k, m, o) staining is able to differentiate new and old mineralized bone. New mineralized bone appears in intense violet-blue, and old mineralized bone in the defect borders can be seen with a paler tone. Bone substitute is shown in grey. Magnification ×40

subcutaneous and muscular planes along with the periosteum were dissected to expose the calvaria. Once the parietal bones were exposed, the bone defect was created using a trephine bur of 5 mm of external diameter connected to an electric motor at 22.4 G-force and irrigated by sterile saline to avoid overheating of the margins and to clean the bony layer generated during the ostectomy. Two bicortical critical-sized circular defects were created in both parietals (Gomes & Fernandes, 2011). A total of 30 defects were filled with one of the two study materials (β-TCP or β-TCP-Fn), leaving 30 empty defects as controls in the contralateral side. The graft material was randomly assigned and introduced until the defect was full without compacting to respect the critical distance between the particles to allow the vessels penetration, due to the different thickness of the rat's calvarial defect, no standardized amount of particles was calculated as Donos et al. (2004 protocol. The surgeon was blinded to the material grafted. All defects were covered with a native bovine collagen membrane (Collagen-Klee®, Medical Biomaterials Products GmbH, Neustadt, Glewe, Germany), and the surgical field was closed by primary intent with 3–0 Vicryl sutures (Laboratorios Aragó, Barcelona, Spain; Figure 2). Meloxicam (Metacam®, Boehringer Ingelheim, Sant

Cugat del Vallés, Barcelona, Spain) 2 mg/kg was administered subcutaneously every 24 hr for 2 days.

The animals were kept in a heated mat to prevent post-surgery hypothermia and were controlled visually until they waked up from the anaesthesia; when they were brought back to their room in individual cages, water and food ad libitum were given to them. Two weeks later, the stitches were removed. All of them received a 25 mg/kg dose of tetracyclines one week later. One week before sacrificing the animals, they received a dose of fluorochrome (calcein green) subcutaneously (Figure 3). Animals were sacrificed by CO_2 at 6 and 8 weeks after surgery.

A total of four study groups were obtained according to the time of euthanasia and type of filling: β-TCP-Fn/6 weeks, β-TCP/6 weeks, β-TCP-Fn/8 weeks and β-TCP/8 weeks.

2.4 | Specimen retrieval and histological preparation

The skull portion surrounding the defect was extracted and fixed in a 4% neutral-buffered formalin solution at 4°C for 1 week. The skulls were half divided using a precise saw band blade (Exakt, Norderstedt, Germany). The samples were dehydrated in ascending concentrations of alcohol and posteriorly infiltrated in a glycol methacrylate base resin polymerizable with light (Technovit 7200 VLC, Heraeus-Kulzer, Wehrheim, Germany). The polymerized blocks were processed with Exakt cutting and polishing equipment (Exakt, Norderstedt, Germany) following a standardized method (Donath & Breuner, 1982). The obtained sections were of the central region of the defect and had a parallel orientation to the sagittal suture. Blocks were cut at 200 μm thickness and then reduced by polishing to approximately 40 μm. Slides were stained following the method of Laczko and Levai (1975).

2.5 | Histomorphometric analysis

Histomorphometric studies were performed by an experienced investigator (F.M-G) who was blind to the experiment. To capture the images, an optical microscope was used (BX51, Olympus, Tokyo, Japan) connected to a colour digital camera (DP71, Olympus, Japan), assembled to a motorized stage (Märzhäuser, Steindorf, Germany). The images obtained were automatically aligned and fused, obtaining complete images of the defect with a magnification of ×100.

Proportions occupied by bone, biomaterials and soft tissue present in the defects were identified from the digital histological images using a pen computer (Cintiq companion, Wacom, Germany), coloured (Photoshop, Adobe, USA) and digitally measured using two automated image analysis systems (CellSens, Olympus Corporation, Japan; Image-Pro Premier, Media Cybernetics, USA). For each central section, the following variables were assessed following guidelines previously published (Benic et al., 2016) (Figure 1):

- Defect area diameter or former defect: area occupied by bone that was extracted during the surgery (descriptive variable).
- Augmented area (AA) within the former bone defect (primary outcome variable).
- Area of mineralized bone matrix (MBM; primary outcome variable).
- Non-mineralized tissue (NMT) and residual bone substitute (BS; mm^2 and percentage) within AA (secondary outcome variables).

