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Comparison of osteoconductive properties of three different β-tricalcium phosphate graft materials: A pilot histomorphometric study in a pig model

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ABSTRACT

Aims: The aim of this study was to compare the de novo bone formation ability and osteoconductive effects of three different β-tricalcium phosphate (β-TCP) graft materials. The micro-architectural parameters of the newly formed bone tissues were also compared among the different graft materials.
Material and methods: Eight male Swiss domestic pigs were used in the study. Five bony defects were made with a trephine bur. Three of the defects were filled with Cerasorb®, Kasios® and Poresorb®. The fourth defect was filled with an autogenous bone graft. The last defect remained empty. All subjects were sacrificed after 8 weeks.
Results: When compared to a negative control group, significant healing was observed in all the groups except the Cerasorb group. The osteoconductivity of the Poresorb group was better than that of the other groups ($p < 0.05$). The difference in the osteoconductivity of the Kasios and Cerasorb groups was statistically significant ($p < 0.05$). Comparison of the micro-architectural properties of newly formed bone tissues retrieved from the defects showed that those filled with Poresorb were the best.
Conclusion: β-TCP materials show different results in terms of the volume and characteristics of new bone formation, although they have a similar chemical structure.

1. Introduction

Bone regeneration techniques include surgical interventions performed to improve the quantity and quality of the bone at sites with inadequate bone volume. Autogenous bone grafts and other bone substitutes, including allogeneic, xenogeneic and synthetic biomaterials, are frequently used to induce osseous regeneration in oral and maxillofacial practice (Bernstein et al., 2006).

Currently, autogenous bone grafts are considered the gold standard (Hjorting-Hansen 2002). However, the amount of autogenous bone grafts that can be obtained is limited. Moreover, these grafts have disadvantages, such as morbidity and the need for additional surgical intervention (Noia et al., 2011).

Allografts were developed as an alternative to autogenous grafts. However, they have disadvantages, including demineralization and use of frozen tissue. The use of dehydrated human bone is also controversial. In addition, allografts carry the risk of transmission of infectious diseases (Buser et al., 1998). This risk led to the development of bone-like synthetic materials.

Natural and synthetic bone substitutes are widely used for bone regeneration due to their biocompatibility, osteoinductive/osteoconductive effects and lack of risk for antigenicity. The graft materials serve as a scaffold, and they are replaced by newly formed osseous tissue following graft resorption. These biomaterials provide both mechanical support and osteoinductive/osteoconductive activity to regenerating tissues (Artzi et al., 2004), which are favourable features of bone substitutes.

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Various calcium phosphate bone substitutes have been developed in recent years. These include hydroxy apatite, β-tricalcium phosphate (β-TCP), bioglass and mix structures. Pure β-TCP ceramics are commonly used in bone regeneration due to their excellent biocompatibility, bioresorptive ability and osteoconductive effects (Goel et al., 2013; Winter et al., 1969). β-TCP and similar synthetic bone substitutes have various advantages, including ease of availability, no risk of transmission of infectious diseases and no need for a second surgical intervention (Bucholz 2002). β-TCP completely resorbs within 6–12 months and is replaced by newly formed osseous tissue (Palti and Hoch, 2002).

The composition, geometry, porosity, granule size and microstructure of the material are very important for bone induction (Yuan et al., 2001) and clinical success. Bone regeneration activity may differ among different bone substitutes, even when they are made of the same biomaterial. To determine the contribution of graft materials to healing, several authors investigated resorption and transformation to natural bone structure with different types of graft materials and techniques (Feichtinger et al., 2006; Nolff et al., 2010).

In the present study, we compared the osteoconductive effects, level of resorption and de novo bone formation of defects repaired with three different β-TCP graft materials, each of which has a distinct granular structure and size, using histomorphometric analysis, in addition to the micro-architectural parameters of the newly formed bone.

2. Materials and methods

The ethical review committee of Çukurova University Medical Scientific Research Center approved the study.

2.1. Bone substitutes

Cerasorb M (Curasan AG, Kleinostheim, Germany) is a mixed material that consists of an organic element and pure β-TCP material, with a porosity rate of 62%. This material serves as a porogen. The particle size and amount of the porogen affect the pore size and porosity of the material. β-TCP is a porous material. It has a macroporous structure, with the pores interconnected with micro-pores. The macro-pores vary from 50 to 500 μm, whereas the micro-pores are smaller than 50 μm (Peters and Reif, 2004).

