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In vivo evaluation of an injectable Macroporous Calcium Phosphate Cement

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Abstract Although Calcium Phosphate Cements (CPC) are highly biocompatible and osteconductive materials, its resorption rate still remains too slow for some applications. In this work the introduction of Macroporosity in an injectable CPC is evaluated as a way to accelerate resorption and to increase bone ingrowth. A Macroporous and a standard CPC were injected just after preparation in a defect drilled in rabbit femur for their *in vivo* evaluation. The foaming agent used was Albumen, which gave up to a 75% porosity. Sodium Alginate was added to promote the cohesion of the foamed paste after implantation. In the case of the Macroporous Cement, bone growth and neovascularisation was observed inside the pores of the material, not only at the margins of the cement but also in some central pores. After 12 weeks of implantation, the residual material volume of the Macroporous Cement was approximately 35% of the initial value, whereas only the outer layers of non-Macroporous CPC were resorbed, being the residual material volume close to 100%. The higher resorption rate was due to the higher surface contact with body fluids which increased the dissolution rate, and to the enhancement of the cellular activity

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1 Introduction

The use of Calcium Phosphate materials as bone substitutes has increased in the last years [1–3]. Although the autologous bone grafts remain as the gold standard, the low disponibility and the morbidity of the patient donor site have made Calcium Phosphate materials the best bone substitutes for certain applications, as bone grafting, bone fillers in trauma, fracture repair or in dental applications. Their chemical structure close to bone mineral, their osteoconductivity and osteoinductivity, the ability to be resorbed by the organism and the biocompatibility of the degradation products as a source of calcium and phosphate ions in the implant site, make them very suitable biomaterials.

The use of a malleable paste that fills and adapts to the bone defect and that can be injected trough a nozzle is of great advantage in applications in minimal invasive surgery. In this field, there are actually two main strategies in Calcium Phosphates: the use of Hydroxyapatite or Biphasic Granules in a polymer gel matrix. or the use of self-setting Calcium Phosphate Cements (CPC) which are able to set and harden within the body [4–6]. Although there are some CPC formulations in the market, there is still much ongoing research directed mainly to improve their mechanical properties and to obtain a faster resorption rate. The incorporation of drugs or bioactive molecules is also a promising field which is being explored [3, 7, 8].

CPC are highly reactive and indeed very porous materials (the porosity ranges generally between 40–50%). However, they still present a very low resorption rate, specially apatitic cements [9, 10]. It has been shown for instance that some CPC could remain as long as 78 weeks when implanted in dog femurs [11]. The bone tissue grows on the surface of the cement, but is unable to penetrate inside the material,

due to the small size of the porosity in the order of some micrometers.

In this sense, it has to be clarified that the terms micro and macroporosity used in this field differ from that proposed by IUPAC (International Union of Pure and Applied Chemistry) in relation to the characterization of porous solids used in other fields such as catalysis, where macroporosity is applied to pores above 50 nm, mesoporosity to pores with diameter ranges between 2 and 50 nm and microporosity to pores smaller than 2 nm. CPC have an intrinsic porosity produced by the spaces created between the precipitated crystals, which generally are smaller than few microns, and which we will identify as microporosity. The porosity introduced by the foaming agent, which is larger in size, will be referred to as macroporosity. The starting hypothesis of this study is that the introduction of macroporosity in CPC could enhance its resoprtion rate by allowing the bone tissue to grow inside the material.

There are different techniques to produce interconnected macroporous ceramic bodies. However, most of these techniques are not applicable in the case of the CPC. Most of them need either the elimination of the porous material during sintering or they use toxic materials. For CPC, the requirements to produce a porous body are quite demanding as they have to be prepared *in situ* and the cements have to withstand the presence of liquid medium without disintegrating before setting. Some attempts have been made to introduce macroporosity in CPC by using soluble particles [12, 13], resorbable polymers [14], fast resorption phases [15] or foaming agents [16, 17].

In previous studies, it was shown that an injectable Macroporous CPC can be obtained by using a natural foaming agent, Albumen, the mixture of proteins that form the egg-white [16, 18]. The purpose of this work was to characterize the material and to evaluate the *in vivo* performance of this injectable Macroporous CPC.

