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MBCP® biphasic calcium phosphate granules and tissucol® fibrin sealant in rabbit femoral defects: The effect of fibrin on bone ingrowth

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An ageing population implies an increase in bone and dental diseases, which are in turn a source of numerous handicaps. These pathologies are an expensive burden for the European health system. As no specific bioactive materials are efficient enough to cope with this burden, we have to develop an injectable, mouldable, self-hardening bone substitute to support bone tissue reconstruction and augmentation.

New, highly bioactive and suitable biomaterials have been developed to replace bone grafts in orthopedic revision and maxillofacial surgery for bone augmentation. These mouldable, self-hardening materials are based on the association of MBCP® Biphasic Calcium Phosphate Granules and Tissucol® Fibrin Sealant. The *in vivo* evaluation of ingrowth in relation to the composite was made in an experiment on rabbits. The results indicate that in the presence of fibrin sealant, newly-formed bone developed at a small distance from the surface of the calcium phosphate ceramic. Two different bone apposition processes were identified. Without the fibrin component (MBCP group), bone rested directly on the surface of the granules. This observation is commonly described as osteoconduction in calcium phosphate materials. On the contrary, the presence of the fibrinogen component seemed to modify this standard osteoconduction phenomenon: the newly-formed bone essentially grew at a distance from the surface of the granules, on the fibrillar network, and could be considered as an inductive phenomenon for osteogenic cell differentiation from mesenchymal stem cells.

1. Introduction

Many bone substitutes have been developed in the last few years to replace autogenous bone grafts in bone and joint surgery. **Calcium phosphate ceramics** are bone graft substitutes that have been commonly used in clinical situations for many years. Their bioactivity makes it possible to use them as bone substitutes following a dissolution-precipitation process [1, 2]. The association of calcium phosphate ceramics with a sealant such as fibrin sealant has made them available for a greater number of indications. **Fibrin sealants** are biological adhesives derived from blood that mimic the last step of coagulation. They are usually used to promote hemostasis and tissue adhesion in a wide field of medical applications, such as cardio-vascular medicine, orthopedics, neurosurgery, plastic and dental surgery [3–7].

The fibrin sealant-biphasic calcium phosphate composite seems promising in the field of bone substitution because of the combination of their respective properties. Calcium phosphate ceramics are indeed widely used despite certain limitations: in the form of granules, they are difficult to handle; in the form of blocks, they do not fit tightly to the implantation site. The adhesive properties of fibrin sealants improve the mechanical properties of calcium phosphate ceramic granules [1, 8] and make it possible to: (1) mould them even to complex forms of bone defect, (2) improve the handling of the composite. Biologically, vascular proliferation inside the biomaterial is accelerated thanks to the presence of growth factors in the sealant [9, 10].

Fibrin sealants are based on the activation of fibrinogen by thrombin, leading to the polymerization of soluble fibrinogen into insoluble fibrin, which has adhesive properties [4]. Each component of the sealant can interact with the associated calcium phosphate ceramic.

The purpose of this study was to demonstrate the effects of the fibrinogen component and its role in

the bioactivity of a calcium phosphate ceramic-fibrin sealant composite.

2. Materials and methods

2.1. Composites

Micro-macroporous Biphasic Calcium Phosphate (MBCP®) ceramics (Biomatlante, Vigneux de Bretagne, France) were used in the form of 1 or 2 mm diameter granules (0.45 g corresponding to 1 cc for each defect). The MBCP® was composed of 60% of hydroxyapatite and 40% of ß-TCP. The total porosity was 70%, of which 50% were macropores with a diameter of 300 to 600 μm and 30% were micropores of less than 10 μm in diameter. The fibrin sealant (Tissucol®, Tisseel® Baxter Biosciences Biosurgery, Vienna, Austria) was presented in a frozen kit with two syringes. The first syringe contained fibrinogen, fibronectin, factor XIII and aprotinin. The second syringe contained 4 U of thrombin and calcium chloride. One ml of the reconstituted sealant was mixed with 0.45 g of granules (previously moistened in sterile water) (i.e. a final total volume of around 2 ml) for each implant, in order to obtain a granule/sealant volume ratio of 1:1.

