BONE REGENERATION USING β-TRICALCIUM PHOSPHATE IN A CALCIUM SULFATE MATRIX

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The aim of the study was the histomorphometric comparison of the osteogenic potential of β-tricalcium phosphate (β-TCP) alone or in a calcium sulfate matrix. Three round defects, 10 mm (diameter) × 5 mm (depth), were created on each iliac crest of 4 dogs. The defects were divided into 3 groups. Ten defects were filled with β-TCP in a calcium sulfate (CS) matrix (Fortoss Vital; group A), 10 defects were filled with β-TCP alone (Fortoss Resorb; group B), and 4 defects were left ungrafted to heal spontaneously (group C). All defects were left to heal for 4 months without the use of a barrier membrane. Histologic evaluation and morphometric analysis of undecalcified slides was performed using the areas of regenerated bone and graft remnants. All sites exhibited uneventful healing. In group A sites (β-TCP/CS), complete bone formation was observed in all specimens, graft granules dominated the area, and a thin bridge of cortical bone was covering the defect. Group B (β-TCP) defects were partially filled with new bone, the graft particles still dominated the area, while the outer cortex was not restored. In the ungrafted sites (group C), incomplete new bone formation was observed. The outer dense cortical layer was restored in a lower level, near the base of the defect. The statistical analysis revealed that the mean percentage of new bone regeneration in group A was higher than in group B (49.38% and 40.31%, respectively). A statistically significant difference existed between the 2 groups. The beta-TCP/CS group exhibited significantly higher new bone regeneration according to a marginal probability value ($P = .004 < .05$). The use of β-TCP in a CS matrix produced significantly more vital new bone fill and preserved bone dimensions compared with the use of β-TCP alone.

Key Words: bone regeneration, β-tricalcium phosphate, calcium sulphate

INTRODUCTION

A prerequisite for achieving a successful outcome using dental implants is the adequate bone volume and quality at the recipient site.[1] However, this is not usually the case due to postextraction trauma, bone resorption, or periodontal defects. Guided bone regeneration (GBR) is a well-established method to exclude soft-tissue cells by means of barrier membranes.[2–4] One of the alternatives to overcome membrane collapse is the use of a graft material to support the membrane by filling the space beneath, which may also act as a scaffold of bone ingrowth.[5–9] Nowadays, a large number of filling materials are available, among which autogenous bone is still considered to be the gold standard. However, harvesting autogenous bone has its disadvantages: secondary donor site surgery, extended operating time, risk of complications, as well as limited amount of graft material.[10,11] Furthermore, one of the main advantages of using autogenous bone, that is, its osteogenic and osteoinductive potential, has been questioned lately, since studies have shown that it undergoes necrosis.[12–14] As an alternative, bone graft substitutes such as xenografts, allografts, or alloplastic materials have been proposed. Among the most

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promising is the tricalcium phosphate (TCP), an alloplastic ceramic material studied and used extensively in the past decades.[15–19] It is considered to be bioactive (by means of inducing specific biologic reactions) and biocompatible (not stimulating inflammatory or foreign-body giant cell activity).[16,20,21] This is mainly because TCP is composed of Ca and P ions, which are the most commonly found elements in bone. However, TCP cements have a slower resorption rate than bone and are usually too dense to allow bone tissue to grow into the defect in a limited period of time.[22–24] By adding a faster resorbing material, pores may be created, ensuring new bone tissue growing into the defect.

Calcium sulfate (CS) has been used as a bone filler for many decades[25,26] and is considered to be highly biocompatible and bioresorbable.[27,28] However, CS alone is not an effective material as bone filler since its resorption rate is considerably faster than bone growth, resulting in an absence of the appropriate scaffold within the defect. This means that CS has time-limited osteoconductive properties, as documented by many studies.[29,30] By mixing CS with other bone graft materials, the osteogenesis is accelerated, by accomplishing increased calcification and quantity of new bone in a shorter period of time.[31,32]

The aim of the present study is the histological evaluation of the osteogenic potential of β-TCP (β-TCP) alone or in combination with a CS matrix without using a membrane, in bony defects of a canine model.