2 Materials and methods

2.1 *In vitro* study and characterisation

2.1.1 Materials preparation

The CPC consisted of α-Tricalcium Phosphate with 2% of Precipitated Hydroxyapatite (PHA, Merck, Ref. 2143) as seed material. The α-Tricalcium Phosphate was prepared by heating at 1400°C a mixture of $CaCO_3$ (Sigma-Aldrich C-4830) and $CaHPO_4$ (Sigma, C-7263), followed by quenching in air and milled as described elsewhere [19]. For the *in vivo* studies the CPC powder was sterilized with γ-rays at 25 kGrays.

Table 1 Compositions of the different CPC studied

Sample	Sodium alginate in the Liquid phase	Foaming agent (14wt%)	
1	−	−	Non foamed
2	+	−	Non foamed
3	−	+	Foamed
4	+	+	Foamed
5*	+	+	Non foamed

*The Group 5 is equal to Group 4 but unfoamed.

The liquid phase was a 1% Na_2HPO_4 (Merck 1065860 500)solution, and in some formulations 1% Sodium Alginate (Panreac 373059) was added as cohesion promoter, to avoid disintegration of the cement when immersed in water. The liquid to powder ratio was L/P = 0.40 ml/g.

The foaming agent used was dehydrated Albumen (Igreca), which was mixed with distilled water in a ratio 1:7 to give a 12.5 wt% which is the protein content in natural Albumen. The foaming of the egg white was made by mechanical stirring with the use of a domestic food mixer. The porous samples were obtained by gently mixing the cement paste with the foamed Albumen as described elsewhere [18]. Table 1 summarizes the different cement formulation characterized in terms of setting and hardening properties and cohesion behavior. The rationale was to analyze the effect of each additive used either as a cohesion promoter (Sodium Alginate) or as a foaming agent (Albumen).

For the in vivo studies, the different liquid phases were sterilized by filtering through a 0.22 μm membrane (Millex GP-Millipore). The egg white solution was sterilized with the aid of a Stericup Filter (Millipore, SC00B02) under vacuum in a laminar flow cabin. Its protein content after filtration was determined with a Micro BCA Protein Reagent Kid (Pierce), being finally 6 wt%.

2.1.2 Cohesion, setting and hardening of the CPC

The Cohesion Time of the samples was studied by immersing samples of 12 mm diameter and 6 mm height in Ringers Solution at 37°C as described elsewhere [20]. In order to evaluate the evolution of the mechanical properties and the setting kinetics of the different CPC, samples of 6 mm diameter and 12 mm height were prepared. The evaluation times were 2, 8, 24, 72 and 128 h. The samples were tested in compression, in a universal testing machine at a crosshead speed of 1 mm/min. Just after the mechanical test, the samples were immersed in acetone to stop the setting reaction and then dried. Two samples were kept for microstructural characterization by Field Emission Microscopy (FEM) and the other samples were crushed to perform XRD. The reaction kinetics was studied by quantifying the different phases

occurring in the cement on the basis of an external standard method [19].

2.1.3 Porosity and microstructure characterization

The porosity of the samples were characterized by means of mercury picnometry as described elsewhere [12, 17]. The macroporosity morphology was characterized by FEM after coating the samples with graphite. Pore size distribution and interconnection was measured by mercury porosimetry (Micromeritics Autopore IV 9500), which allows detecting the open porosity in the range 0.003–350 μm.

2.2 *In vivo* experiments

2.2.1 Surgical procedures

36 Adult Female New Zealand rabbits of 8 months and 5.5 kg were used in this study, distributed in two groups, one for the Dense Cement and the other one for the Macroporous Cement. The implantation times were 1, 4 and 12 weeks. 6 rabbits were used for each group and implantation time. As a control, a cavitary defect with the same geometry was drilled in the contralateral femur of the rabbits used for the Dense Cement group, where no material was implanted.

The animals were induced general anesthesia by injection of medetomidina (50 μg/kg IM) and ketamine (25 μg/kg IM). Isofluorane at 4% was used as inhalated anesthesiscs. After shaving and disinfecting, the femoral condyles were exposed by a lateral longitudinal incision. A critical size defect of 6 mm diameter was created in the distal part of the femur with a refrigerated drill to avoid necrosis. A defect of 6 mm was chosen as it does not undergo spontaneous healing [21]. Defects were filled by injection trough a 2 mm nozzle with the two finally chosen cements. After filling the defect, the muscle, the subcutaneous tissue and the skin were closed in layers. The animals were sacrificed with an overdose of sodium pentobarbital.

The use and handling of the animals was performed according to the European Union Guidelines for the Care and Use of Laboratory Animals (86/609/CE).