New bone formation expressed as percentage to homogenize all the measures and mm^2 of the target area was calculated as AA and GT. The term "mineralized bone matrix" was adopted according to the recommendations of the American Society for Bone and Mineral Research (ASBMR) to standardized bone histomorphometric nomenclature (Dempster et al., 2013). The present reporting of in vivo experiments followed the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2014).

2.6 | Statistical analysis

In order to compare both active treatments, the sample size was established at 15 animals per group in order to obtain 80% of statistical power to detect, by means of a bilateral *t* test for independent groups and fixing the significance level to 5%, an effect size of 1.1. To ensure study internal validity, each animal was also measured under a control condition. Quantitative variables are expressed as mean and standard deviation (*SD*) and categorical variables as frequencies and percentages. To compare treatments and analyse the effect of time and their interaction, a mixed model was used considering the animal as a random factor. For each follow-up time, pairwise comparisons between groups were performed using Tukey's correction for multiplicity of contrasts. All models have been validated qualitatively exploring graphically the distribution of the residuals. The R software v3.1.2 (Development Core Team, 2008) was used for statistical analysis. Statistical significance was set at $p < 0.05$.

3 | RESULTS

Of the 30 rats included in the study, one animal died after 48 hr of surgery due to postoperative complications. Therefore, 29 animals were studied. However, one sample recovered at 8 weeks from the group of β-TCP-Fn had an extremely irregular shape and was discarded. Histomorphometric analysis was performed in 58 samples, 28 collected at 6 weeks and 30 at 8 weeks. There were 13 (22.4%) samples in the β-TCP-Fn group, 16 (27.6%) in the β-TCP group and 29 (50%) in the untreated controls.

The mean (*SD*) diameter of the bone defect was 4.95 (0.41) mm and comparable regarding treatment groups ($p = 0.147$) and weeks ($p = 0.786$). The mean target area was 4.79 (1.24) mm^2, with significant differences ($p = 0.0004$) among the study groups, in particular between β-TCP-Fn and controls (5.66 [1.48] vs. 4.23 [0.92] mm^2; $p = 0.0007$) and between β-TCP and controls (5.11 [1.13] vs. 4.23 [0.92] mm^2; $p = 0.029$; Table 1).

TABLE 1 Results of histomorphometric variables in the three study groups Data expressed as mean (*SD*); weeks: weeks; NA: not applicable