Materials and Methods

Graft material

Two types of bone substitutes were tested.

Fortoss Resorb (Biocomposites Ltd, Keele, Staffordshire, England) is a porous β-TCP synthetic graft in a granular form with a particle size of 250 to 500 μm.

Fortoss Vital (Biocomposites Ltd) is a synthetic composite biomaterial based on a porous β-TCP in a matrix of calcium sulfate.

Animal model

The protocol of the study was approved by the standing committee on Animal Research at Veterinary Headquarters of Karditsa Prefecture, Thessalia, Greece.

Four adult Beagle dogs were used. The dogs were housed in Surgery Clinic (Faculty of Veterinary Medicine, University of Thessaly, Karditsa, Greece) and maintained according to E.U.–Guide for the Care and Use of Laboratory Animals.

Surgical procedure and experimental design

The dogs were not fed for 12 hours before general anesthesia to prevent aspiration of stomach contents.

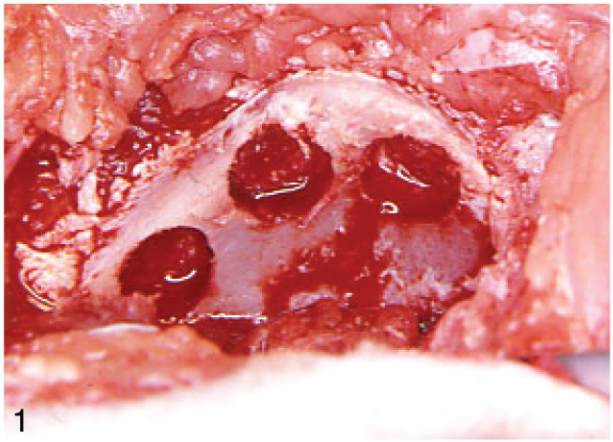
All operating procedures were performed under general anesthesia and sterile conditions in an animal operating theatre. The dogs were premedicated with 0.7 mg/kg xylazine (Rompun; Bayer, Leverkusen, Germany) intramuscularly. Anesthesia was induced with 5 mg/kg sodium thiopentone (Pentothal; Abbott Laboratories, Chicago, Ill) intravenously and maintained with a mixture of isoflurane (Forenium; Abbott Laboratories) and oxygen in a semiclosed breathing circuit.

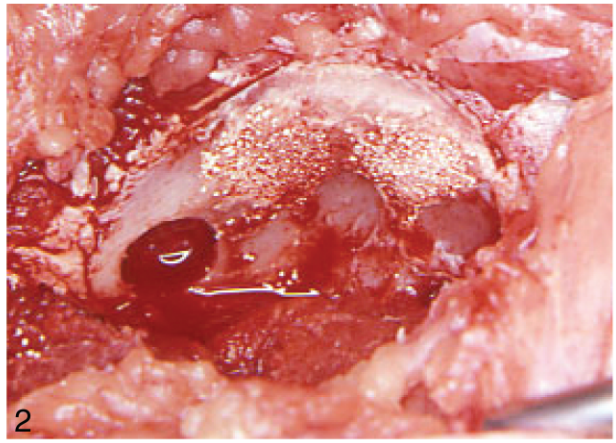
Artificial bony defects were created between the cranial and caudal dorsal iliac spine of the iliac wing of the animals by the aid of trephine burs. In each ilium, 3 defects of 10-mm diameter and 5-mm depth were prepared (Figure 1). In this way, a total of 24 experimental defects were made that were divided into 3 groups: in group A, 10 defects were filled with Fortoss Vital; in group B, 10 defects were filled with Fortoss Resorb; and a control group of 4 defects (1 in each dog) were left unfilled for spontaneous healing (Figure 2). All surgical sites were covered by the periosteum, muscles, fat tissues, and skin without using any barrier membrane and sutured (Vicryl, Ethicon GmbH, Norderstedt, Germany).