2.2.2 Histological preparation of the samples

The implants were cut using an autopsy saw. The surrounding tissues were removed and the samples were fixed 1 week in 10% formol. The samples were dehydrated in different graded ethanol series (70–100%), and infiltrated with 4 different graded mixtures of ethanol and infiltrating resine, glicometacrilate (Technovit 7200®, VLC - Heraus Kulzer GMBH) with 1% of Benzoyle Peroxide (BPO®**:** Heraus Kulzer GMBH). The last infiltration was perfomed with pure infiltrating resine under vacuum. The samples were then polymerized, first under low intensity UV light for 4 h, followed by a polymerization under high intensity UV light for 12 h and finally by keeping the samples heated for 24 h to assure complete polymerization.

The samples were glued to a sample holder. Longitudinal sections of 200 μm were cut with a band saw (Exakt 400, System, Aparatebau GMBH), and were polished with 1200 and 4000 silicon carbide papers (Exakt-Micro Griding System®) until a samples thickness of 70 μm was obtained. The embedded specimens obtained from the histological preparation were mounted on a scanning electron microscope (LEO-435VP).

2.2.3 Histomorphometric studies

For the histomorphometric studies, the samples were stained with Lévai Laczcó staining and Kossa staining. The samples were studied using a current optical microscope clouped with a digital camera. The images were analyzed with an Olympus Micro Image 1.0 software. The definitions of the different parameters are given below (Histomorphometry Nomenclature Committee upon request of the American Society for Bone and Mineral Research [22]):

- Implant section area (ISA): is the surface of the formed bone defect during the operation.
- Bone tissue volume (BTV): is the area occupied by the trabeculae in the osseous walls of the defect (mm^2).
- Proportion of tissue penetration (PTP): measures the mean growth of the trabecula from the periphery to the center of the defect (mm).
- Residual material volume (RMV) is the surface occupied by the non-rebsorbed implant (mm^2).
- Osteoid tissue volume (OV): obtained by multiplying the Osteoid total length by the Osteoid medium width (μm^2).

2.3 Statistical analysis

The statistical analyses were performed with the aid of the SPSS 12.0 software for windows. The two-way analysis of variance was chosen. When any statistical significance was found the Tukey test was chosen for multiple comparisons.

3 Results

3.1 Cohesion, setting and hardening of the CPC

Groups 1 and 2, which were non-foamed CPC, had a good cohesion behavior. They could be immersed in water immediately after preparation without suffering disintegration. However, when foamed Albumen was added to the cement paste (Group 3) the macroporous paste obtained was not stable

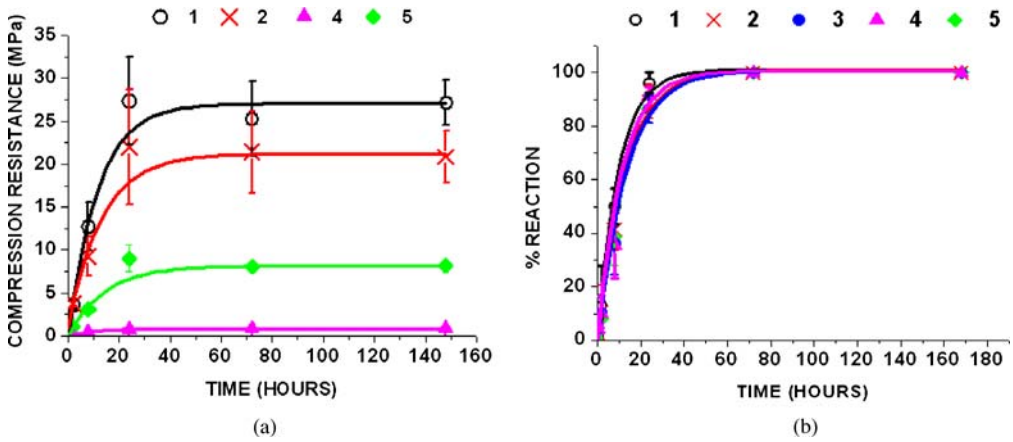

Fig. 1 (a) Evolution of the Mechanical Properties with the time. (b) Evolution of the setting reaction of the different CPC with time

after immersion and it disintegrated. The addition of Sodium Alginate reversed this situation, and the foamed cement paste obtained in Group 4 had a good cohesion, maintaining the foamed structure also when it was immersed in water immediately after mixing.

