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Influence of calcium chloride and aprotinin in the in vivo biological performance of a composite combining biphasic calcium phosphate granules and fibrin sealant

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Abstract Highly bioactive biomaterials have been developed to replace bone grafts in orthopedic revision and maxillofacial surgery for bone augmentation. A mouldable, self-hardening material can be obtained by combining TricOs® Biphasic Calcium Phosphate Granules and Tissucol® Fibrin Sealant. Two components, calcium chloride and antifibrinolytic agents (aprotinin), are essential for the stability of the fibrin clot. The ingrowth of cells in composites combining sealants without calcium chloride or with a low concentration of aprotinin was evaluated in vivo in an experiment on rabbits. Bone colonization was compared using TricOs alone or with the composite made from TricOs and the standard fibrin sealant. Without the addition of calcium chloride, the calcium ions released by the ceramic component interacted with the components of the sealant too late to stabilize the clot. With a low concentration of aprotinin, the degradation of the clot occurred more quickly, leading to the absence of a scaffold on which the bone cells could colonize the composite. Our results indicate that a stable fibrin scaffold is crucial for bone colonization. The low calcium chloride and low aprotinin groups have shown lower bone growth. Further studies will be necessary to determine the minimal amount of antifibrinolytic agent (aprotinin) necessary to allow the same level of osteogenic activity as the TricOs-fibrin glue composite.

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Introduction

Autogeneous bone grafts are considered to be the gold standard for bone replacement in bone and joint surgery. To bypass their limited availability, calcium phosphate ceramics have been developed and used with success as bone graft substitutes in various types of bone surgery [1–4]. Their main limitations are their lack of true osteoinductive properties [5, 6], limited osteoconduction and their degradation in blocks in large bone defect reconstruction. Calcium phosphate ceramics are presented in a variety of forms, such as blocks or granules. Although easy to handle, blocks cannot fit closely to cavity surfaces. Granules, on the other hand, are difficult to handle and have a tendency to migrate when inserted into a cavity. In this context, the adjunction of a binding agent such as fibrin glue facilitates the stability of the granules at the site of implantation [7, 8].

Fibrin glues are biological adhesives that mimic the last step of the coagulation cascade through the activation of fibrinogen by thrombin leading to a clot of fibrin with adhesive properties [9–11]. These components are biocompatible and biodegradable without any negative effects. These glues are widely used in a number of fields of surgery (abdominal, thoracic, vascular, oral, endoscopic) [9, 10, 12].

The combination of ceramic and fibrin glue makes a cumulation of the properties of the two components possible. The resistance of this composite is greater than that of ceramic alone, with adhesion to the walls of the bone defect [13, 14]. In biological terms, the bone substitution of such a composite should be quicker due to the role of the fibrin on vascular proliferation [15, 16] and greater degradation of the ceramic due to increased recruitment of macrophages. In addition, the presence of growth factors in the fibrin glue should have a positive impact during the first stages of bone colonization [16]. Different studies on the fibrin glue-ceramic combination have nevertheless shown variable results [17]. For some authors [7, 18–20], this combination had a negative impact on bone colonization compared to ceramic alone. For others [16, 21–24], bone neoformation was similar to that observed with ceramic alone.

Two components of fibrin glue are essential for the stability of the fibrin clot. First of all, antifibrinolytic agents slow down the early degradation of fibrin clots by means of proteolytic enzymes present in situ. Of these agents, a polypeptide called aprotinin is often used in fibrin glues, despite its bovine origin and its potential allergic reactions [25–29]. Secondly, calcium chloride ($CaCl_2$) acts on the continuance of the glue by influencing the stability of the bindings between the fibrin chains [10, 27]. Although both these components are required for fibrin glue preparation and efficacy, we cannot assert that they are necessary when the main components (fibrinogen + thrombin) are mixed with calcium phosphate, which is able to release large amounts of Ca and PO_4 ions.

The aim of this study was to investigate the influence of $CaCl_2$ and aprotinin on the bioactivity of a fibrin glue-biphasic calcium phosphate ceramic composite as a means of developing a specific composite for bone reconstruction by apposition.

