Macroporous biphasic calcium phosphate ceramics *versus* injectable bone substitute: a comparative study 3 and 8 weeks after implantation in rabbit bone

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Macroporous biphasic calcium phosphate ceramics (MBCP) and a calcium phosphate injectable bone substitute (IBS), obtained by the association of biphasic calcium phosphate (BCP) ceramic granules and an aqueous solution of a cellulosic polymer, were compared in the same animal model. The two tested biomaterials were implanted in distal femoral osseous defects in rabbits. Qualitative and quantitative histological evaluation was performed three and eight weeks after implantation to investigate bone colonization and ceramic biodegradation associated with the two bone substitutes.

Both biomaterials expressed osteoconduction properties and supported the apposition of a well-mineralized lamellar newly-formed bone. Bone colonization occurred much earlier and faster for IBS than for MBCP implants, although the respective rates of newly-formed bone after eight weeks of implantation did not differ significantly. For both biomaterials, ceramic resorption occurred regularly throughout the implantation period, though to a greater extent with IBS than with MBCP implants.

The associated polymer in IBS produced intergranular spaces allowing body fluids to reach each BCP ceramic granule immediately after implantation, which may have favored osteoblastic activity, new bone formation and ceramic resorption. This completely interconnected open macroporosity could account for the earlier and more satisfactory bone substitution achieved with IBS.

1. Introduction

Calcium phosphate ceramics are totally biocompatible, bioactive materials generally available as blocks or granules of dense or porous ceramics [1–3]. Macroporous blocks have proved brittle and difficult to sculpt and do not fit tightly to the surface of bone defects, and granules are difficult to handle and to keep in place after implantation. Calcium phosphate bone substitutes, especially in macroporous forms, show biodegradability and osteoconduction properties, and biphasic calcium phosphate (BCP) ceramics, an association of hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP), have proved to be efficient bone substitutes that respond well to material resorption/bone substitution events [4, 5]. Such BCP ceramics are now currently used for bone filling in spinal, tumoral, orthopedic and periodontal applications [3, 6–8].

Injectable biomaterials are considered to improve the biological behavior and enlarge the indications of calcium phosphate bone substitutes. A calcium phosphate injectable bone substitute (IBS), obtained by associating a biphasic calcium phosphate ceramic mineral phase with an aqueous solution of a cellulosic polymer, has recently been developed [9–13].

The present study compared the *in vivo* bone substitution process in macroporous biphasic calcium phosphate (MBCP) ceramics and a calcium phosphate IBS in the same animal model. Histological studies and scanning electron microscopy (SEM) were used to compare their respective biological efficiency after implantation in rabbit bone.

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2. Materials and methods

2.1. Biomaterials

2.1.1. BCP ceramics

For both materials, the mineral phase was composed of BCP ceramics with a 60/40 HA/β-TCP weight ratio. BCP was obtained by hydrolysis of commercial dicalcium phosphate dihydrate, calcination at 550 °C to form a calcium-deficient apatite (CDA) and subsequent sintering of the formed CDA to obtain BCP.

2.1.2. Macroporous BCP implants

Macroporous implants were manufactured by isostatic compression of the CDA powders after incorporation of porogen agents (naphtalen) and final sintering at 1150 °C. Macroporous BCP implants were 6 × 6 mm cyclinders, with a 50% starting macroporosity and a 565 µm mean macropore diameter. Implants were individually packed and steam-sterilized at 121 °C for 20 min.

2.1.3. Injectable bone substitute

The IBS was a composite biomaterial obtained by the association of a polymer and a calcium phosphate mineral phase composed of BCP granules. The granules were obtained after granulation of CDA powders and sintering at 1150 °C. The resulting BCP granules were sifted to select only those with a 200 to 500 µm diameter.

The carrier phase was a 3% aqueous solution of a cellulosic derivative polymer with total biocompatibility (hydroxy-propyl-methyl-cellulose = HPMC) [11, 14, 15]. The composite IBS was obtained by mixing the 3% aqueous HPMC solution with the selected BCP granules in a 50/50 weight ratio. The IBS was placed in ready-to-use glass flasks and steam-sterilized at 121 °C for 20 min.