The evolution of the mechanical properties and the reaction kinetics is shown in Fig. 1. The maximum strength was reached at 24 h after immersion in Ringers solution, corresponding to approximately 80% of the degree of reaction. There was no significant delay in the reaction kinetics due to any of the different additives, Albumen or Sodium Alginate. A slight decrease in the compression resistance could be observed in the non-macroporous samples (Group 2) due to the use of Sodium Alginate. The decrease in Groups 4 and 5 was mainly due to the presence of a high macroporosity in these samples, even if group 5 had not been mechanically foamed. Although the setting reaction of the samples of Group 3 could be monitored, they could not be tested mechanically, since due to their poor mechanical properties, they were broken on demolding.

3.2 Porosity

The density, macro and microporosity values for the different samples studied by mercury picnometry are reported in Table 2. The porosity of Groups 1 and 2 is due to the microporous structure of the CPC. The porosity of Group 5, even if

Table 2 Apparent density (D_{app}), macroporosity (P_{macro}), total porosity (P_{total}) and Compressive strength (C) .Standard deviation between brackets

Sample	D_{app} (g/cm^3)	P_{total} (%)	P_{macro} (%)	C (MPa)
1	1.63 (0.07)	48.03 (2.32)	–	27.16 (2.63)
2	1.64 (0.05)	47.57 (1.66)	–	20.99 (2.92)
4	0.81(0.04)	74.141 (1.34)	49.51 (2.55)	0.86 (0.26)
5	1.17(0.04)	62.72 (1.17)	28.27 (2.53)	8.15 (0.77)

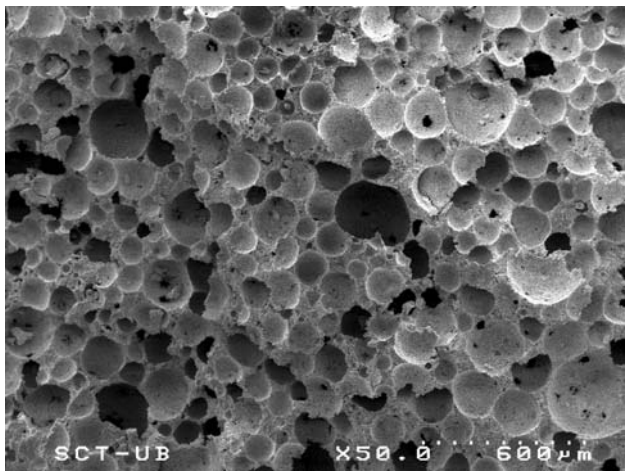

Fig. 2 SEM micrograph of a Macroporus Cement (Group 4)

Albumen was not foamed, was attributed to the air entrapped during mixing the powder with the liquid phase, showing the good foaming properties of Albumen. Group 3 samples could not be measured due to the reasons mentioned above.

Figure 2 shows the morphology of the macropores obtained by mixing the foamed Albumen with the cement paste. The obtained pores are spherical, reproducing the structure of the Albumen foam. The diameter of the pores falls between 100–300 μm although bigger pores could be observed; some of the pores are interconnected as can be seen in the picture, through small windows usually less than 50 μm. Mercury porosimetry confirmed these results as shown in Fig. 3.

After the *in vitro* experiments, a Microporous or Dense CPC (Group 2) and a Macroporous CPC (Group 4) were chosen for the *in vivo* study.

3.3 Histomorphometry

Figure 4 shows the evolution with implantation time of the different histomorphometric parameters. No bone healing

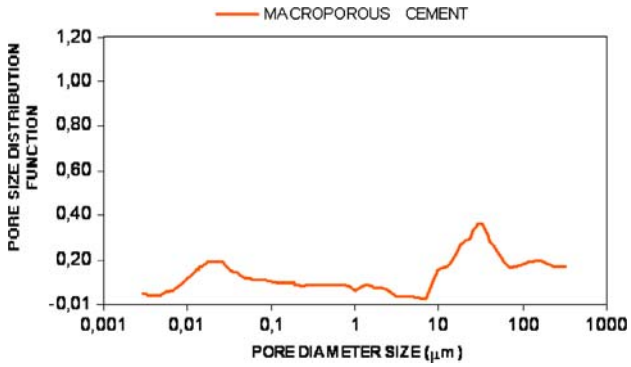

Fig. 3 Pore size distribution function vs. entrance pore diameter of Macroporous Cement as determined by mercury intrusion porosimetry

could be observed in the control defect due to the critical size, although bone remodeling and new bone formation could be observed in the border of the defect.