2.2. Surgical procedure

Under general anesthesia, performed by intramuscular injections of xylazine (5 mg/kg) and ketamine (35 mg/kg), lateral-bilateral knee arthrotomies were performed on fifteen female adult New Zealand White rabbits (Charles River, Saint Aubin les Elboeuf, France). A drilled bone defect with a diameter of 6 mm and a depth of 10 mm was centered on the lateral condyle. The cavity was thoroughly rinsed with physiological saline solution. The defects were filled with three different composites carefully compacted to prevent the formation of dead spaces. The composites were (i) Control group, MBCP alone; (ii) F+T group, association of fibrin sealant with MBCP; (iii) F group, association of Tissucol's fibrinogen component alone with MBCP. The cavity was closed with an MBCP ceramic plug with a diameter of 6 mm and a length of 3 mm. The wound was sutured in three layers.

Under general anesthesia, the rabbits were euthanized 3 and 6 weeks after implantation by intracardiac injection of a barbiturate (Dolethal®, Vetoquinol, France).

The care and use of these laboratory animals was in compliance with French law on animal experimentation.

The distribution of the implants was made randomly.

2.3. Histological examinations

The femoral condyles were harvested and the peripheral soft tissue removed. The samples were X-rayed to localize the implant. The specimens were fixed in 4% neutral formol solution for 7 days, rinsed in water, dehydrated in ethanol of increasing concentration (from 70% to 100%) and embedded in glycol methyl methacrylate. For each implant, two sections with a thickness of 100 μm were made with a diamond circular saw (Isomet®, Buehler LTD, Lake Bluff, USA); the first section was stained with Movat's pentachrom [11], the other was used for polarized light microscopy.

After polishing and gold-palladium sputter-coating, global histomorphometry ($\times$50) was carried out on the residual surface of the block using scanning electron microscopy (SEM) with backscattered electrons (BSE) (Jeol JSM 600, Tokyo, Japan), connected to an image processing system (Quantimet 500MC, Leica, Cambridge, Great Britain). To cover the whole surface of the implant, a series of contiguous images was automatically made for each implant.

On each image, the newly-formed bone and MBCP ceramic in each field were identified by semi-automatic binary treatment within the limits of the bone defect. Their respective surfaces were expressed as a percentage of the whole surface of the bone defect [12].

A centripetal image analysis was made for each sample. After exact juxtaposition of each binary image of the same implant, a global binary image representing the whole surface defect was reconstructed (Adobe Photoshop 5.0). On this reconstructed image, an original histomorphometrical routine developed for the study was applied to evaluate the kinetics of the bone growth along the depth of the bone defect [13]. The analysis surface was defined by semi-automatic detection of the outer limits of the bone defect (with possible correction by the operator). Through iterative transformation by erosion of the previously defined analysis surface, the centripetal analysis routine divided it automatically into concentric bands. For each band, the routine calculated the percentage of bone and ceramic, structures easily detected after the prior binary treatment. The width of the band was initially defined as 40 pixels corresponding to 0.43 mm.

Statistical analysis was performed using Statview (Deltasoft, Grenoble, France). On each group of data, a non parametric analysis of an independent series was performed (Kruskall-Wallis H test). Once the statistical significance was determined ($p < 0.05$), a parametric analysis was performed to compare the data 2 by 2 (ANOVA).

3. Results

3.1. Histological observations

The distribution of the residual MBCP granules was homogeneous on the surface of the bone defect, regardless of group (MBCP, F+T, F).

For the **MBCP group**, lamellar bone was apposed in close contact at the surface of the granules, either in the macropores or between the MBCP granules. Consequent bone ingrowth could be observed after 3 weeks of implantation (Fig. 1), and in direct contact with the MBCP surface (Fig. 2). All the granules presented a regular outline, with cells lining the surface. Collageneous fibers were present between the granules.