The animals were followed postoperatively; 12 mg/kg of amoxicillin and clavulanic acid (Synulox; Pfizer, New York) were administered for 5 days, and the wound was left to heal for 4 months. At the end of this period, the whole iliac crest was removed intact by the aid of burs, scalpels, and chisels without killing the animals and processed for histological analysis. Location of the defects' site during retrieval surgery was proven to be uneventful because of their large diameter.

Histological processing

The bone samples, after their removal, were cleaned from the soft tissues, rinsed with saline, and placed in a fixative consisting of 10% neutral buffered formalin. The specimens were dehydrated in increasing grades of ethanol, ending in absolute 100% alcohol, infiltrated in resin (Technovit 7200, Heraeus Kulzer GmbH, Wehrheim, Germany) and polymerized for 12 hours under blue light. Using a high-speed rotating diamond blade microtome (Accutom II, Struers, Copenhagen, Denmark), 200- to 250-μm-thick sections were obtained, which were further reduced by a grinding unit (DAP-V, Struers) to a final thickness of about 60 to 80 μm. Sections were stained with a solution of toluidine





Figures 1–2. Figure 1. Three experimental defects, 10 × 5 mm, were created in each iliac crest. Figure 2. One defect in each iliac crest was left unfilled for spontaneous healing.

blue and pyronin G. The histological sections were evaluated using a transmission light microscope (Axiostar Plus, Zeiss, Göttingen, Germany) with an integrated color video camera (DC88AP, Sony, Tokyo, Japan) and a frame grabber. The ActioVisio (Zeiss, Göttingen, Germany) image analysis software was used to digitize the selected images for the histometric analysis. Bone graft area (BGA) and the total volume of the regenerated bone (BV) were measured and expressed as a percentage of the total defect area. The statistical comparison of the measurements between the groups was made using the Student *t* test. The level of significance was set at $P \leq .05$.

Results

Histological evaluation

In the β-TCP/CS combination group, complete regeneration of the defects was observed in all specimens. A thin bridge of cortical bone was covering the defect, resembling the outer cortex. Graft granules dominated the sites and were always restricted within the defect limits. In most of the cases, they were located within the lacunae of the new cancellous bone under the cortex or in some regions were impacted in the cortical bridge (Figure 3). The graft particles were partially or completely embedded in new lamellar bone with osteons in various developmental phases (Figure 4). In higher magnification (×100), close contact of the material with new bone may be detected as well as the resorption activity (Figure 5).