The BTV was similar in both cements and significantly higher than in the control at 1 and 4 weeks. No statistically significant differences ($p < 0.05$) were found between the three groups at 12 weeks. The PTP and OV for the Macroporous and Dense Cements increased in all the time periods, but the increase was more pronounced for the Macroporous Cement. Statistically significant differences were found in the PTP at week 12 between the Macroporous and the Dense Cement. No statistically significant differences were observed between both cements in the OV values, although the same tendency as in the PTP was observed. The RMV decreased with time for both cements, smoothly for the Dense Cement and much more drastically for the Macroporous one, being the differences between both cements statistically significant at all time periods. These results indicate that the Macroporous Cement presented higher material degradation and proportion of osseous tissue penetration.

3.4 Qualitative histology

Figure 5 shows a Control Defect, a Dense Cement and a Macroporous Cement after twelve weeks of implantation. It can be observed that no bone was formed in the case of the control. The Dense Cement showed bone growth on the surface but almost no resorption. The Macroporous Cement showed much more resorption and bone growth. It can be observed also that the macroporosity was not homogeneously distributed in the Macroporous Cement, and that

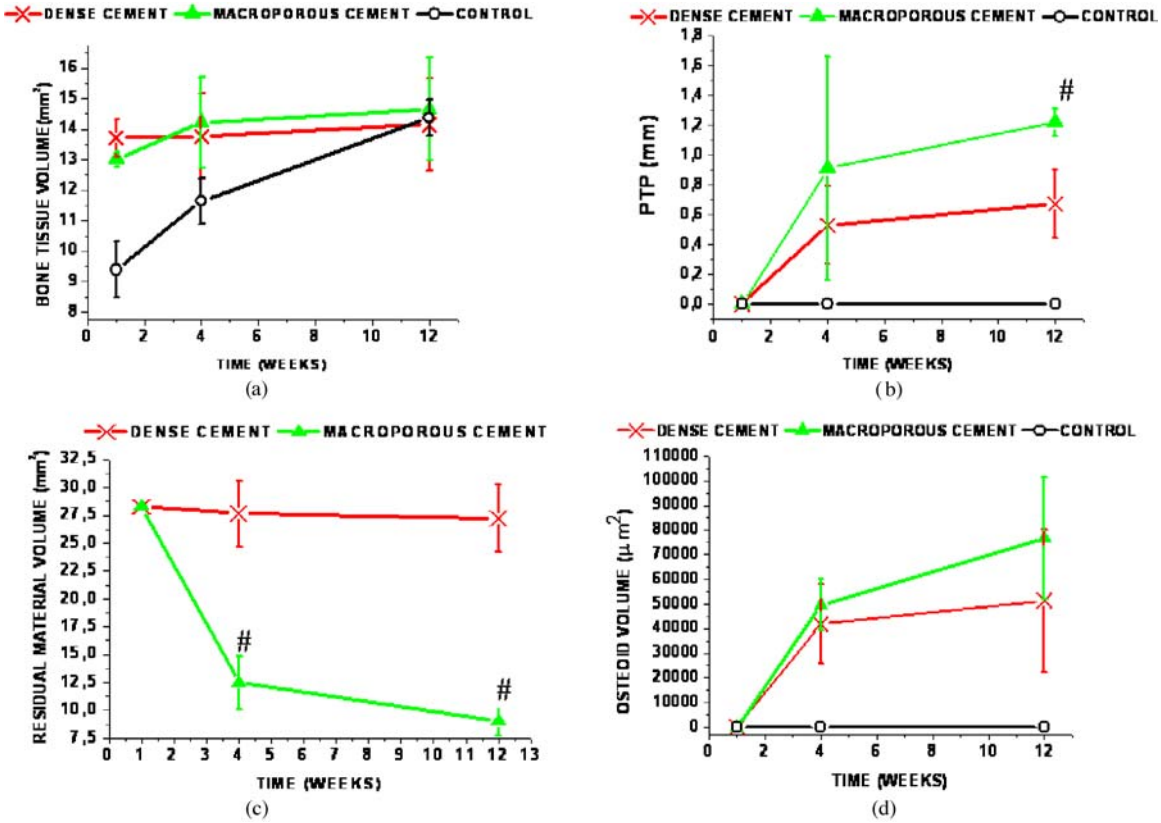

Fig. 4 (a) Bone Tissue Volume (BTV), (b) Proportion of Tissue Penetration (PTP), (c) Residual Material Volume (RMV) and (d) Osteoid Volume (OV) for the Dense and Macroporous Cements and the Control Defect. The symbol # indicates the time points where statistically significant differences were found between the Dense and the Macroporous Cement