	Study groups				Between-group comparison			Within-group comparison		
Histomorphometric variables	β-TCP-Fn	β-TCP	Controls	*p* value overall	*p* value β-TCP-Fn versus β-TCP	*p* value β-TCP-Fn versus controls	*p* value β-TCP versus controls	*p* value β-TCP-Fn 6 versus 8 weeks	*p* value β-TCP 6 versus 8 weeks	*p* value controls 6 versus 8 weeks
Defect diameter, mm										
Overall	5.10 (0.09)	5.03 (0.19)	4.84 (0.54)	0.1470	0.8918	0.1847	0.3315			
6 weeks	5.10 (0.09)	5.07 (0.09)	4.89 (0.37)	0.4543	0.9889	0.5124	0.6121			
8 weeks	5.11 (0.09)	4.99 (0.25)	4.80 (0.67)	0.2955	0.8736	0.3366	0.5187	0.9786	0.7164	0.5763
Target area, mm^2										
Overall	5.66 (1.48)	5.11 (1.13)	4.23 (0.92)	0.0004	0.2714	0.0007	0.0291			
6 weeks	5.30 (1.07)	5.41 (1.21)	4.45 (0.93)	0.0657	0.9969	0.1609	0.1362			
8 weeks	6.16 (1.94)	4.87 (1.07)	4.02 (0.89)	0.0013	0.0803	0.0012	0.1616	0.1990	0.3178	0.3042
Augmented area within target area, mm^2										
Overall	5.03 (2.04)	4.71 (1.26)	2.46 (1.02)	<0.0001	0.6834	<0.0001	<0.0001			
6 weeks	4.37 (1.87)	4.97 (1.33)	2.32 (0.85)	0.0003	0.6939	0.0062	0.0006			
8 weeks	5.94 (2.08)	4.51 (1.24)	2.60 (1.18)	<0.0001	0.1473	0.0001	0.0053	0.0544	0.5062	0.5722
Augmented area, %										
Overall	87.0 (20.1)	92.5 (14.0)	58.6 (20.4)	<0.0001	0.8424	0.0001	<0.0001			
6 weeks	80.8 (25.0)	91.2 (8.7)	52.0 (14.0)	<0.0001	0.5512	0.0036	0.0001			
8 weeks	95.6 (4.7)	93.5 (17.6)	64.7 (23.8)	0.0003	0.9702	0.0052	0.0014	0.1684	0.7806	0.0750
Mineralized bone matrix, mm^2										
Overall	1.60 (1.23)	1.00 (0.70)	1.25 (0.73)	0.1071	0.0908	0.2442	0.5558			
6 weeks	1.14 (1.14)	0.95 (0.85)	1.22 (0.76)	0.7113	0.8391	0.9902	0.6896			
8 weeks	2.23 (1.16)	1.04 (0.62)	1.27 (0.72)	0.0496	0.0486	0.0720	0.7975	0.0414	0.7335	0.8588
Mineralized bone matrix, %										
Overall	27.0 (18.1)	19.8 (13.7)	30.3 (15.6)	0.0847	0.3080	0.9362	0.0712			
6 weeks	19.6 (14.2)	16.2 (13.9)	28.1 (14.3)	0.1658	0.8741	0.4523	0.1848			
8 weeks	37.4 (19.1)	22.7 (13.5)	32.3 (17.0)	0.2272	0.2630	0.8105	0.3289	0.0669	0.3351	0.4677
Bone substitute mm^2										
Overall	1.65 (0.92)	1.59 (0.61)	NA	0.7860	0.7860	NA	NA			
6 weeks	1.39 (0.67)	1.92 (0.48)	NA	0.1817	0.1817	NA	NA			
8 weeks	2.02 (1.18)	1.34 (0.59)	NA	0.1024	0.1024	NA	NA	0.1458	0.1245	NA
Bone substitute, %										
Overall	29.2 (9.8)	31.0 (10.2)	NA	0.6649	0.6649	NA	NA			

(Continues)

TABLE 1 (Continued)

Histomorphometric variables	Study groups			p value overall	Between-group comparison			Within-group comparison			
	β-TCP-Fn	β-TCP	Controls		p value β-TCP-Fn versus β-TCP	p value β-TCP-Fn versus controls	p value β-TCP versus controls	p value β-TCP-Fn 6 versus 8 weeks	p value β-TCP 6 versus 8 weeks	p value controls 6 versus 8 weeks
6 weeks	27.7 (10.0)	33.3 (8.2)	NA	0.3074	0.3074	NA	NA			
8 weeks	31.5 (10.1)	29.2 (11.7)	NA	0.6986	0.6986	NA	NA	0.5283	0.4337	NA
Gained tissue, mm^2										
Overall	3.25 (1.75)	2.59 (0.85)	1.25 (0.73)	<0.0001	0.1202	<0.0001	0.0001			
6 weeks	2.53 (1.69)	2.87 (0.80)	1.22 (0.76)	0.0009	0.7992	0.0160	0.0020			
8 weeks	4.25 (1.39)	2.38 (0.88)	1.27 (0.72)	<0.0001	0.0037	<0.0001	0.0268	0.0044	0.3365	0.8801
Gained tissue, %										
Overall	56.3 (19.6)	50.9 (15.1)	30.3 (15.6)	<0.0001	0.4631	<0.0001	0.0004			
6 weeks	47.2 (20.2)	49.5 (12.5)	28.1 (14.3)	0.0058	0.9617	0.0325	0.0149			
8 weeks	68.9 (9.9)	52.0 (17.5)	32.3 (17.0)	<0.0001	0.1471	0.0001	0.0140	0.0243	0.7619	0.4804

Note. *p*-values from the correspondent mixed model considering week, treatment and their interaction as fixed effects and animal as a random factor. Tukey's correction for multiple testing was applied.