Kasios (Kasios, L'Union, France) is a synthetic material used as a bone substitute. It contains pure β-TCP. Similar to natural bone mineral, β-TCP contains calcium phosphate in a proportion of 99.9%. β-TCP particles are replaced by newly formed osseous tissue as they undergo resorption. Kasios is a graft material with interconnected macro-pores of 200–500 μm and micro-pores of 1–5 μm. It has a porosity rate of 60–80%.

Poresorb (Lasak, Prague, Czech Republic) is a product with a mean porosity of 30–40%, macro-pores of 100 μm and micro-pores of 1–5 μm. Micro-pores provide a convenient surface for cellular attachment by enabling rapid penetration of blood vessels into the material. The gross structure of Poresorb conducts the newly generated blood vessels required to supply blood to newly formed bone tissue, and the spaces between the granules are filled by biochemical components. The resorption of calcium phosphate ceramics is based on the amount of bone tissue that replaces the material. In general, Poresorb is totally replaced by newly formed bone tissue within 6–12 months after resorbing (Sponer et al., 2011).

2.2. Animal model

Eight male domestic pigs (*Sus scrofa domesticus*), aged 6–9 months with a mean weight of 42.8 ± 6.3 kg, were included in the present study. The animals were bred at Çukurova University Medical Scientific Research Center and were kept in a compound under appropriate conditions. Water and fodder were provided ad libitum.

2.3. Anaesthesia protocol

All of the animals were premedicated with intramuscular injections of 20 mg/kg of ketamine (Ketalar®, Phizer, Turkey) and 2 mg/kg of xylazine (Rompun®, Bayer, Turkey). All pigs underwent surgery under general anaesthesia, produced by intravenous injection of 40 mg kg^{-1} h^{-1} of thiopental sodium (Pental Sodium®, I.E. Ulagay, Turkey). Following prone positioning of the pigs, the posterior edge of the frontal bone was palpated and then prepared and draped under aseptic conditions. Local anaesthesia and haemostasis were provided by supraperiosteal injection of 4% Articaine with 1:200,000 epinephrine (Ultracain-DS®, Hoechst Marion Roussel, Turkey).

2.4. Surgical protocol

The bone was exposed by a skin incision down to the periosteum, and periosteal flaps were raised. Five bony defects were made with a trephine bur 1 cm in diameter and 4 mm in depth. Three of the defects were filled with Cerasorb® (500–1000 μm), Kasios® (1000–2000 μm) and Poresorb® (600–1000 μm). Bone harvested from the defects was used as autogenous bone graft in the fourth defect as positive control group. The last defect remained empty to be filled with a blood clot as negative control (Fig. 1). The distribution of the defects was assigned randomly by the drawing straws method. All of the defects were covered with a resorbable collagen membrane (Collagen AT, Italy). The wound was sutured with 3/0 resorbable sutures (Vicryl, Ethicon, USA).

2.5. Postoperative care

All of the pigs received analgesic (Tramadol, 1 mg/kg) (Contramal®, Abdiibrahim, Turkey) and antibiotic injections (Cefazolin 25 mg/kg) (Cefamezine®, Eczacibaşi, Turkey) intramuscularly preoperatively and twice per day during the first three postoperative days. The animals were given a normal diet.

All of the animals were sacrificed after 8 weeks with an intravenous injection of 100 mg/kg of sodium pentobarbitone (Pental®, Bilim, Turkey). The operated sections of the calvarial bone of the pigs were harvested, and soft tissues were stripped off. The

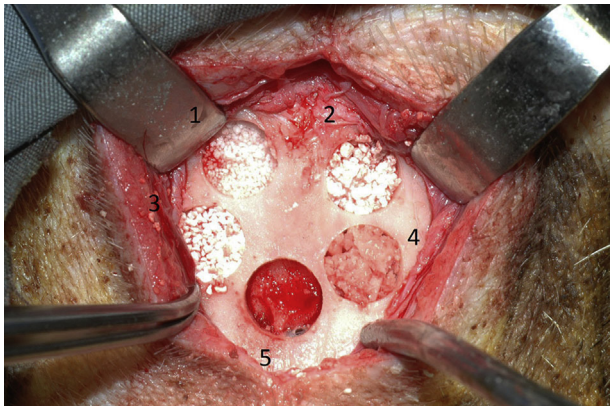

Fig. 1. Intraoperative view of graft placement. 1: Cerasorb®, 2: Kasios®, 3: Poresorb®, 4: autograft, 5: blood clot.

harvested bone was then carefully sectioned to have each defect with the surrounding 1 mm of bone as the specimen.