Materials and methods

Composites

Granules of 1 or 2 mm in diameter of Microporous Macroporous Biphasic Calcium Phosphate TricOs® (Baxter BioSciences BioSurgery) ceramics (Biomatlante manufacturer, Vigneux de Bretagne, France) were used in association with fibrin glue Tissucol® (Baxter BioSciences BioSurgery, Vienna, Austria). The TricOs granules consisted of 60% Hydroxyapatite and 40% ß TCP with 70–75% of total porosity. The total porosity was constituted of 70% macroporosity with macropores from 300 to 600 µm and by 30% microporosity with micropores from 1 to 10 µm. The glue was presented in a frozen kit with two syringes. The first syringe contained fibrinogen, fibronectin and factor XIII with aprotinin. The second syringe contained 50 UI of thrombin with or without $CaCl_2$. Immediately before implantation, two cm^3 of granules (0.45 g) were mixed with one cm^3 of fibrin glue to obtain a granule/glue volume ratio of 2:1. The composite obtained were then carefully compacted in the bone defects. The density of the composite after reticulation was 33% of spaces occupied by granules, 30% of macropores in the granules and 37% of intergranule spaces filled by the fibrin glue as determined by microtomography microCT (Skyscann 1072) (Fig. 1) and confirmed with 2D statistical evaluation performed by image analysis using scanning electron microscopy SEM.

Surgical procedure

Twenty female adult New Zealand White rabbits (Charles River, Saint Aubin les Elboeuf, France) were randomly divided into four groups. Under general anesthesia performed by intramuscular injections of xylazine (5 mg/kg) and ketamine (35 mg/kg), bilateral femoral implantations were performed in aseptic conditions. A cylindrical bone defect (6 mm diameter and 10 mm depth) was created at the distal femoral end and rinsed with physiological saline solution. The defects were filled with one of the composites carefully compacted to prevent the formation of dead spaces. The composites were: TricOs alone (TricOs group), the combination of standart fibrin glue with biphasic calcium phosphates (F group), the combination of TricOs and fibrin glue without calcium chloride (no $CaCl_2$ group) and the combination of TricOs and fibrin glue with a low concentration of aprotinin (low aprotinin group). The aprotinin concentration of the low aprotinin group was the minimal concentration compatible with the manufacturer's recommendations. A cylindrical plug 6 mm in diameter and 3 mm in thickness of the same micro macroporous ceramic than TricOs were machined (Biomatlante France) to close the cavity. Under general anesthesia, the animals were sacrified 3 and 6 weeks after implantation by intracardiac overdose of a barbiturate (Dolethal®, Vetoquinol, France).

The care and use of these laboratory animals complied with French animal experimentation laws, and have been approved by the animal ethics committee of the National Veterinary School in Nantes.

Histological examinations

Femoral condyles were harvested and soft tissue removed. Radiographs were performed to localize the implants. The specimens were fixed in 1/9 neutral formol solution for 7 days, rinsed in water, dehydrated in ethanol of increasing