3.1 | Augmented area

Considering the area composed by mineralized bone matrix and non-mineralized tissue and/or bone substitute, expressed as a percentage of the target area, significant difference between the three study groups was found, with significantly higher mean values for the two treatment groups (β-TCP-Fn and β-TCP) as compared with controls (87.0% [20.1] and 92.5% [14.5], respectively, vs. 58.6% [20.4]; $p < 0.001$; Table 1). Also, at 6 and 8 weeks, the augmented area was also significantly higher in the two grafted treatment groups than in the controls, particularly in the β-TCP-Fn group (Table 1, Figure 4). However, within-group differences for the comparison of the mean augmented area between 6 and 8 weeks were not observed in any group.

3.2 | Mineralized bone matrix

In relation to the effect of the graft materials on MBM, significant differences between the treatment groups as compared with controls were not found when MBM was assessed as a percentage of the target area (Table 1, Figure 5). However, in the β-TCP-Fn group, there was a significant trend for an increase in MBM area from 6 to 8 weeks (1.14 [1.14] mm^2 and 2.23 [1.16] mm^2; $p = 0.04$). Also, the increase in MBM area from 6 to 8 weeks was of a higher magnitude in the active treatment group than in controls without achieving statistical significance (β-TCP-Fn from 19.6% to 37.4%; β-TCP from 16.2% to 22.7%; controls from 28.1% to 32.3%; Table 1). When considering absolute MBM expressed in mm^2, there was a significant trend for an increase in MBM area in the β-TCP-Fn group from 6 to 8 weeks (1.14 [1.14] mm^2 and 2.23 [1.16] mm^2; $p = 0.041$).

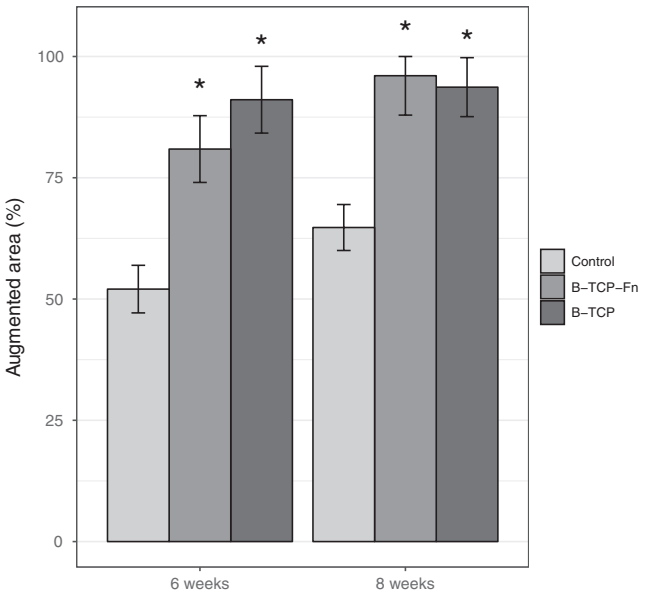

FIGURE 4 Percentage of augmented area (AA) within the target area by treatment groups and study periods (data expressed as mean and standard deviation; *statistically significant differences for the comparison of β-TCP-Fn and β-TCP vs. controls)