For the **F+T group**, the newly-formed bone was formed at distance from the surface of the granules (about 50 μm). This phenomenon was clearly evident at 6 weeks and could be seen with the BSE. The large MBCP granules had an irregular outline, with the presence of 100 μm wide small calcium phosphate

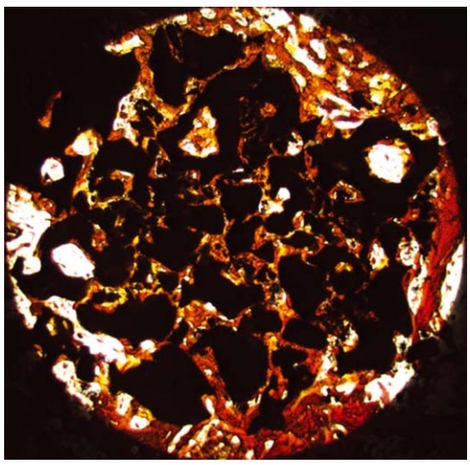


Figure 1 Histology image of the MBCP group at 3 weeks, implanted in the femoral epyphysis. Note the amount of bone ingrowth near the ceramic granules (Stain: Movat; magnification ×12.5).

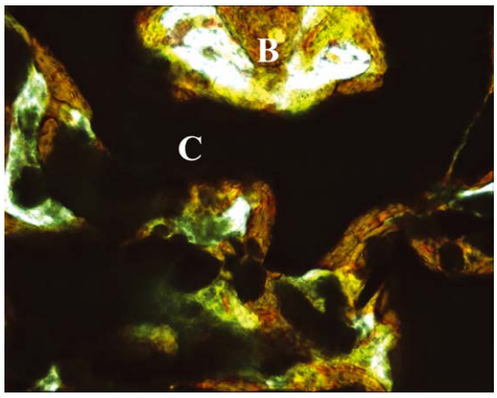


Figure 2 Histology image of the MBCP group at 3 weeks, implanted in the femoral epyphysis. Note the lamellar bone apposed in close contact at the surface of the granules, 3 weeks after implantation. (Stain: Movat; magnification ×50; C = ceramic, B = bone).

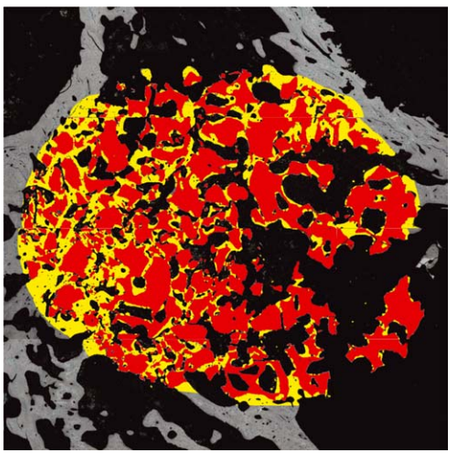
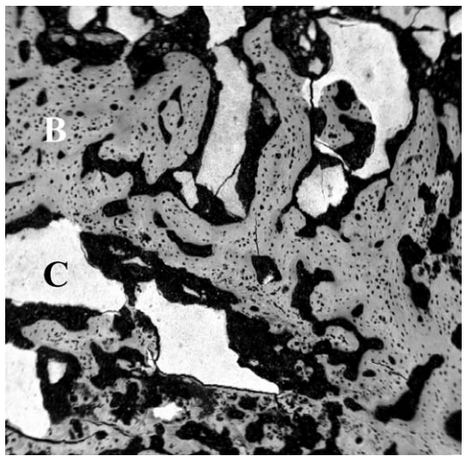
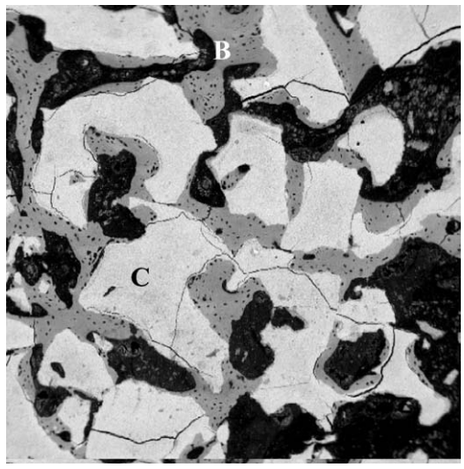