Undecalcified sections of seven intact samples from each group were prepared for histomorphometric analysis. Due to damage that occurred during histomorphometric section preparation, one sample from the control group (blood clot group) and one sample from the autograft group were excluded. Thus, six samples from the blood clot and autograft groups and seven samples from the Cerasorb, Kasios and Poresorb groups were included in the analysis.

2.6. Specimen fixation

Histomorphometric examination was performed as described in our previous studies (Erdogan et al., 2006; Tatli et al., 2011). The specimens were fixed in 10% buffered formalin, dehydrated in increasing concentrations of ethanol, from 70% to 99%, for 10 days and embedded in methylmethacrylate (Technovit 7200VLC; Haerus Kulzer GmbH, Wehrheiam/Ts, Germany).

2.7. Slide preparation

Sagittal sections (50 μm thick) were prepared using an electric diamond saw and grinding system (Exakt; ExaktVertriebs, Norderstedt, Germany). Histological sections were immersed in toluidine blue working solution (Toluidine Blue O, Sigma Aldrich) for 3 min at ambient temperature and stained with toluidine blue. The samples then washed with distilled water, dried and cover slipped.

2.8. Evaluation

Digital images of the sections were obtained with a digital camera (Camedia C4040; Olympus Corp, Tokyo, Japan) attached to a microscope (Olympus BX50, Olympus Corp, Tokyo, Japan) at a magnification rate of ×2. The images were transferred to a personal computer, and the bone defect areas filled with the bone substitutes were examined. The measurements were made by histomorphometry software (TAS V 1.2.9; Steve Paxton, University of Leeds, Leeds, West Yorkshire, UK). First, the tissue compartments (new bone, marrow space and bone substitute) within the limits of the bone defects defined by the defect walls and the barrier membrane were calculated in percentage (the total volume of the membrane-covered defect was taken as a reference of 100%). Second, an 'osteoconductivity test' was performed by calculating the percentage of graft surface covered by newly formed bone. This measurement was done with intersection counts using image-analysing software (ImageJ, version 1.33u; Wayne Rasband, National Institutes of Health, Bethesda, MD). Third, micro-architectural analysis of newly formed bone matrix; including the trabecular thickness (TbTh), trabecular width (TbWi) and trabecular separation (TbSp), was performed. The nomenclature and calculations for bone histomorphometry were applied in accordance with the report of the American Society for Bone and Mineral Research (Dempster et al., 2013).

2.9. Statistical evaluation

Data were analysed using MedCalc statistical software (version 10.1.6, Mariakerke, Belgium). As the data were not normally distributed, the Kruskal–Wallis one-way analysis of variance and the Mann–Whitney *U*-test were utilised. When employing multiple comparisons, *p*-values were corrected using the Bonferroni adjustment procedure. In all statistical tests, the significance level was defined as $p < 0.05$.

3. Results

No surgery- or drug-related complications were observed in any of the animals. However, one pig was excluded due to frontal sinus perforation, which occurred during preparation of the defect in the frontal bone.

The percentages of newly formed bone tissue, marrow space and residual bone substitute obtained by the histomorphometric analysis of the bone substitutes are given in Table 1. When compared to the negative control group, all of the groups showed significant healing except the Cerasorb group ($p < 0.001$) (Figs. 2 and 3). However, in terms of multiple comparisons, the difference between the Cerasorb and Kasios groups was not significant ($p > 0.05$). The volume of residual graft was significantly decreased in the Cerasorb group compared to the other groups ($p < 0.05$). However, the residual graft volumes in the Kasios and Poresorb groups were comparable ($p > 0.05$) (Fig. 3).

The osteoconductivity level was better in the Poresorb group compared to the other groups ($p < 0.05$). The difference in the level of osteoconductivity between the Kasios and Cerasorb groups was statistically significant ($p < 0.05$; Table 1).

With regard to the micro-architecture of de novo bone formation, TbTh, TbWi and TbSp were best in the positive control group ($p < 0.05$) (Fig. 4). Regarding TbTh and TbWi, bone tissue formation was better in the Poresorb group compared to the other groups ($p < 0.05$). However, there was no significant difference in TbSp between the positive control and Poresorb and Kasios groups ($p > 0.05$). The bone quantity and quality in the Cerasorb group were significantly poorer compared to those in the other group. Nevertheless, the characteristics of the newly formed bone in the Cerasorb group were significantly better than those of the negative control group ($p < 0.05$) (Table 1 and Fig. 4).