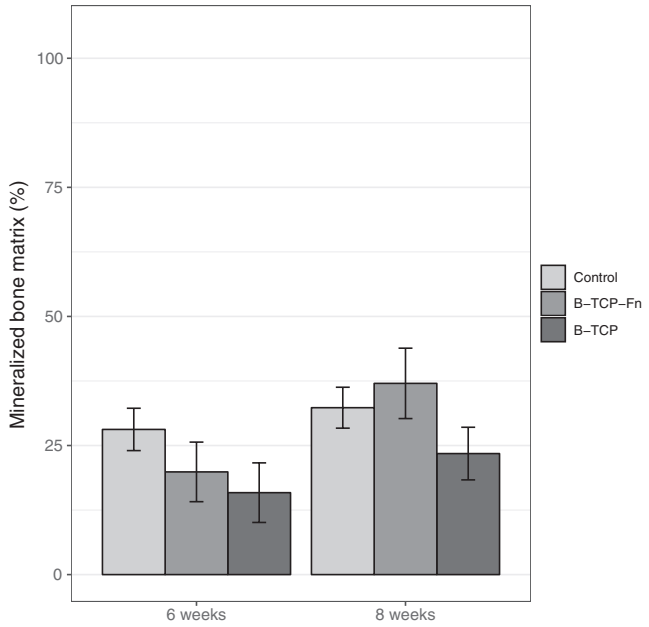

FIGURE 5 Percentage of mineralized bone matrix (MBM) within the target area by treatment groups and study periods. Both β-TCP-Fn and β-TCP groups showed higher values than in controls but differences were not statistically significant (data expressed as mean and standard deviation)

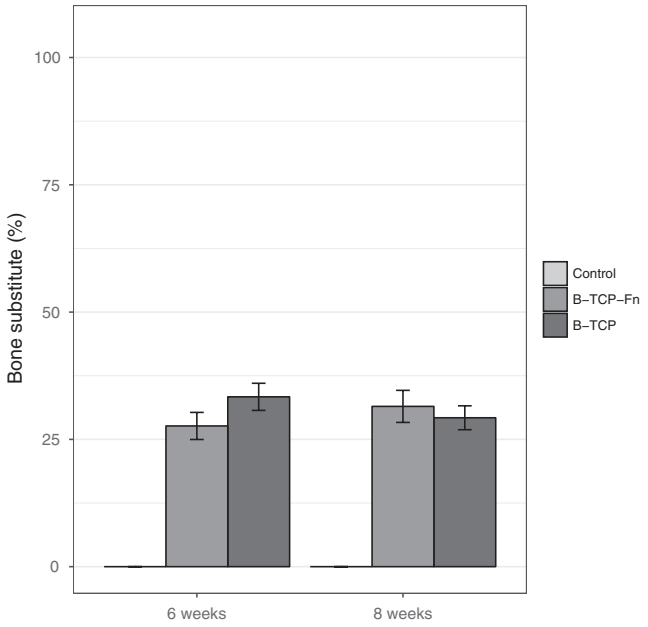

FIGURE 6 Percentage of bone substitute (BS) within the target area by treatment groups and study periods (data expressed as mean and standard deviation)

3.3 | Bone substitute

Bone substitute was only evaluated in the two active treatment groups. Although there were no statistically significant differences between the grafted defects with β-TCP-Fn or β-TCP, the percentage of bone substitute within the target area at 8 weeks was somewhat greater in the β-TCP-Fn (31.5% [10.1]) than in the β-TCP group (29.2% [11.7]; Table 1, Figure 6).

3.4 | Gained tissue

Gained tissue is a total volume of mineralized bone matrix (mining hard tissue as ASBMR suggest) and residual bone substitute, measured in mm^2 and as a percentage of the target area. GT within the target area was significantly higher in the active treatment groups than in the controls (β-TCP-Fn vs. control; $p < 0.001$ and β-TCP vs. control; $p < 0.001$; Table 1). Differences between the use of β-TCP-Fn or β-TCP were not observed except when compared β-TCP-Fn versus β-TCP groups at 8 weeks and measured in mm^2 ($p = 0.003$). Between-group comparisons with controls, at all study periods, were statistical significant in favour of each active comparator groups (β-TCP-Fn and β-TCP). In addition, CSDs treated with of β-TCP-Fn showed significant increases in gained tissue between 6 and 8 weeks ($p = 0.02$; Table 1, Figure 7). Expressing GT in absolute area (mm^2), the same statistical differences were obtained when comparing each group to controls. Finally, differences between the use of β-TCP-Fn or β-TCP were observed at 8 weeks (4.25 [1.39] mm^2 and 2.38 [0.88] mm^2, respectively; $p = 0.004$).