Figure 4 BSEM image with semi-automatic binary treatment of the F+T group 6 weeks after implantation in the femoral diaphysis. Note the slight decrease in bone ingrowth compared with MBCP, with centripetal bone colonization. (magnification ×12; yellow = bone; red = ceramic).

particles within the intergranular spaces. There were bone cells lining the collageneous fibers present between the granules (Figs. 3(a) and 3(b)). A slight decrease in bone ingrowth towards the center of the



(a)



(b)

Figure 3 (a) BSEM image of the F+T group 6 weeks after implantation in the femoral diaphysis. Note the bone growth at a small distance (50 μm) from the surface of the ceramic (magnification ×50; C = ceramic, B = bone, black = intergranular space). (b) BSEM image of the MBCP group 6 weeks after implantation in the femoral diaphysis. Note the bone growth in close contact with ceramic. (magnification ×50; C = ceramic, B = bone, black = intergranular space).

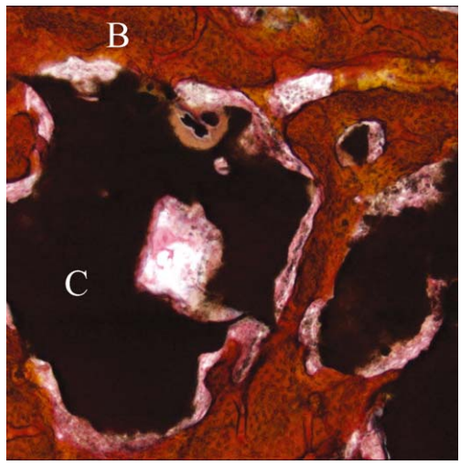


Figure 6 Histology image of the F group at 6 weeks, implanted in the femoral epyphysis. Note the space of about 50 μm between bone and ceramic, 6 weeks after implantation. (Stain: Movat; magnificant ×50; C = ceramic. B = bone.

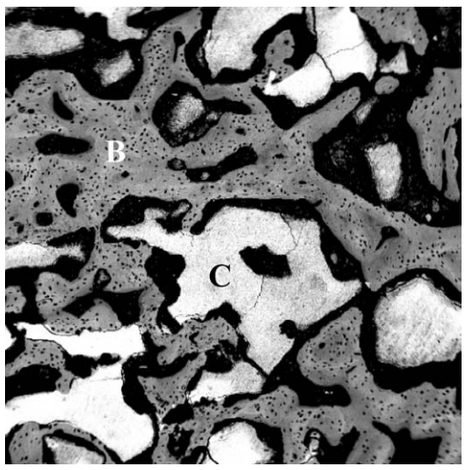


Figure 5 BSEM image of the F group 6 weeks after implantation in the femoral diaphysis. Note the presence of bone at a small distance from the surface of the ceramic. (magnification ×50, C = ceramic, B = bone, black = intergranular space).

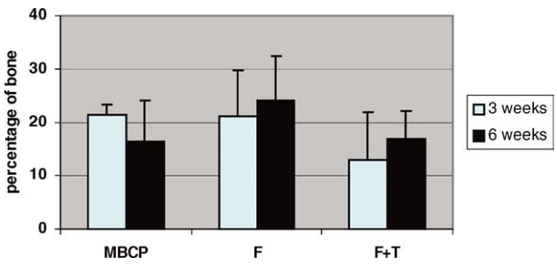


Figure 7 Percentage of bone growth at 3 and 6 weeks in the different groups MBCP, F, F+T. The percentage of bone growth seems similar in MBCP and F and in contrast, decreased for F+T.

implant could be observed compared to the MBCP group (Fig. 4).

For the **F group**, as with the F+T group, newly-formed bone appeared at a small distance (around 50 μm) from the surface of the ceramic granules after 6 weeks of implantation (Figs. 5 and 6). The amount of bone ingrowth was greater than for the F+T group. Small particles (<100 μm) of ceramic were found between the larger ones. As with the F+T group, there were bone cells lining the collageneous fibers present between the granules.