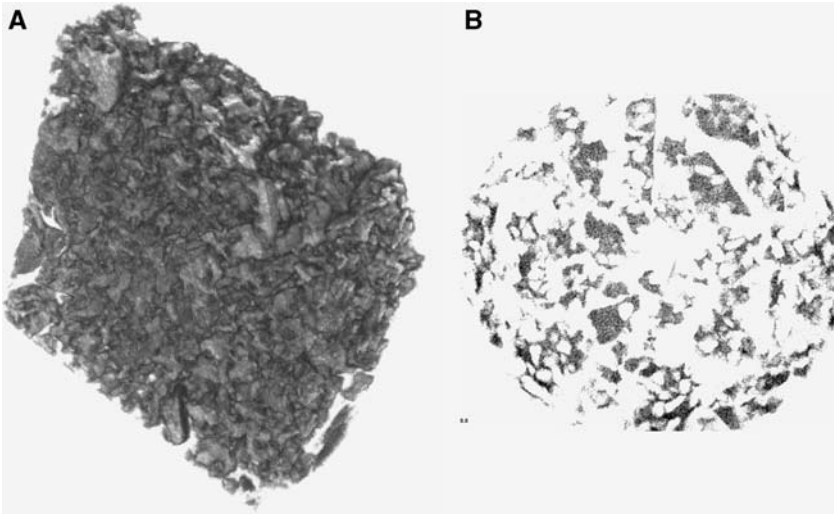

Fig. 1 3D reconstruction using microCT of (**A**) TricOs and (**B**)TricOs/Fibrin glue

concentration (from 70% to 100%) and embedded in glycol methyl methacrylate.

For each sample, two 100 μm-thick sections were made using a diamond circular saw (Isomet®, Buehler LTD, Lake Bluff, USA). Qualitative and quantitative analyses were performed on the residual blocks according to a specific method developed using SEM. Briefly, after histological sectioning, polishing and gold–palladium metallization of the remaining blocks, images were obtained by means of SEM using backscattered electrons (Jeol JSM 600, Tokyo, Japan) at a magnification of 50. The percentage of bone formation and ceramic was then determined using a semi-automatic image analyzer (Quantimet 500MC, Leica, Cambridge, Great Britain). The total implant area was divided into series of automatically-acquired contiguous images.

On each image, semi-automatic binary treatment within the limits of the bone defect made it possible to identify the newly-formed bone and TricOs ceramic. The results were expressed as a percentage of the whole surface of the bone defect.

Juxtaposition of each binary image made it possible to reconstruct a global binary image of the whole surface of the implant. On the reconstructed image, the kinetics of bone growth along the depth of the implant was evaluated by means of centripetal bone analysis [30]. The percentage of bone and ceramic was calculated in concentric analysis bands of a width of 0.43 mm (i.e. 40 pixels), obtained by iterative transformation by erosion of the post-operative surface of the bone defect.

The quantitative results for the newly-formed bone and ceramic were studied for statistical purposes by a Kruskall–Wallis H test (non parametric analysis on independent series) on each group of data. When statistical significance was determined with p values < 0.05, a parametric analysis was performed to compare the data in pairs (ANOVA).

Results

Histological observations

For the *TricOs group*, lamellar bone was apposed in close contact at the surface of the granules. These granules presented a regular outline. Bone was localized in the macropores or between the ceramic granules (Fig. 2A, B and 3A).

For the *F group*, a space of about 50 μm separated the surface of the granules and the newly-formed bone. This phenomenon was clearly evident at 6 weeks (Fig. 2C, D and 3B). Small particles of ceramic (about 100 μm large) were observed between large granules of TricOs with an irregular outline.

For the *no $CaCl_2$ group*, the bone growth was limited, mostly at the periphery of the bone defect (Fig. 2E, F). When observed, new bone formation was systematically at a small distance from the surface of the ceramic (about 50 μm). Small particles (100 μm) were also present between the bigger-sized granules (Fig. 3C).

As in the previous group, for the *low aprotinin group*, the bone formation was limited and located at the periphery of the implant (Fig. 2G, H). Bone formation at a distance from the surface of the ceramic (Fig. 3D) and small particles were observed, similar to the no $CaCl_2$ group.

Histomorphometric analysis

Global analysis

At 3 weeks, the percentage of bone ingrowth was statistically higher for TricOs compared to the other groups ($p = 0.02$). At 6 weeks, no statistical differences were observed between any of the samples. Between 3 and 6 weeks, no statistical differences were observed in any