2.2. *In vivo* implantations

Bilateral implantations were carried out on 12 mature female New Zealand White rabbits (3 to 3.5 kg). Lateral arthrotomy was performed at the knee joint, and a cylindrical defect was created at the distal femoral end. After saline irrigation, the osseous cavity was carefully dried and filled with a macroporous BCP implant or the injectable biomaterial.

After injection of the IBS, a macroporous BCP plug was positioned over the defect to close the femoral cavity and maintain the injected material in place [13]. Subcutaneous tissues and skin were closed in different layers.

Animals were implanted for three and eight weeks, providing six samples per biomaterial and implantation time.

For IBS, two others animals were implanted bilaterally in the same conditions and sacrificed immediately after surgery to provide four samples for control data relating to the initial quantity of implanted ceramic. For macroporous BCP implants, the percentages of starting macroporosity and implanted ceramic were determined before implantation on cylinder surfaces studied by SEM and image analysis.

All rabbits were sacrificed by an intracardiac overdose of sodium pentobarbital. Femoral extremities were immediately excised from the animals, fixed in glutaraldehyde solution and dehydrated in graded ethanol and acetone. Finally, non-decalcified bone specimens were impregnated and embedded in glycomethylmethacrylate.

2.3. Histological evaluation

Qualitative and quantitative studies were performed to compare the ceramic resorption-bone substitution process associated with each of the tested biomaterials.

For each sample, serial sections were cut perpendicularly to the drilling axis of the femoral cavity for light microscopy observations. Sections 10 µm-thick for IBS and 20 µm-thick for macroporous specimens were stained with solochrome-cyanine and hematoxylin-eosin. The resin blocks were then carbon-coated for SEM examination.

The rates of newly-formed bone and resorbed ceramic were evaluated quantitatively by image analysis (Leica Quantimeter 500, Cambridge, UK) of SEM observations of implant surfaces using backscattered electrons (Jeol JSM 6300 microscope, Tokyo, Japan). Successive analysis of the contiguous fields composing the whole implant area allowed us to determine the respective distribution of the three tissue components (BCP ceramic, soft tissues, newly-formed bone) and to express them as a percentage of the total implant area, according to a previously described method [16]. Results are expressed as the mean percentage $\pm$ SD of newly-formed bone, remaining ceramic and BCP degradation rate, for two surfaces from at least five specimens for both tested materials and implantation times.

Electron probe microanalysis (EXL Oxford Link System, Bucks, UK) was performed using an X-ray energy dispersive system from images of the implant surfaces obtained with backscattered electrons. This analysis compared the maturation state of newly-formed bone associated with both biomaterials three weeks after implantation. The main components of bone tissues (calcium, phosphorus, magnesium, sodium and oxygen) were studied and calcium phosphorus (Ca/P) ratio of newly-formed bone was determined.

For each tested biomaterial and implantation time, quantitative results for the percentages of newly-formed bone and remaining ceramic and for the BCP degradation rate were studied statistically by one-way analysis of variance followed by a post-hoc Duncan test. The Ca/P ratio of newly-formed bone was also compared in both materials after three weeks of implantation. p values < 0.05 were considered statistically significant.

3. Results

3.1. Qualitative studies

Both biomaterials expressed notable osteoconductive properties, and newly-formed bone was clearly identified in association with IBS and MBCP implants. On stained sections and SEM images, newly-formed bone appeared to be qualitatively similar for both IBS and MBCP, regardless of the implantation period. Well-mineralized, newly-formed lamellar bone developed in close contact

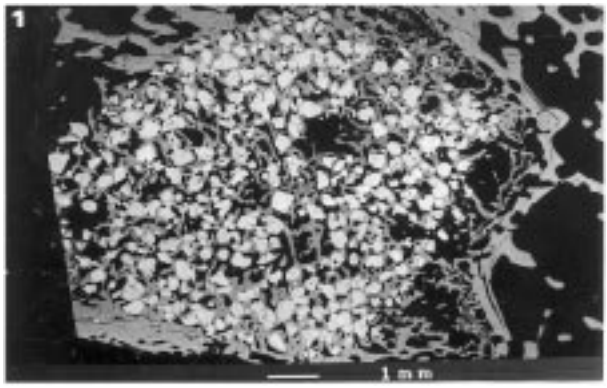

Figure 1 SEM of newly-formed bone in a femoral defect filled with IBS containing 200 to 500 µm BCP particles three weeks after implantation. New bone appears in gray, BCP ceramic in white and soft tissues in black. Centripetal new bone formation occurred both in close contact with the surface of the BCP particles and in intergranular spaces.