Table 1
Histomorphometric comparisons of the graft groups. In regard to "Tissue compartments"; percentages of new bone, marrow space and residual graft materials were shown for each group. In regard to "Osteoconductivity"; the percentages of the graft surface covered by newly formed bone were shown for each group. In regard to "Micro-architectural parameters"; trabecular thickness, trabecular width, and trabecular separation values of the new bone were shown for each group. The results were shown as means ± standard deviations.

Histomorphometric parameters	Groups					*p*
	Autograft ($n = 6$)	Blood clot ($n = 6$)	Cerasorb ($n = 7$)	Kasios ($n = 7$)	Poresorb ($n = 7$)	
Tissue compartments (%)						
New bone	64.83 ± 2.85	38.66 ± 4.54	41.28 ± 4.02	43.85 ± 3.07	48.71 ± 2.56	0.0001
Marrow space	35.16 ± 2.85	61.33 ± 4.54	29.28 ± 5.28	17.28 ± 3.45	14.14 ± .28	<0.0001
Residual graft	0	0	29.42 ± 6.29	38.85 ± 4.87	37.14 ± 3.38	<0.0001
Osteoconductivity (%)	–	–	58.09 ± 1.88	61.44 ± 2.01	63.91 ± 2.91	0.0018
Micro-architectural parameters (μm)						
Trabecular thickness (TbTh)	95.72 ± 3.13	43.23 ± 3.18	66.44 ± 2.55	76.19 ± 2.74	81.35 ± 4.37	<0.0001
Trabecular width (TbWi)	97.08 ± 6.18	52.29 ± 3.63	57.23 ± 2.81	62.66 ± 4.45	72.33 ± 3.30	<0.0001
Trabecular separation (TbSp)	33.85 ± 3.55	54.26 ± 4.69	40.58 ± 4.31	35.40 ± 4.01	34.21 ± 3.00	0.0003

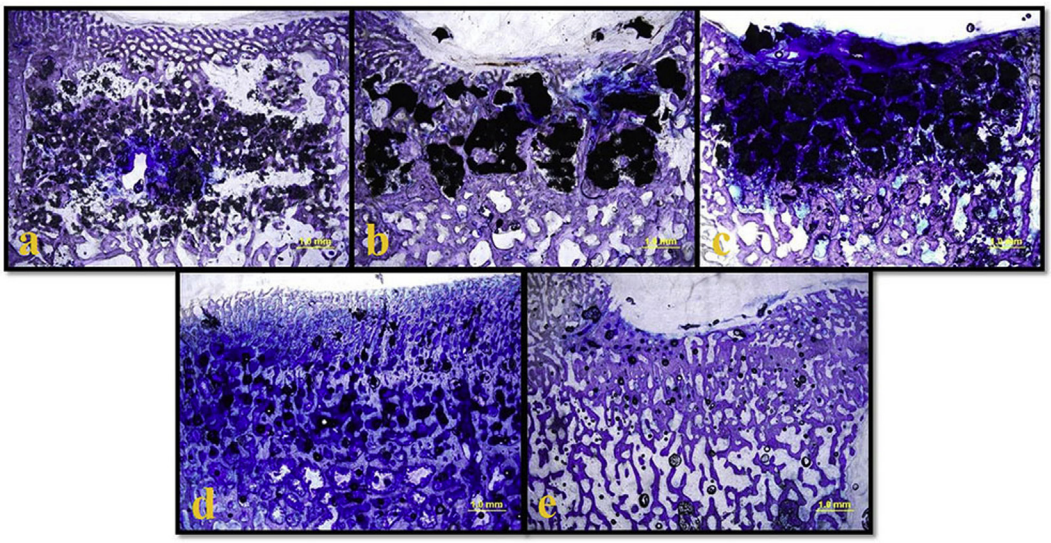

Fig. 2. A 50-μm-thick histologic section from all groups. a: Cerasorb®, b: Kasios®, c: Poresorb®, d: autograft, e: blood clot (Toulidine blue stain, original magnification ×2).