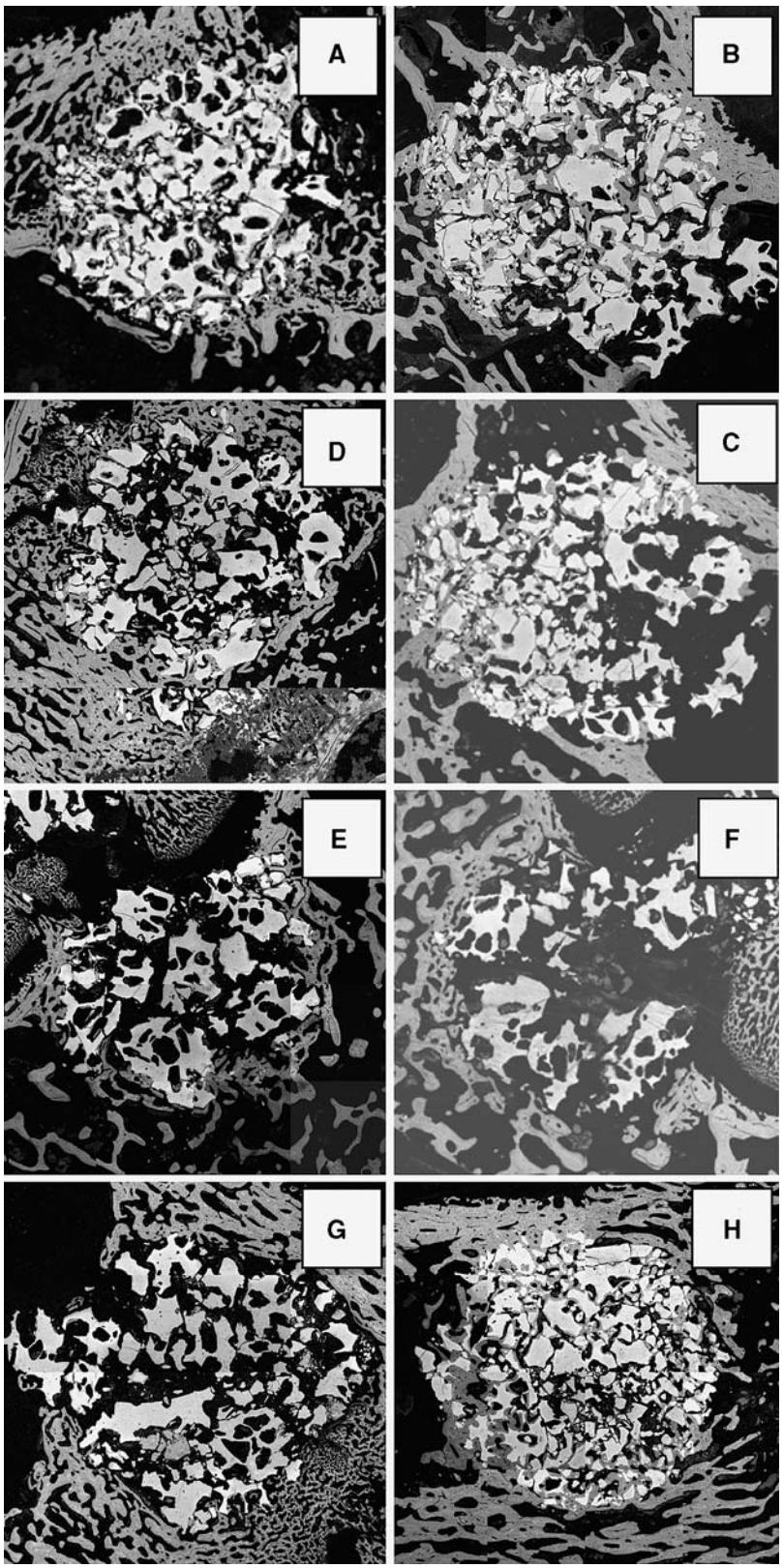

Fig. 2 BSEM micrographs of TricOs group (**A** and **B**), F group (**C** and **D**), no $CaCl_2$ group (**E** and **F**) and low aprotinin group (**G** and **H**) at 3 (left column) and 6 weeks (right column)

group. Despite no statistical differences, at 6 weeks, there was more bone neoformation in the TricOs and F groups than in the no $CaCl_2$ or low aprotinin groups (Fig. 4).

Regardless of the group and delay of implantation, the ceramic charges (Fig. 5) were homogeneous. No statistical differences were observed ($p = 0.35$).