with the ceramic surface in MBCP implants and with IBS. However, with IBS, newly-formed bone was also observed far from BCP granules in intergranular spaces (Fig. 1). Bone ingrowth was quite homogeneous over the defect area three weeks after IBS injection, whereas newly-formed bone was only detected in the peripheral macropores of MBCP implants (Fig. 2a and b). After eight weeks, bone ingrowth was complete over all defects filled with IBS (Fig. 3a and b), whereas only a low amount of newly-formed bone was found on the surface of the central macropores of MBCP implants (Fig. 4).

3.2. Quantitative bone colonization

Bone ingrowth was more intense and faster for IBS than for MBCP during the first three weeks of implantation (Figs 1 and 2). The amount of newly-formed bone observed in bone defects filled with IBS after three weeks of implantation ($21.4\% \pm 4.7$) was similar to that obtained with MBCP implants after eight weeks ($22.0\% \pm 5.3$). Newly-formed bone in MBCP implants three weeks after implantation represented only $8.2\% \pm 2.2$ of the implant surface.

After three weeks of implantation, there was more than twice as much new bone formation with IBS as with MBCP implants ($p < 0.05$). With IBS, bone ingrowth remained nearly unchanged between three and eight weeks, so that the final newly-formed bone rates with IBS and MBCP were not significantly different (Fig. 5).

3.3. Quantitative ceramic biodegradation

On control animals implanted with IBS, BCP ceramic represented only $39.4\% \pm 0.6$ of the femoral defect surface immediately after implantation (four analyzed surfaces for each control implant), despite a 50/50 BCP/HPMC weight ratio. In macroporous implants, the initial quantity of BCP ceramic represented $50\% \pm 2$ of the implants surface, as determined by SEM and image analysis before implantation.

With IBS, ceramic biodegradation showed the same pattern as with macroporous implants, though to a higher extent. With IBS, the percentage of BCP ceramic decreased from $39.4\% \pm 0.6\%$ to $33.2\% \pm 3.6$ after three weeks and to $31.2\% \pm 3.8$ after eight weeks. With MBCP implants, the percentage of ceramic decreased from $50\% \pm 2$ to $46.8\% \pm 2.9$ after three weeks, to $45.5\% \pm 2.0$ after eight weeks.

With IBS, BCP degradation appeared faster than with MBCP. After eight weeks of implantation, the BCP degradation rate for IBS was more than twice as high as that for MBCP implants (22% *versus* 9%, $p < 0.05$) (Fig. 6).

3.4. Microanalysis

EDX microanalysis confirmed that bone colonization was accelerated with IBS as compared to MBCP implants. Three weeks after implantation, the Ca/P ratio of newly-formed bone for IBS was similar to that of normal host bone, but significantly lower for MBCP implants (Table I).

4. Discussion

This study compared the biological behavior of massive macroporous BCP ceramic and an injectable particulate one. In both IBS and MBCP implants, bone colonization progressed from the periphery of the defect toward the center. With IBS, bone ingrowth was homogeneous over the entire implantation site after three weeks, whereas with MBCP implants only a very small amount of newly-formed bone was observed in central macropores after