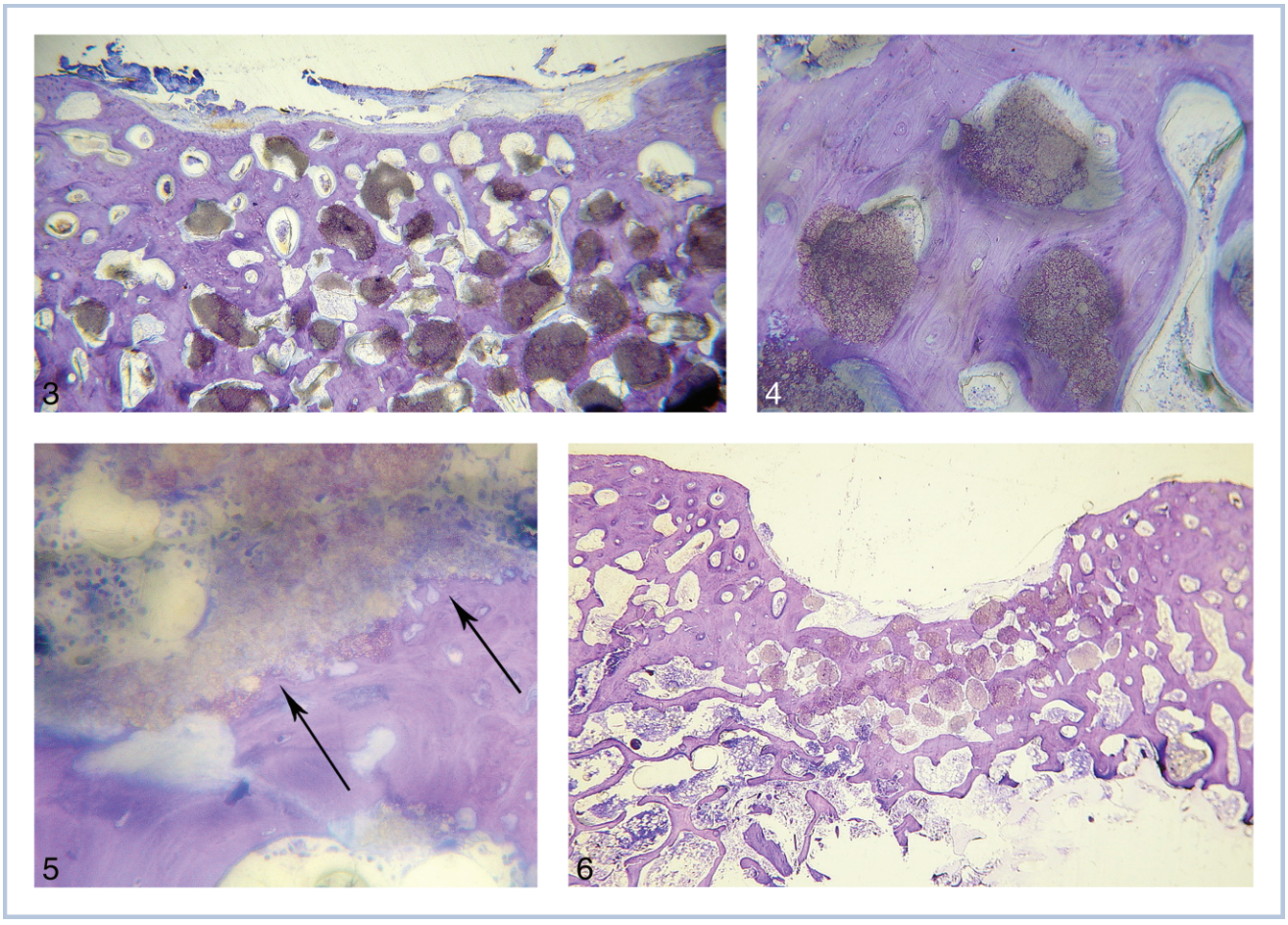
The defects of the β-TCP group were dominated by the graft granules; however, they were also partially filled with new bone, while the outer cortex was not restored. Concavities of various sizes with epithelial tissue ingrowth can be seen in the center of the artificial defect (Figure 6). Some particles were based at the superficial bony layer and protruded toward the soft tissues (Figure 7). In some cases, the outer bony layer had a tendency to bridge the defect, but it was still interrupted by soft connective tissue invasion (Figure 8). Fortoss Resorb granule remnants were found within the soft tissues adjacent to the defect. Specimens that showed greater regeneration capability were also found, without the presence of the dense cortex that was characteristic of the β-TCP/CS group, however. However, numerous osteons were present in this area, indicating the high remodeling activity of the new bone. Despite the quantitatively inferior bone augmentation, the level of maturation and arrangement of the new bone had the same characteristics as the samples of the previous group.

In both grafted groups, bone substitute showed closed contact with the young lamellar bone, with no signs of inflammatory or rejection reactions.

The histological evaluation of the ungrafted sites revealed incomplete new bone formation presenting a characteristic concavity (Figure 9). The outer dense cortical layer was restored but in a lower level near the native bony base and walls of the defect.