3.2. Histomorphometric analysis

3.2.1. Global analysis

At 3 and 6 weeks, bone ingrowth was similar between the MBCP and F groups, however a slight (but not significant, $p = 0.4$) decrease was observed for the F+T group at 3 weeks (Fig. 7). No difference was observed at 6 weeks. Regardless of the group or implantation time, no statistical difference was observed for the amount of ceramic ($p = 0.6$) (Fig. 8).

3.2.2. Centripetal analysis

Bone substitutes are recognized through bone growth from the host bone to the implant, and progressing from the outer part to the core by osteoconduction. This process of bone ingrowth is centripetal.

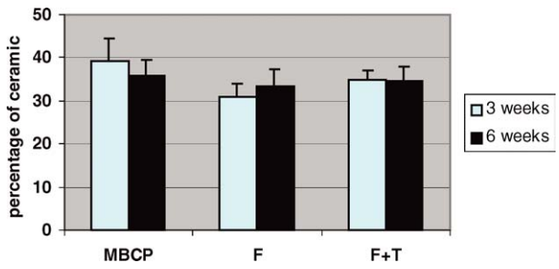


Figure 8 Percentage of ceramic at 3 and 6 weeks in the different groups (MBCP, F, F+T). The percentage of ceramic is similar for the 3 groups.

For the **MBCP group**, as early as 3 weeks, bone colonization was homogeneous along the depth of bone defect, with no evolution at 6 weeks. For the **F+T** and **F groups**, centripetal bone colonization from the outer margin of the defect was evident at 3 weeks. The kinetics of the bone colonization toward the center of the defect appeared accelerated for the F group compared to the F+T group. This phenomenon was still present at 6 weeks: for the F group, deep bone colonization seemed to be equal to that of the MBCP group, and a slight delay was still observed for the F+T group compared to the MBCP and F groups (Figs. 9 and 10).

4. Discussion

The results of this study reveal the differences in the nature of bone colonization depending on the addition or not of fibrin sealant components to MBCP granules. For the F+T and F groups, bone developed at a small distance from the surface of the ceramic, whereas for the MBCP group, the bone was apposed, in close contact with the ceramic. Differences in the kinetics of deep bone colonization were also observed. Despite the fact that the final amount of bone ingrowth was similar in the different groups, deep bone colonization appeared to be slower for the F+T or F groups compared to the MBCP group. This could be due to the spaces containing no organic materials between the granules in the MBCP group compared to the spaces filled with fibrin in the F+T group or fibrinogen in the F group.

In previous studies, the association of fibrin sealant-calcium phosphate ceramic has given variable results. For some studies [9, 14–16], this association has had a positive effect on bone colonization. In others [10, 17, 18], a negative effect has been observed. The composition of the sealant appears important for the bioactivity of such associations [15]. In this study, the implantation of coral granules in the rabbit's femoral condyles seems to produced more deep bone ingrowth in the presence of Autocolle® (Centre de transfusion sanguine, Tours, France) compared to Tissucol® (Baxter Biosciences, Vienna, Austria). These results were observed during the first month postoperatively and were more pronounced at 2 months. The main difference between these two composites was the concentration of fibrinogen, which varied between 50 to 65 mg/L for Autocolle® and 70 to 100 mg/L for Tissucol®/Tisseel®, reticulated with 500U of thrombin It was largely demonstrated that the high amount of

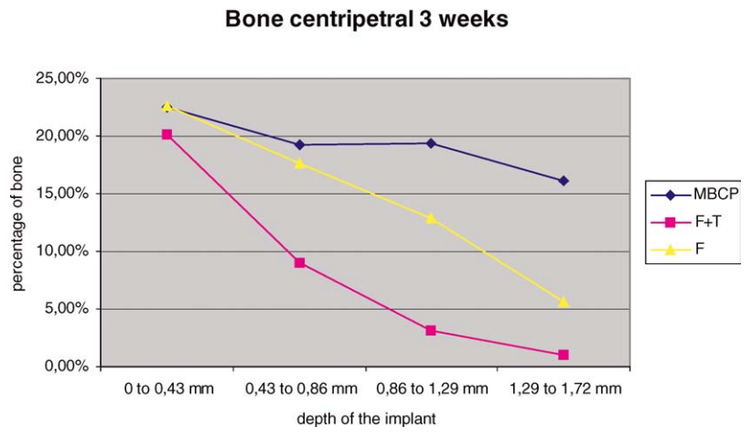


Figure 9 Bone centripetral at 3 weeks.