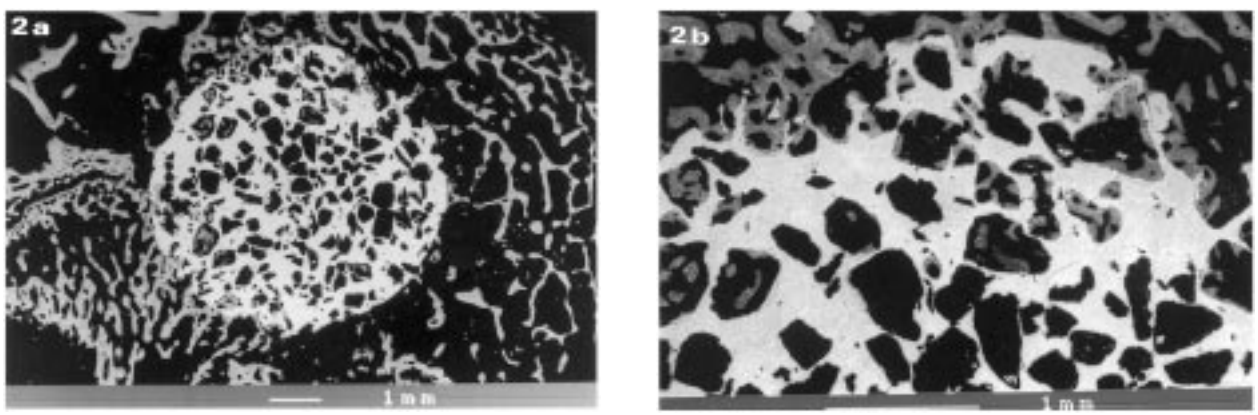

Figure 2 SEM of newly-formed bone in a femoral defect filled with a MBCP implant three weeks after implantation. (a) General features of the implant. (b) Higher magnification image of bone ingrowth. Newly-formed bone occurred only on the surface of the peripheral macropores.

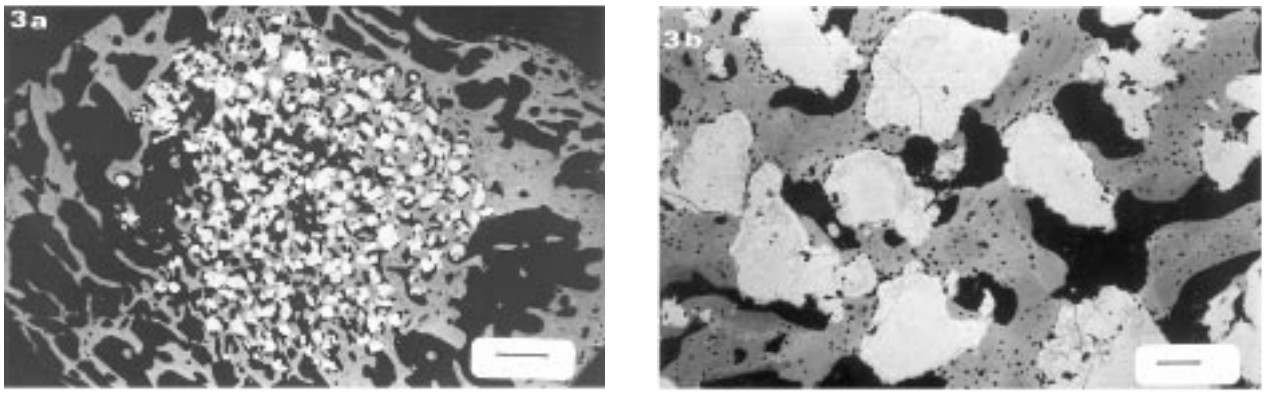

Figure 3 SEM of newly-formed bone in a femoral defect filled with IBS containing 200 to 500 μm BCP particles eight weeks after implantation. (a) New bone formation is homogeneous throughout the defect area (bar: 1 mm). (b) New bone formation is particularly important in the center of the defect (bar: 100 μm)

three and eight weeks of implantation. With IBS, all cellular and consecutive tissular processes seemed to have developed during the first three weeks of implantation, and bone ingrowth did not increase between three and eight weeks of implantation. The newly-formed bone rate obtained with both tested materials was not significantly different after eight weeks of implantation.

It seems that the superiority of IBS is not really due to greater bone ingrowth but to its capacity to undergo early and intense bone substitution in early implantation times, as confirmed in SEM and EDX microanalysis. This capacity, which seems to be related to specific cellular events we already reported [17, 18], suggests that IBS is more favorable than massive implants for these events as well as consecutive ceramic resorption-bone substitution.

A previous study performed on IBS showed that BCP particles were not completely degraded after long-term (78-week) implantation. Ultrastructural studies indicated that HA was the remaining calcium phosphate phase. This suggests that a biphasic ceramic similar to the one used here can associate sufficient bioactivity and biodegradability with adequate *in vivo* stability to allow extensive and viable bone substitution [4, 19].