Morphometric results

Histomorphometric analysis of the bone grown in the β-TCP/CS group showed a mean value of 49.38% (SD, 6.73; SE, 2.13), whereas in the β-TCP group, it measured 40.31% (SD, 5.42; SE, 1.71). Statistical



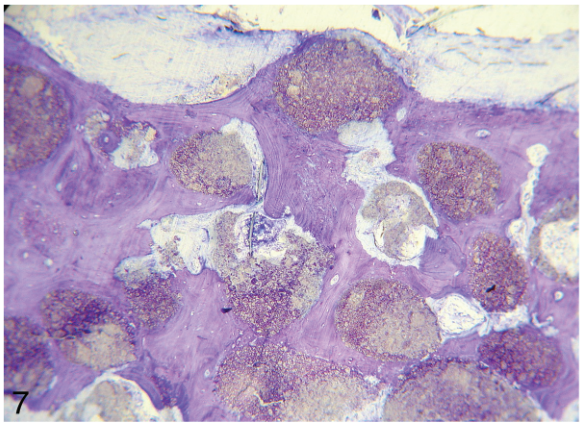
FIGURES 3–6. FIGURE 3. All β-tricalcium phosphate (β-TCP)/calcium sulfate (CS) group defects were filled with newly formed bone covered by a thin bridge of cortical bone (original magnification ×25). FIGURE 4. β-TCP/CS graft particles embedded in new lamellar bone (original magnification ×40). FIGURE 5. In high-magnification resorption of β-TCP/CS, graft material may be detected in close contact with new bone, red arrows (original magnification ×100). FIGURE 6. The defects of the β-TCP group were partially filled with new bone while the outer cortical bridge was absent (original magnification ×10).

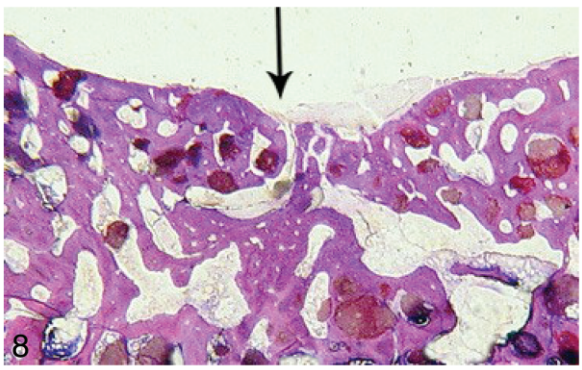
analysis of these data demonstrated a significant difference between the 2 groups ($P = .004 < .05$). The ungrafted sites demonstrated a mean bone growth of 17.77% (SD, 2.98; SE, 1.49). The remaining graft volume in the β-TCP/CS group was measured at 21.62% (SD, 4.27; SE, 1.35), and in the β-TCP group, it was 19.69% (SD, 7.39; SE, 2.34). Results are shown in Tables 1 and 2.

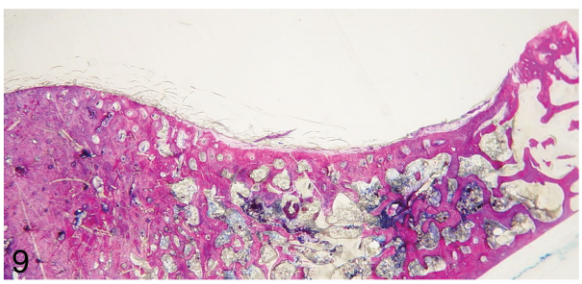
DISCUSSION

Tricalcium phosphate as a bone graft substitute has been evaluated at length in previous studies. It binds to bone by means of mechanical anchorage with no formation of intermediate apatite layer.[33–35] Bioresorption of TCP granules occurs due to chemical dissolution in biological fluids and cellular degradation. Solubilization is induced by mesenchymal cells, which are also actively involved in the degradation process.[36,37] Studies have shown the capability of osteoblastic cells,[38] fibroblasts,[39] and osteoclasts[40] to degrade TCP ceramic material. Monocyte/macrophage participation is well documented in vivo[41] as well as in vitro.[42]

It seems that the more soluble a CaP ceramic, the more rapidly it is resorbed by osteoclasts. However, the increased number of released calcium ions may, on one hand, inhibit osteoclasts' activity,[40] while on the other hand, it provides a good environment for osteogenesis.[35] Therefore, it seems that TCP resorption is performed at a rather unpredictable rate that does not always correspond to the new bone formation rate. This behavior is evident in the