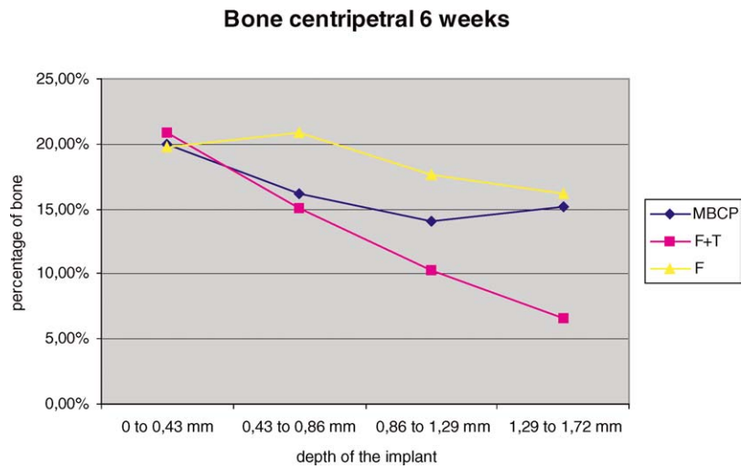


Figure 10 Bone centripetral at 6 weeks. At this time, the bone growth is homogeneous from the periphery to the center of the implant for MBCP and F. For F+T, the bone ingrowth decreases from the periphery to the center of the implant.

thrombin (500U) involved a fast transformation of fibrinogen to fibrin and creates a very dense 3D network unsuitable for homogenous mixture and further cell deep colonization. For this reason the suitable mixture of CaP granules with fibrin sealant must be made with low concentration of thrombin (4U). This phenomenon in the study of Kania *et al.* [15] may be related to the density of the 3 dimensional fibrin network, which is more dense in Tissucol used. This was confirmed, in our experiment, in group 3 (MBCP with Fibrinogen alone, which is little or not reticulated). The network was also more deeply invaded by bone compared to the MBCP and reticulated fibrin in group 2. The results of the present study revealed differences in relation to the presence or absence of reticulated fibrin, this data justify the 4U of thrombin for the combination of MBCP and fibrin sealant.

First of all, two different bone apposition processes have been identified. Without the fibrin component (MBCP group), the bone rested directly on the surface of the granules. This observation has been commonly described for calcium phosphate materials used in osteoconduction [19]. Immediately after implantation, contact with biological fluid induces a dissolution process of the calcium phosphate material leading to the precipitation of the biological apatites with an epitaxic growth by secondary nucleation [20, 21]. Secondly, an osteoformation process is detectable only a few days after implantation [18]. Calcium phosphate ceramic in fact serves as scaffolding allowing new bone to progress from the periphery to the center of the implant [20, 22]. On the contrary, the presence of the fibrinogen component (F, F+T groups) seemed to modify this standard osteoconduction phenomenon: the newly-formed bone almost always grew at a distance from the surface of the granules, on the fibrillar network and could be considered as an inductive phenomenon for osteogenic cell differentiation from mesenchymal stem cells.

Arnaud [17] has already observed this modified osteoconduction with the association of coral granules and fibrin sealant. Transformation of the surface of the granules in contact with fibrinogen might explain this phenomenon. Previous studies have suggested that various non collagenous proteins play a role in the mineralization process as nucleators or inhibitors of hydroxyapatite formation [23]. Of these proteins, fibronectin (which is one of the components of the sealant) has been described as a nucleating protein in early calcification. Fibronectin modifies interface energy between the surface/solution and the ion/cluster/solution, so biological apatites are formed by secondary nucleation and not by crystal epitaxy at the surface of the ceramic [24]. *In vitro*

studies of the calcium phosphate ceramic-fibrin sealant association have demonstrated that the fibrinogen component encircles the granules. As regards fibronectin or non collagenous proteins, the fibrinogen component may modify the interface between the MBCP granules and the solution, resulting in modified osteoconduction. Furthermore, cell modifications have been described. Bone cells seem to rest not on the granules but on the 3 dimensional network produced by the transformation of the fibrinogen into fibrin, which attracks the fibroblasts to produce the collagen extracellular matrices present between the granules. Experimental studies have shown that these collagenous fibers are present for at least 4 days after the date of implantation with a degradation process that can last for over 14 days [3, 25, 27]. The fibers are thus present for enough time to act as scaffolding for the bone cells, making deep bone colonization possible.