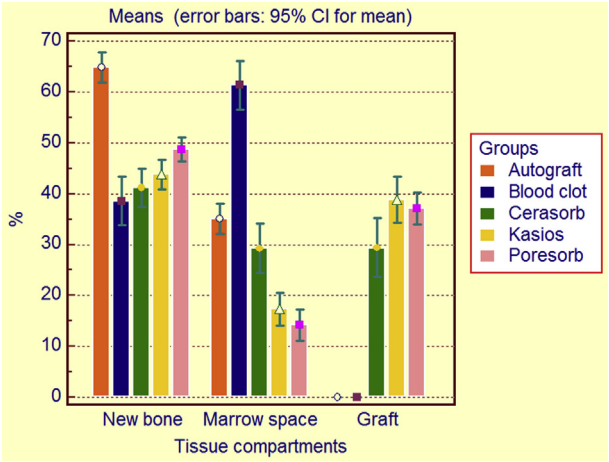

Fig. 3. Osteoconductivity of all groups.

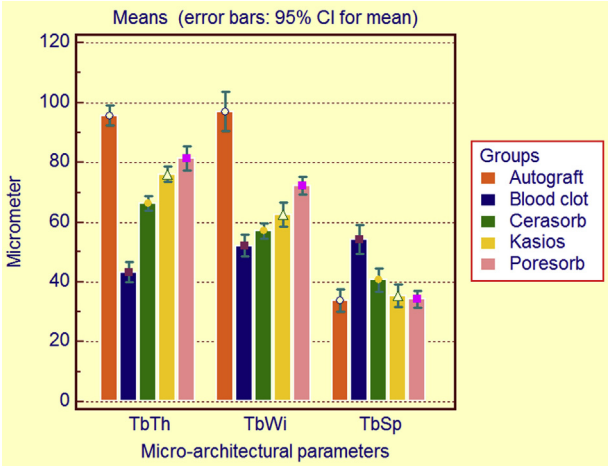

Fig. 4. Micro-architecture of de novo bone formation in all groups.

4. Discussion

Autogenous bone grafts remain the gold standard in the reconstruction and restoration of bone defects. These grafts are harvested from iliac bone, tibia, calvarial bone, ribs, mandibular symphisis, zygomatic buttress and ramus in oral and maxillofacial surgery (Garg 2004). Biocompatible bone substitutes that can serve as a scaffold in bone regeneration are being developed to reduce or alter the need for autogenous bone grafts. In the present study, we evaluated the efficiency of three different porous, pure β-TCP materials in bone regeneration of frontal bone defects in a porcine model using histomorphometry. De novo bone formation was observed after healing in all of the study groups.

In this pilot study, we used the frontal bone in a porcine model due to the amount of available bone, favourable bone micro-architecture and similarity to human bone, as we demonstrated in a previous paper (Erdogan et al., 2013). The utilisation of maxillofacial bones through an intraoral approach has disadvantages, including an increased risk of postoperative complications because of the high microbial content of the oral cavity and difficulty in examining wound healing during the observation period. Therefore, we preferred an extraoral approach, which may represent the maxilla or the mandible. Previous studies using the same animal model and similar defect types demonstrated that an observation period between 4 and 12 weeks was suitable for evaluation of osseous healing (Schlegel et al., 2006; Tudor et al., 2008). Thus, we selected an average (8-week) observation period after the treatment of the defects with graft materials.

The bone formation rate is calculated by determination of the mineralised surface and mineral apposition (how did osteoblasts form bone tissue regarding form and mineralisation) in newly formed bone tissue (Ott 2002). A previous study that compared β-TCP, autogenous graft and bovine bone graft observed no significant difference between β-TCP and autogenous bone graft at the end of 8 weeks and greater amounts of de novo bone formation compared to the bovine bone graft (Jensen et al., 2006). However, we detected a significant difference in de novo bone formation between the autogenous bone graft and β-TCP grafts in the present study. The difference can be explained by graft resorption. β-TCP, a synthetic material with a calcium phosphate component, is replaced by newly formed bone tissue after resorption (Kamitakahara et al., 2008). In animal studies of β-TCP, residual graft particles were

encountered at the defect site, although resorption was observed at all follow-up times over 8 weeks (Alfotawei et al., 2014; Artzi et al., 2004; Buser et al., 1998). In our study, the residual particle ratio was 38.85 ± 4.87% in the Kasios group, 37.14 ± 3.38% in the Poresorb group and 29.42 ± 6.29% in the Cerasorb group. β-TCP material seems to favour this ratio, which allows bone to undergo remodelling with a slow resorption rate (Kamitakahara et al., 2008). We also observed greater amounts of de novo bone formation in the Poresorb group compared to the other β-TCP groups, a finding that may be due to slow resorption features of this graft material.