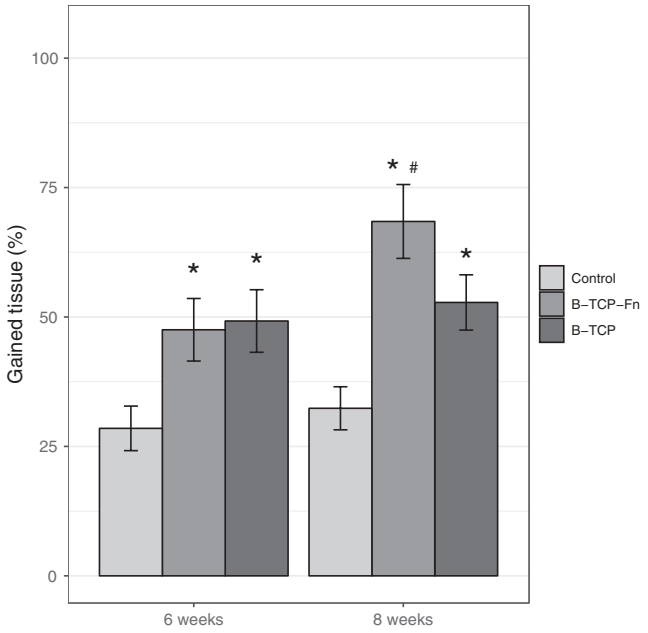

FIGURE 7 Percentage of gained tissue (GT) within the target area by treatment groups and study periods (data expressed as mean and standard deviation; *statistically significant differences for the comparison of β-TCP-Fn and β-TCP vs. controls; #statistically significant difference for within-group comparison between 6 and 8 weeks)

4 | DISCUSSION

In relation to the primary objective of the study, the addition of fibronectin to β-TCP grafted material was effective to improve bone regeneration in calvarial CSDs in a rat model as compared to

negative controls. However, significant differences as compared to β-TCP alone were not observed; the percentage of augmented area within the CSD and the amount of bone regenerated were also similarly reflected. Also, in the group of the β-TCP-Fn, the amount of gained tissue in the target area, defined as the sum of MBM and bone substitute areas, increased its percentage significantly over time ($p = 0.024$), whereas in the β-TCP group seemed to stabilize at 6 weeks with no further significant increases ($p = 0.762$).

In relation to the secondary objectives of the study, a significant difference in favour of β-TPC-Fn compared to β-TPC alone ($p = 0.04$) was found in MBM in mm^2 at 8 weeks. Changes in bone regeneration according to the materials used and the times of analysis also suggest a more favourable effect of β-TCP-Fn as the percentage of MBM in the target area further increased from week 6 to week 8 ($p = 0.067$).

In a previous experimental study using a dog model study, in which the effect of β-TCP, β-TCP-Fn and β-TCP-Fn-ADSCs on alveolar bone dehiscences was compared, only the defects treated with β-TCP-Fn-ADSCs showed a significant increase in the bone regeneration area when the animals were sacrificed at 3 months versus at 1 month ($p = 0.006$) (Alvira-González et al., 2016).

Autologous bone remains the gold standard until a new material with the same osteoinduction, osteoconduction, biocompatibility and safety properties will be found (Guskuma et al., 2010). However, the stability of volume grafted with autologous bone might be compromised by a high rate of reabsorption (Damron et al., 2013). In a comparison of allografts and β-TCP in 95 patients with solitary bone cysts, the application of β-TCP showed an advantageous alternative for lacunar bone defect repair (Wang et al., 2013). In our experimental study, the combination of β-TCP with Fn generally appeared more advantageous than β-TCP alone but with very few significant differences (MBM in mm^2 at 8 weeks).