Figures 7–9. Figure 7. Occasionally β-tricalcium phosphate (β-TCP) graft particles protruded superficially toward the soft tissues (original magnification ×40). Figure 8. Numerous soft connective tissue invasions from the surface toward the center of the defect were observed in the β-TCP group specimens, red arrows (original magnification ×25). Figure 9. Ungrafted site: incomplete bone formation near the native bony walls with restoration of the outer cortex (original magnification ×10).

conflicting results of many studies on the bioresorption of TCP.[43–47] The β-phase isomer of TCP (β-TCP), however, is characterized by physiologic pH, homogenous microporosity, increased solubility, and a more predictable resorption rate that resembles the new bone remodeling rate. Variations in composition or impurities may affect solubility, whereas the pure phase seems to be resorbed in 5 to 6 months.[21,48] It

Table 1

New bone volume values (%)*

	β-TCP/CS	β-TCP	Without Graft
	50.94	40.46	17.60
	48.67	40.15	16.70
	61.67	33.96	21.89
	39.45	38.87	14.87
	46.56	49.98	
	47.47	39.79	
	55.3	44.66	
	40.57	30.35	
	48.37	41.89	
	54.78	43.03	
Mean	49.38	40.31	17.77
SD	6.73	5.42	2.98

*β-TCP indicates β-tricalcium phosphate; CS, calcium sulfate.

should be noted that a faster resorbable material might allow soft-tissue cells to prematurely intrude into the defect, while nonresorbable or slowly resorbable materials that remain for a long time may inhibit new bone deposition.[30]

Material microporosity seems to regulate its degradation rate and provides the right environment for the deposition of new bone by the adjacent living bone.[49,50] The presence of CS increases the porosity of the grafting material by its early resorption, while it facilitates the circulation of biological fluids and growth factors. Nevertheless, the exact period of time that CS remains in a bony defect without being resorbed has not yet been estimated. It is reported, however, to be approximately 4 to 5 weeks[28,51,52]; however, other studies report 4 to 10 weeks,[53] 16 weeks,[54] 6 months,[55] or even 9 months.[56] In any case, the CS degradation rate depends on many factors such as the vascularity and the size and shape of the defect.

Schenk[57] stated that a stable material surface plays

Table 2

Remaining graft volume values (%)*

	β-TCP/CS	β-TCP
	30.44	6.98
	23.76	12.53
	18.46	20.44
	22.67	18.96
	20.53	29.56
	17.89	28.89
	25.67	17.45
	19.48	26.98
	15.76	21.36
	21.56	13.78
Mean	21.62	19.69
SD	4.27	7.39

*β-TCP indicates β-tricalcium phosphate; CS, calcium sulfate.

an important role in GBR procedures. That is, the more solid the scaffold of the graft, the more successful the outcome. Covering the defect by a barrier membrane, specially a reinforced one, increases immobilization of the bone substitute, avoids its displacement, and improves its osteoconductive properties.[58] In contrast, several studies suggest that a membrane is not absolutely necessary and may even interfere with bone regeneration because it compromises blood supply from the periosteum and impedes its osteogenic effect, which is attributed to the inner cambial layer.[59,60] Salata et al[61] found that the use of GBR membrane in combination with bone substitutes did not significantly improve bone formation compared with the use of bone substitutes alone. The findings of the present study do not support the opinion that a membrane in bone regeneration procedures is superfluous. This would ignore many significant studies that clearly show the benefit of using a barrier membrane even without using bone filler.[2,4,5,62] In any case, a membrane group was not used for comparison. The results should be interpreted only as an out-performance of the β-TCP/CS group compared with the β-TCP alone.