In another study, β-TCP led to smaller amounts of de novo bone formation, although it was associated with more rapid resorption compared to bovine graft materials (Artzi et al., 2003). In a later study, the authors observed a negative correlation between the resorption capacity and de novo bone formation of β-TCP (Artzi et al., 2005). They suggested that bone remodelling differs in defects treated with β-TCP, a synthetic, porous ceramic material, regarding both chemical and functional manner. In the present study, the finding that resorption was more rapid in the Cerasorb group (29.42% of residual bone substitute after 8 weeks; Table 1) but smaller amounts of de novo bone formed compared to the other groups, is in agreement with the above-mentioned conditions.

Bone augmentation materials should have biocompatibility, osteoinductive and/or osteoconductive properties, remodelling capacity and mechanical features similar to bone, supporting de novo bone formation (Theler 2011). Osteoconduction is defined as bone formation on the surfaces of the material implanted (Albrektsson and Johansson, 2001). Multi-porous granular β-TCP has a matrix structure with osteoconductive activity, leading to osteogenic bone formation (Horch et al., 2006). Ogose et al. (2002) observed de novo bone formation in a histological evaluation performed 4 weeks after implementation of β-TCP in human femur. In addition, they reported that de novo bone formation occurred at the periphery rather than the centre of the graft. Thus, the authors concluded that the graft provided a persistent osteoconductive effect. In our study, all three materials had osteoconductive effects, enabling de novo bone formation. However, osteoconductivity was significantly higher in the Poresorb group (63.91 ± 2.91%) compared to the other test groups (61.44 ± 2.01% and 58.09 ± 1.88% in the Kasios and Cerasorb groups, respectively).

In addition to providing a strong scaffold, biomaterials with larger particles enable earlier regeneration by forming a more stable structure compared to graft materials with smaller particles, which can cause aseptic inflammation in soft tissues (Tadic and Epple, 2004). A previous study demonstrated that the microporous structure of calcium phosphate ceramics accelerated bone formation and increased bone volume (Yuan et al., 2001). The Poresorb material has larger granules and smaller micro-pores than the Kasios and Cerasorb materials. We think that the better osteoconductivity of the Poresorb material is attributed to these properties and makes it more effective in de novo bone formation.

The assessment of the micro-architecture of de novo bone is crucial in evaluations of bone quality. Several methods are available to assess the micro-architecture at the trabecular level. These include histomorphometry, qualitative computed tomography (CT), high-resolution CT, volumetric qualitative CT and high-resolution magnetic resonance imaging (Baum et al., 2012; Vandeweghe et al., 2013). In the present study, we used the histomorphometric technique to evaluate the morphology of the newly formed bone and bone healing. Measurements of all distances were achieved by two methods: The wall thickness was measured with a direct method, and the TbTh, TbWi and TbSp were measured with an indirect method. The indirect method minimises sampling errors, resulting in less effort (Eriksen 1986). The TbTh is the distance obtained from three-dimensional imaging of the bone, and the TbWi is the distance obtained from the same region in two-dimensional images of the bone. Although thickness and width are identical in a numeric manner, TbTh is calculated by dividing TbWi by $4/\pi$ or 1.2 (Dempster et al., 2013). TbSp refers to the distance between margins of bone marrow rather than the distance between two mid-points in bone marrow (Dempster et al., 2013). In the micro-architectural analysis of the de novo bone formation, in the Poresorb group, TbTh and TbWi values were 81.35 ± 4.37 and 72.33 ± 3.30 μm, respectively. These values were closest to those observed in the positive control group and are considered high. The second best TbTh and TbWi values were achieved in the Kasios group, followed by the Cerasorb group. There was no significant difference in the TbSp values of the Poresorb and Kasios groups, whereas the values in the Cerasorb group were higher than those observed in the Poresorb and Kasios groups. Regarding the histomorphometric characteristics of the new bone formation, the bone micro-architecture in the Poresorb group was the closest to that of autogenous bone graft.

5. Conclusion

In conclusion, clinical results in terms of volume and the characteristics of new bone formation may vary with different β-TCP materials, although they have a similar chemical structure. The different clinical results are likely due to the variation in granular size and porosity of the β-TCP graft materials used. Further studies are necessary to find the optimal parameters for β-TCP bone substitutes to achieve de novo bone with better characteristics for use in augmentation procedures.

Conflict of interest

The authors have no financial interest to declare in relation to the content of this article. No grants were received.

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