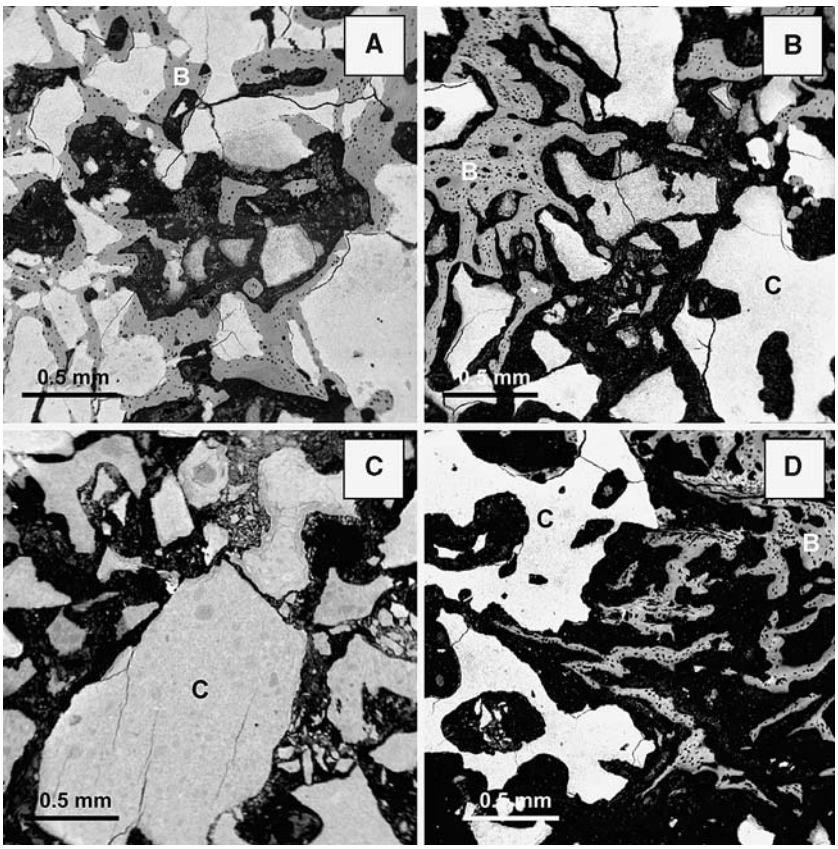

Fig. 3 BSEM micrographs at a higher magnification of : (**A**) TricOs group 6 weeks after implantation showing newly-formed bone in close contact with ceramic granules (B = bone, C = ceramic); (**B**) F group showing the newly-formed bone at about 50 µm from the surface of the ceramic 6 weeks after implantation (**C**) no $CaCl_2$ group, 6 weeks after implantation showing the small particles of degradation (**D**) low aprotinin group 3 weeks after implantation showing the bone growth at a distance from the surface of the ceramic (*On the images, B = bone, C = ceramic*)

Centripetal analyses

Regardless of the group (TricOs, F, without $CaCl_2$ and low aprotinin), ceramic distribution was homogeneous all along the depth of the bone defect.

For the *TricOs group* (Fig. 6), homogeneous bone colonization was observed at 3 weeks, with no evolution at 6 weeks. For the *F group* (Fig. 6), centripetal bone ingrowth from the outer margin of the defect was evident at 3 weeks with less bone in the center of the defect. Although less pronounced, this phenomenon was still present at 6 weeks. Compared to TricOs, a delay in new bone formation was observed in the depth of the bone defect. For the *no $CaCl_2$* and *low aprotinin groups* (Fig. 6), new bone formation occurred at the periphery of the bone defect. At 3 weeks, bone colonization was limited and appeared negligible in the center of the bone defect, up to a depth of 0.43 mm from the edge of the defect. At 6 weeks, the same tendency was observed, despite a slight increase in bone formation in the outer parts of the bone defect compared to 3 weeks. Compared to other groups (TricOs and F groups), a pronounced delay in deep bone colonization was observed at 3 and 6 weeks (Fig. 6).