Many composite calcium phosphate biomaterials are now being investigated in order to broaden the applications of calcium phosphate ceramics. HPMC has been proposed to improve the injectability, cohesion after injection and mechanical properties of calcium phosphate cements [20, 21]. The addition of fibrin to HA bone cement favors the setting reaction of the resulting composite, whereas the real biological influence of fibrin on new bone apposition on the cement surface does not appear to be significant since the cement remains hermetic to biological fluids [22]. Although the addition of gelatin to such cements improves their viscosity and injectability and preserves their physicochemical properties, the biological advantages are not

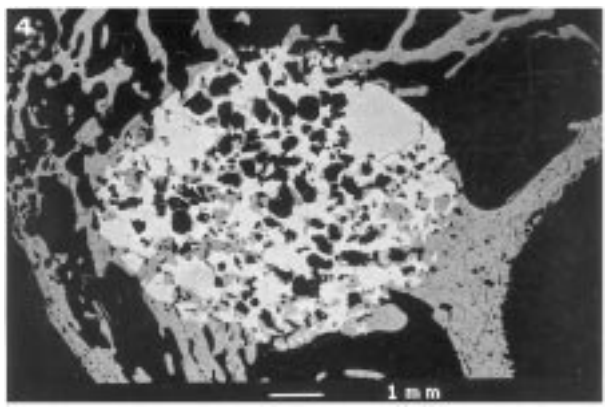

Figure 4 SEM of newly-formed bone in a femoral defect filled with a MBCP implant eight weeks after implantation. A small amount of newly-formed bone was observed on the surface of the central macropores.

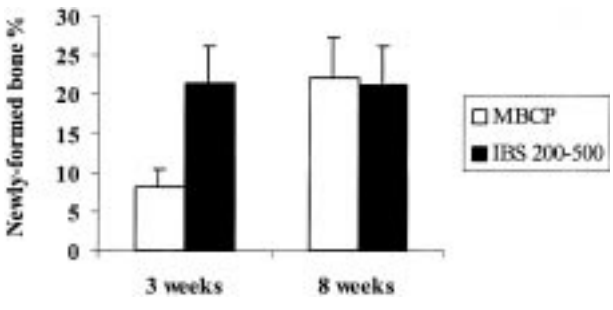

Figure 5 Changes in the rate of newly-formed bone within femoral defects filled with the two tested biomaterials. Results are given as the percent age of newly-formed bone (mean ± SD) relative to the total implant area for the different implantation periods. With IBS, bone ingrowth was significantly greater than with MBCP implants after three weeks, but the newly-formed bone rate obtained with both tested materials was not significantly different after eight weeks of implantation.

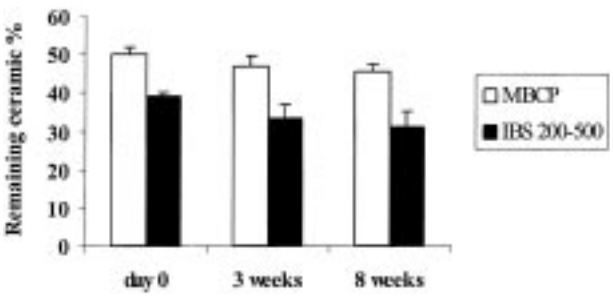

Figure 6 Changes in BCP ceramic percentage within the femoral defects filled with the two tested biomaterials. Results are given as the percent age of remaining ceramic (mean ± SD) relative to the total implant area for the different implantation periods. With IBS, approximately 40% of the defect was filled with BCP granules immediately after injection and the percentage of remaining ceramic significantly decreased from the implantation to eight weeks.

TABLE I Calcium-phosphorus ratio in host bone and in newly-formed bone 3 weeks after implantation. Results are means ± SD for 10 points in host bone or newly-formed bone per implant (Ca/P in weight ratio; * = $p < 0.05$ compared with host bone)

	Host bone	Newly-formed bone in MBCP implants	Newly-formed bone in IBS 200-500
Ca/P	2,16 ±0,05	2,05* ±0,07	2,17 ±0,10

clear [23]. The addition of 500 to 1000 μm β-TCP granules to calcium phosphate cements has been proposed to improve their osteoconductive properties and bone colonization [24, 25]. Finally, the presence of a resin acrylic organic matrix or bioglass particles in calcium phosphate bone cements improved their bone-bonding properties and mechanical resistance, but had no significant influence on biological integration and progressive substitution by newly-formed bone [26, 27].