Microporosity, crystallinity and size of the β-TCP particle seem crucial to provide an optimal structure for vascular growth and bone formation. Microporosity (pore size) <10 μm increases macromolecular adhesion and favours fluid penetration, although a highly porous β-TCP material (>100 μm) also supported new bone formation, creating a bridge between borders and facilitating bone ingrowth in critical size defects in rabbits' tibiae (Calvo-Guirado et al., 2012). On the other hand, reducing the size of β-TCP granules to nanometres may also contribute to induce higher porosity and larger specific surfaces, leading to an improved regenerative effect (Lee, Lim, et al., 2016). In bony defects of a pig model in which three β-TCP graft materials were compared, it was concluded that the optimal grain size of the particle should be between 160 and 300 μm or even smaller (Damlar et al., 2015). In our study, the size of KeraOs® was smaller (100–250 μm), so future studies should be focused on assessing the performance of β-TCP materials with even smaller particle sizes. In addition, different materials such as calcium phosphate glass and high-density polyethylene have been used in β-TCP composites to enhance proliferation and adhesion of osteoblasts and fibroblasts cells (Cai et al., 2009; Homaeigohar et al., 2008, 2005). Porous beta-calcium silicate ceramics when compared with β-TCP ceramics implanted in rabbits' calvarial defects resulted in more newly formed bone with beta-calcium silicate than with β-TCP (Xu et al., 2008). However, no other previous studies comparing beta-calcium silicate ceramics with β-TCP-Fn have been performed.

Fn has been used to stimulate mineralization and cell adhesion in tricalcium phosphate scaffolds, resulting in early differentiation of osteoblasts (Ball et al., 2009). In a novel multilayered chitosan–hydroxyapatite composite, the addition of Fn (25 or 50 μg/ml) improved osteoblast cell adhesion and proliferation (Fernández et al., 2012), demonstrating the potential of Fn to improve the ability of composites as bone grafting materials. In our study, the concentration of 1 g of Fn is fixed in strict conditions to a β-TCP scaffold resulting in 10 μg/ml. Comparisons regarding concentrations cannot be calculated because the data in the study of Fernández et al. (2012) are expressed in mm^3 for the scaffold and in 10 μl/ml for the Fn. However, all data suggest that Fn used in vitro (F20—Fn-derived oligopeptide) induces osteoblast differentiation as it is mediated by BMP-2, and, therefore, it can be used as a therapeutic biomolecule to facilitate even periodontal regeneration (Cho et al., 2017).

Other attempts to improve β-TCP scaffolds have been carried out. Simvastatin stimulates BMP-2 expression in osteoblasts and has been proved in rat calvarial defects, combined with three different calcium phosphate biomaterials: α-TCP, β-TCP and hydroxyapatite to enhance bone regeneration. The results showed that simvastatin also affected the α-TCP and β-TCP degradation, and especially when combined to α-TCP, which showed a higher degradation rate allowing more bone formation (Rojbani et al., 2011). In another study of a combination of simvastatin and α-TCP, 0.1 mg was the optimal dose for stimulation of the maximum bone regeneration in rat calvarial defects (Nyan et al., 2009). In rat skull defects, the combination of α-TCP and 0.2 mg epigallocatechin-3-gallate (green tea catechin) stimulates maximum bone regeneration (Rodriguez et al., 2011). These combinations would be potentially effective as bone graft materials.

Some other biomaterials that have been associated to β-TCP and have been carefully examined are growth factors (Cochran et al., 2016; Li et al., 2011) and dental pulp stem cells (Annibali et al., 2014, 2013), in both cases with inconsistent results. For example, bone morphogenetic protein 2 (BMP-2) did not substantially change the osteoconductive properties of the biomaterials grafted when it was compared to TCP alone (Luvizuto et al., 2011). On the other hand, the utilization of a demineralized bone matrix (DBM) putty appeared to allow complete closure of critical-sized calvarial defects in New Zealand white rabbits displaying viable new bone at 12 weeks (Clokie et al., 2002), and as a consequence, it has been suggested to be used to enhance the protection of intracranial contents following craniofacial surgical procedures (Haddad et al., 2006).