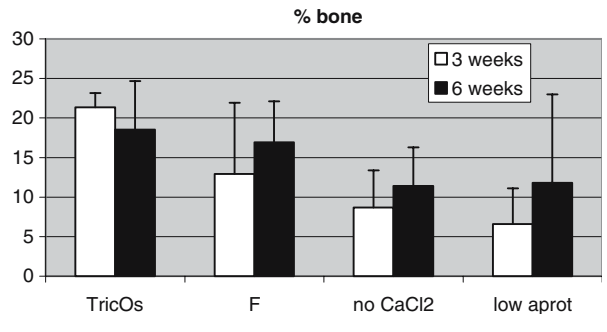

Fig. 4 Percentage of bone growth at 3 and 6 weeks in the different groups (TricOs, F, no $CaCl_2$ and low aprotinin). Bone neoformation appeared greater in the TricOs and F groups than in the no $CaCl_2$ and low aprotinin groups at 6 weeks

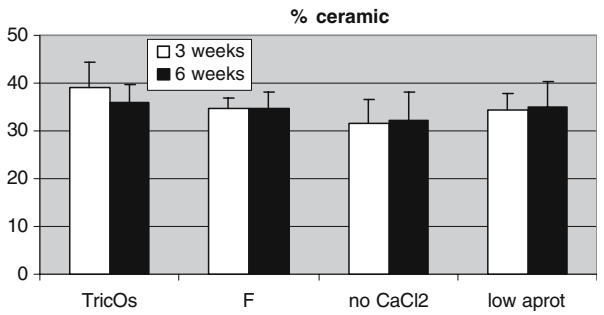

Fig. 5 Percentage of ceramic at 3 and 6 weeks in the different groups (TricOs, F, no $CaCl_2$ and low aprotinin). Regardless of the group and the delay of implantation, the ceramic charges are homogeneous

Discussion

The combination of fibrin glue and biphasic calcium phosphate ceramic used in this study made it possible to make the ceramic granules easier to handle with similar results in terms of global bone colonization. Our results showed that the stability of the glue is an important factor for the bioactivity of such a composite. Despite no significant difference, bone colonization appeared lower in the no $CaCl_2$ and low aprotinin groups. With lower calcium chloride or aprotinin addition peripheral bone colonization of the bone defect was seen less in the center of the defect, demonstrating the essential role of these two components in the stabilization of the clot and the bioactivity of such a composite.

Fibrin glues are based on a reaction between two main components, fibrinogen and thrombin. These two main components are associated with others such as plasmatic proteins and factor XIII for fibrinogen, calcium chloride and aprotinin for thrombin. These associations lead to the polymerization of soluble fibrinogen into insoluble fibrin with adhesive properties [10]. The polymerization is the result of the formation of covalent links between the fibrin fibers acting on the stability of the clot. Calcium ions are essential for the formation of the covalent bindings between the fibrinogen fibers and in this way act on the stability of the clot [10]. Our results showed that in the no $CaCl_2$ group, the lack of stability of the clot due to the absence of calcium chloride decreased the bioactivity of the composite.