Research data suggest that the occlusive properties of barrier membranes may be achieved by other biomaterials such as CS. Calcium sulfate acts as a binder and enhances graft containment, making the mixture more stable and pressure resistant.[27] In a series of studies, CS barrier properties were tested in bone or periodontal defects in conjunction with a variety of grafts. These studies showed that the CS barrier increases the vital bone volume,[63] promotes periodontal regeneration,[64] excludes epithelial and connective tissue cells, and preserves the alveolar ridge dimensions after tooth extraction.[65–67] Payne et al,[68] in an interesting in vitro study, compared the migration ability of human gingival fibroblasts stimulated by chemotactic substances on 3 different barriers: CS, e-PTFE membrane, and polylactic acid membrane. Calcium sulfate proved to be the most compatible, showing the least interference to cell migration. The problem that seems to be related to the use of a CS barrier is the possibility of the early material resorption and the fractures that may occur on the material surface during the initial postoperative period by any kind of pressure exercised on it. Both of these parameters may allow epithelial ingrowth in the defect area. These latter disadvantages may be surpassed by the use of a β-TCP/CS combination. This mixture solidifies in a few minutes' time after mixing and creates a stable mass with a surface that is not vulnerable to fractures. Whether epithelial ingrowth takes place after CS is resorbed is questionable because the main scaffold of the material is preserved and the pores that are left are relatively small.

It should be noted that bone regeneration seems to vary widely between the different species or even between individual animals of the same species. Furthermore, it is differentiated by the type of bone, the age of the individual, and the presence of the periosteum.[69,70] Mainly, however, healing is largely dependent on wound size and shape, which means that a small 5-wall defect may heal spontaneously without the aid of a graft material or a membrane. On the contrary, a critical size defect (CSD) is defined as the smallest intraosseous wound that does not heal spontaneously by bone formation during the lifetime of the animal or human being.[71] In a later study, a CSD was defined as a defect that has less than 10% bony regeneration during the lifetime of the animal.[72] In the case of the canine ilium, the CSD has not yet been identified.

The number of walls of the host bone defect is critical and should always be taken into consideration when comparing study results. In the present study, cylindrical monocortical defects were created. This shape may be compared with an extraction socket, that is, a 5-wall defect model, a situation quite common in everyday clinical practice. A 10-mm-diameter defect was chosen as it was estimated that this would be similar to a CSD for the dog's ilium. These defects failed to heal spontaneously, and, in any case, a defect of that size would be a challenge to regenerate in clinical practice.

In the present study, the β-TCP/CS combination demonstrated complete regeneration up to the cortex in all 10-mm specimens tested, while β-TCP alone did not succeed in regenerating these large-diameter defects. It is not the first time that CS was used in combination with other biomaterials.[27,51,73] However, differences in powder processing lead to changes in elements' ratios, that is, in the specific case, the Ca/P ratio, which alters the surface chemistry. This leads to differences in the surface Z-potential of the graft. The mineral scaffold of Fortoss Vital is a stoichiometric β-TCP with a Ca/P molar ratio of 1.5. The Z-potential assesses the degree of ionic activity of a material's surface, which is considered to be one of the main physical factors that interfere in the biological behavior of a tissue around an implanted material.[74] This potential depends on a variety of factors, among which is the composition of the implanted material and the surrounding biological fluids, the inflammatory situation, and the environmental pH.[75] The degree to which hydroxyl or carboxyl ion groups alter

the ceramic to osteoblast attachment is not well understood.[76] The link between the Z-potential of bioceramics and their resulting attraction to bone and osteoblasts has been tested in previous studies as well as the relation between the modifications in the processing method of CaP powders and their resulting Z-potential and, hence, their suitability for use as bone tissue engineering scaffolds.[77,78] It is well known that protein adsorption plays an important role in graft behavior and implant integration. The relation between Z-potential and protein adsorption has been confirmed in previous studies.[79] This means that by controlling the Z-potential, by means of special graft processing, host proteins may be attracted into the surgical site, and a positive osteoblast activity is created. This shifting of the isoelectric potential of the surface of Fortoss Vital may be an explanation of its positive regenerative behavior that has been demonstrated in the present study.

CONCLUSION

This study demonstrated complete bone regeneration of critical-size cylindrical bone defects 10 mm in diameter using a composite alloplastic graft of β-TCP in a CS matrix, without a membrane barrier. Use of β-TCP alone resulted in partial bone formation in a 4-month control period. The safety of the tested material was demonstrated as well. Further research should follow to define the critical-size defect in the canine ilium and the necessary period of time for this composite material to be resorbed.

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