In the present study, statistically significant differences were found between each grafting group and the control group in which the defect was left empty. In the controls, calvarial defects gained less volume than those in the remaining groups because grafts helped to maintain the original bonny space. The most relevant finding was the significantly higher bone growth at 8 weeks in defects

treated with β-TCP-Fn. Accordingly, it may be assumed that Fn had a delayed boosting growth effect in the β-TCP graft (between 6 and 8 weeks). However, further research with prolonged observation times (e.g. 12 weeks) is needed to better understand the contribution of Fn in the bone repair process.

Undoubtedly, the use of a native bovine collagen membrane to cover the calvarial CSDs has been beneficial for the bone regeneration process, protecting the bonny area of being colonized by soft tissue at an early stage and therefore giving enough time to the bone cells to refill the hard tissue defect (Donos et al., 2015). Damlar et al. (2015) compared the effectiveness of different β-TCP biomaterials and an autologous bone graft in pigs. The negative controls remained empty, and all defects were covered with a resorbable collagen membrane; they found statistically differences in bone formation between filled defects and the negative controls ($p < 0.05$) as in our present study. The barrier membrane in our study did not influence the ability to be an effective negative control, allowing to obtain statistically significant differences between the testing materials and the control groups at different times. In the meticulous study by Donos et al. (2004 using the same experimental model, they concluded that the use of guided bone regeneration has the same efficacy as the use of regeneration materials, although the times of euthanasia of the animals were double (16 weeks). It could be thought that CSDs of 5 mm in diameter are insufficient for their study time; however, it can be considered adequate in the rat model (Vajgel et al., 2014); nonetheless, the authors concluded that the control's healing occurred with a significant regeneration deficit in height.

Findings of the study should be interpreted taking into account some limitations. A potential source of bias when obtaining the microscope samples could be related to the differences between individuals done by the size and thickness of the calvaria or the imprecise cut axis. This drawback was corrected by expression of results of histomorphometric variables as percentages of the target area. The number of animals was calculated to obtain the minimal sample size necessary and animals could not be replaced, refined or reduced, for this reason, the measurements of the variables are more reliable when expressing the results in percentages.

Regarding the bilateral 5 mm diameter for the CSDs, it seems sufficient to obtain relevant data (Vajgel et al., 2014). Although the design used allows to reduce the risk of bias by having the tested material and the control in the same animal, it can be a risk in terms of contamination of the control defect (Vajgel et al., 2014). In our case, a contaminated sample was found in the group β-TPC at 6 weeks (four particles), two samples from the group β-TPC-Fn at 6 weeks (one and five particles) and a sample with completed healing in the group β-TPC-Fn at 8 weeks (one particle).

Although no species fulfils the requirements of an ideal animal model, rodents are one of the best choices because they are easily available, easy to house and to handle. Also, a large number of studies on regeneration of bone defects published in the literature have been carried out in rat models. In addition, the rat calvarial bone model allows establishing a standardized and reproducible defect.

5 | CONCLUSION

The use of β-TCP coated with Fn (β-TCP-Fn) showed a non-significant slight more effective effect than β-TCP without Fn in improving the volume of regenerated bone of critical-sized calvarial defects in a rat model, possibly allowing a more efficient bone remodelling process in the rats' CSDs. Both β-TCP biomaterials with and without Fn supposed an advantage when compared with bone defects left empty in terms of bone volume maintenance, avoiding the invasion of soft tissue into the hard tissue space and accelerating the new bone formation process. No clear differences were found between β-TCP and β-TCP-fn. Further studies extending the follow-up period longer than 8 weeks or shorter than 6 weeks are needed to assess the osteogenic ability of β-TCP-Fn in the reconstruction of critical-sized calvarial rat defects or in bony defects of other animal models.

ACKNOWLEDGEMENTS

The authors are indebted to Marta Pulido, MD, for editing the manuscript and editorial assistance and to Joan Pérez-Guallar for his valuable assistance in the statistical data analysis.

CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest.

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How to cite this article: Escoda-Francolí J, Sánchez-Garcés MÁ, Gimeno-Sandig Á, et al. Guided bone regeneration using beta-tricalcium phosphate with and without fibronectin—An experimental study in rats. *Clin Oral Impl Res*. 2018;00:1–12. https://doi.org/10.1111/clr.13370