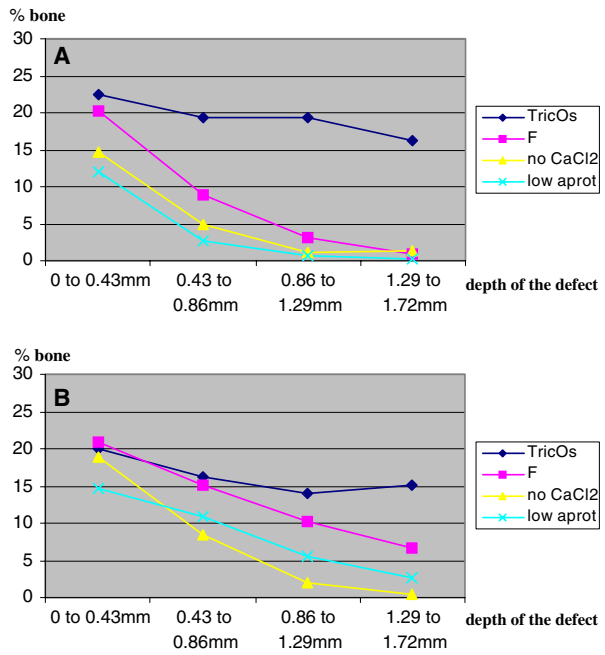

Fig. 6 Bone centripetry at (**A**) 3 and (**B**) 6 weeks. For the TricOs group, the bone colonization was homogeneous along the depth of the implant. For the F group, a slight delay was observed for bone colonization compared to the TricOs group. For the no $CaCl_2$ and low aprotinin groups, the bone colonization was limited to the periphery

Around the calcium phosphate ceramics, the environment was saturated in calcium ions released by pure chemical dissolution in contact with biologic fluids [2, 4, 31]. In a TricOs-fibrin glue composite, the calcium ions released by dissolution of the TricOs could be thought to provide the calcium ions supplied by the calcium chloride of the glue and play a role in the formation of the covalent bindings between fibrinogen fibers. For the no $CaCl_2$ group, the bioactivity was totally modified, suggesting that a high-performance, stable fibrin network was not obtained. The resulting modified structural arrangements led to a quicker degradation of the fibrin clot, meaning that it could not be used as a scaffold for the bone cells. This relatively rapid degradation of the fibrin glue does not allow cell adhesion and colonisation for bone neoformation. The calcium ions released appeared unable to make up for the calcium deficiency of the no $CaCl_2$ group. The calcium ions must be present during the binding reaction that takes place during the mixing of the components if the composite is to attain complete bioactivity. Dissolution studies have demonstrated that few minutes were necessary to objective a release of calcium ions from the ceramic [32]. This difference in timing may explain the weak fibrin net observed for the no $CaCl_2$ group. Moreover, in an in vitro study concerning the culture of bone marrow cells on biphasic calcium phosphate, the concentrations of calcium and phosphate ions in the cell culture medium were significantly lower in the presence of BCP ceramic as compared to plastic [33]. In this situation, it appears that biphasic calcium phosphate ceramics bind the calcium ions from the environment instead of releasing them. These two parameters may explain that the calcium ions necessary for the bindings of the fibrinogen fibers couldn't be supplied by the ceramic component of the composite. A high-performance fibrin net can be obtained only if the right elements of the glue are present in the right place at the right time.

A previous study [34] demonstrated that substituting aprotinin with another antifibrinolytic agent such as tranexamic acid, which acts by inhibiting plasminogen, binding to fibrin leads to approximately the same bioactivity of the composite. Our results showed that in the low aprotinin group, the bioactivity was statistically decreased. The aprotinin used in this study is an inhibitor of several serine proteases, such as plasmin, which degrades fibrin, and is also used to inhibit surrounding tissue fibrinolysis [35]. As a result, the aprotinin allowed the stabilization of the fibrin clot for a longer period. Experimental studies in non osseous sites have shown that in the presence of antifibrinolytic agents, the fibrin clot is maintained over 14 days before its natural degradation occurs. The

decreased bioactivity observed suggests that with an accelerated degradation of the fibrin clot, the collageneous network is build later and therefore cannot serve as a scaffold to enhance bone colonization. Further studies will be necessary to define the minimal aprotinin concentration needed to produce the same bioactivity.

The degradation of the ceramic between the groups with fibrin sealant was the same. In both groups there was higher resorption (10–15%) than with TricOs alone. The difference in bone ingrowth was due to modifications to the structure of the fibrin glue that essentially changed its biological properties.

This in vivo study of bone ingrowth in a calcium phosphate ceramic-fibrin glue composite was characterized by a lack of two components that have an influence on the stability of the clot. This indicates that a stable fibrin scaffold is crucial for bone colonization. Calcium chloride and an antifibrinolytic agent are necessary for obtaining a similar level of bone colonization compared to the TricOs-fibrin glue combination.

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