A rate of newly-formed bone similar to that obtained with our IBS was reported 12 weeks after femoral defects were filled with calcium phosphate bone cements [28]. However, these bone defects were smaller and thus more biologically favorable to bone ingrowth. More surprisingly, these authors observed a rate of newly-formed bone with MBCP implants of only 10% after 12 weeks compared to our rate of 20% after eight weeks. This could have been related to a smaller mean macropore diameter (200 μm *versus* 565 μm in our study).

In our experiment, an IBS associating HPMC polymer and BCP granules in a 50/50 weight ratio was expected to provide a biomaterial with 50% macroporosity and an initial ceramic level of 50%. However, measurements of ceramic quantity showed that BCP represented only 40% of the femoral defect volume, as determined by SEM and image analysis on serial sections from femurs of control animals. Thus, the *in vivo* behavior of the tested IBS should have been closer to that of a macroporous BCP implant with 60% macroporosity than to that of the macroporous implants we used. As the macroporosity parameters for optimal bone colonization had been determined in an earlier study on macroporous implants [16], we attempted to define the most adequate amount of BCP in IBS required for optimal bone substitution and conserved injectability.

Recent studies have found that the mechanical properties of calcium phosphate cements after implantation are equal to or greater than the properties of the trabecular bone and porous BCP [28, 29]. Our IBS displayed bioactive properties, but mechanical properties were inadequate immediately after implantation. Bone ingrowth can only provide mechanical properties once it is homogeneously achieved throughout the defect area. Bone ingrowth in defects filled with IBS seemed to provide an osseous architecture with progressive but early mechanical properties, as suggested by quantitative measurements of newly-formed bone and SEM images. The mechanical characteristics of this restored bone structure now need to be determined.

The potential biological effect of associated HMPC polymer on ceramic resorption-bone substitution process is still uncertain although several studies have shown its perfect biocompatibility for bone substitution applications [11, 14, 17]. However, the IBS composite exhibits physicochemical changes that seem to modify both polymer and calcium phosphate mineral phase, without producing any obvious adverse effects on biological performance [10, 30]. An association of HPMC polymer with 200–400 μm BCP granules has been reported to promote the release of single calcium phosphate crystals and induce a consecutive adverse inflammatory reaction not observed in our experiment [19].

Initial open interconnected macroporosity does not exist in our MBCP implants although macropores are interconnected by micropores, thereby allowing the circulation of body fluids and contributing to the progressive degradation of the ceramic that leads to secondary interconnected macroporosity after implantation.

With IBS, the polymer produced spaces between the injected BCP particles. Despite its fluid consistency, the IBS displayed osteoconductive properties slightly different from those usually observed with macroporous implants [3, 16]. Newly-formed bone not only grew in close contact with the ceramic surface but also in the intergranular spaces. The macroarchitecture of the IBS provided an adequate structure for body fluid circulation, tissue ingrowth and bone colonization. These results appear to be confirmed by a recent study involving implantation of 100 to 300 μm HA particles in rabbit bone defect in which intergranular spaces smaller than 100 μm were necessary to favor bone ingrowth [31].

For IBS, the influence of the interconnective quality conferred by the associated polymer was also apparent on ceramic degradation at eight weeks. The ceramic surface in contact with biological fluids and available for both dissolution and degradation cellular activity was much greater for IBS than for MBCP.

5. Conclusion

This study confirms that BCP particles carried by a cellulosic polymer can conserve bioactivity and are conducive to earlier and more extensive bone substitution than massive ceramics. Early biological efficiency was better with IBS than with MBCP. IBS conserved *in vivo* bioactivity and bone-filling ability and provided suitable injectability.

The presence of intergranular spaces in the IBS seemed particularly favorable since each BCP granule can be reached by body fluids immediately after implantation, which was apparently conducive to neo-angiogenesis, cellular colonization by osteoblastic cells from bone marrow and the resulting bone substitution process. Thus, the IBS seemed to function as a completely interconnected ceramic with total open macroporosity.

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