Furthermore, in qualitative terms, the degradation of the ceramic appeared to differ in relation to the presence, or not, of fibrinogen. For the MBCP group, the surface and texture of the ceramic granules were regular. In the presence of fibrinogen (F, F+T groups), their surface was irregular with the presence of small particles in the intergranular spaces. Several hypotheses can explain this phenomenon, although the presence of small particles does not seem to be caused by the fact that the composite was prepared from a mixture, as preparation was similar for each group. For the MBCP group, the granules were mixed with physiological saline solution in an identical manner and this type of small particle was not found in this group. The presence of small-sized ceramic particles in the implants with fibrinogen could, however, be explained by a modification in the recruitment of the cells engaged in this degradation due to the presence of fibrinogen. Biphasic calcium phosphate ceramics are degraded by osteoclast-like cells, usually found in bone resorption and multinucleated giant cells. Monocytes, macrophages and fibroblasts are only found in pathological processes [27]. On the other hand, fibrin sealants are degraded by macrophages over 14 days [3]. A previous study [26] has shown that the association of biphasic calcium phosphate with fibronectin (one of the components of the fibrin sealant) modifies standard cell interactions. With fibronectin precoated on calcium phosphate ceramic, there are more fibroblastic and macrophagous cell interactions. These cells have been described in the degradation of the sealant. The degradation of the composite therefore seems to modify the standard cells involved in the degradation of the calcium phosphate ceramic. This situation can lead to modifications in the size of the particles resulting from the degradation.

The aim of the organism is to build bone between the granules to restore the physiological bone volume with ceramic and newly-formed bone. Quantitatively, there was no statistically significant difference in the percentage of bone between the groups. We can thus conclude that adding the fibrin sealant has no deleterious effect on bone ingrowth inside calcium phosphate ceramic granules despite different types of osteoconduction processes. Despite this lack of significance, a slight decrease in bone ingrowth, in the following decreasing order: MBCP, F and F+T, was observed. A similar classification was also observed in the centripetal analysis. This demonstrates that the presence of fibrin modifies the standard kinetics of bone colonization toward the center of the implant. By modifying the degradation of the biomaterial, adding fibrinogen leads to the release of small-sized particles. For some authors [29], the presence of these particles has previously been associated with a decrease in osteoblast activity in bone implantation. For others [30], the presence of particles with a diameter of 100 μm was associated with a major inflammatory response at 21 days that could temporarily slow down the bone formation process. Nevertheless, this inflammatory reaction leads to the release of bone ingrowth factors. Thus, ultimately, the inflammatory reaction is favorable for the osteoconduction process. Furthermore, the fact that bone ingrowth was inferior in the F+T group compared to the F group might be explained by the reticulation process. The presence of thrombin (the only component that differentiates these two groups) induced the formation of a network of fibers of relatively high density [3, 4] that was able to oppose the penetration of the cells into the center of the implant. By decreasing the concentration of thrombin, it was possible to obtain a less dense network, thus promoting the penetration of the cells into the center of the implant. In the F group, with no thrombin, the density of the network was weak and seemed more open to a penetration of the cells into the center of the implant.

This *in vivo* study of bone ingrowth in an association of MBCP and fibrin sealant or one of its main components indicated that in the presence of the fibrin sealant, newly-formed bone developed at a small distance from the surface of the calcium phosphate ceramic. With the fibrin network, a small delay in deep bone colonization was observed depending on the fibrin density of the network in association with thrombin concentration. Further studies seem necessary if we are to understand the exact nature of the phenomenon that leads to growth at a distance from the surface of the calcium phosphate ceramic. Determining the ideal fibrinogen/thrombin ratio that will allow enough bone cell penetration is also another possible means of investigation.

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