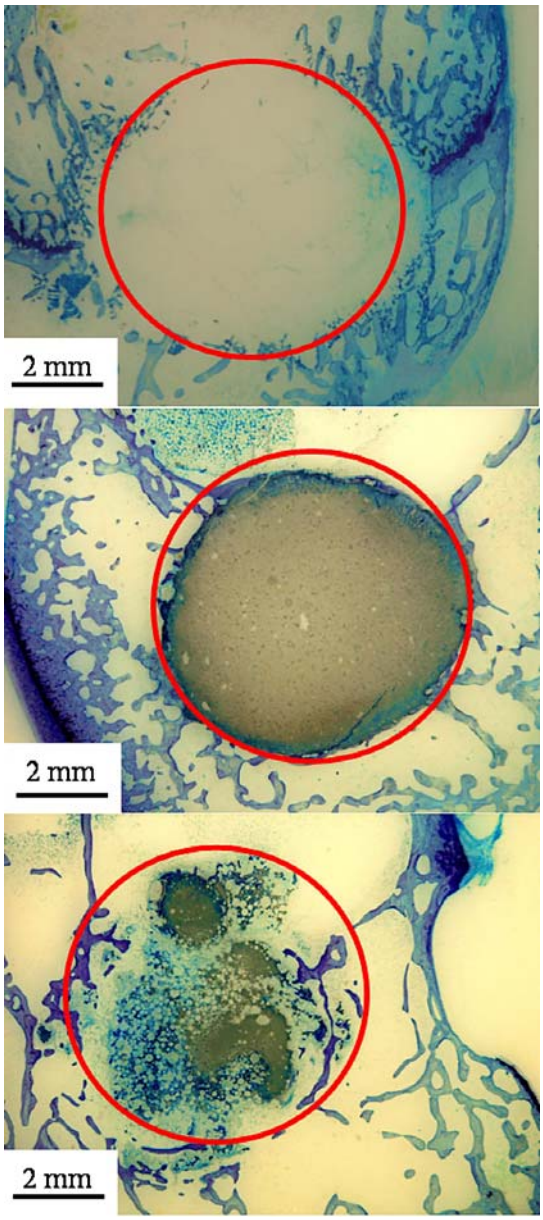

Fig. 5 (a) Control Defect at week 12, No new osteoid tissue is observed after bone remodelling process. The defect became permanent (b) Dense Cement at week 12, where almost no resorption has occurred. (c) Macroporous Cement at week 12, the tissue penetration and the resorption of the cement can be observed. The circle indicates the size of the original defect (stereoscopic microscope images)

some regions were formed. This is due to the preparation and injection process in the operation theater, which should be optimized.

Bone tissue remodeling was observed in the walls of the control defect at week 1, associated with active osteoclastic activity. Osteoid tissue was found at week 4 always near the walls of the defect but almost no bone growth towards the center of the defect was observed. By week 12, the osteoid tissue was not present, since it underwent bone remodeling and calcification (Fig. 5).

No inflammatory reaction was detected at any time period in any of the Cements implanted. Although at week 1, a small gap was seen between the cement surfaces and the bony wall of the drilled hole, no presence of fibrous tissue could be observed.

At week 4, new bone was formed on the surface of the Dense Cement. Active osteoblasts with cuboidal morphology were observed on the surface of the new bone as well as the presence of blood vessels near the surface of the cement. A degradation zone could be already observed at week 4 on the surface of the cement, with the presence of macrophages. Although at week 12, the degradation process continued, it was still on the surface of the implant, with almost no degradation in the bulk, as it was quantified by histomorphometry. SEM observations revealed the formation of a calcium-phosphate (CaP)—rich layer on the periphery (Fig. 6) which was stable in the short term, and acted as an osteoconductive mold for new bone formation.

In the Macroporous CPC, bone growth and new blood vessels were observed on the surface and also inside the pores, at week 4. This bone growth inside the pores was not homogenous through the entire surface, being more pronounced in the outer region. The resorption of the Macroporous Cement was already visible at week 1. A degradation zone was observed on the surface of the cement. At week 4, the resorption of the outer region was much more pronounced in a centripede way, and numerous granules could be observed. Numerous neoconstituted trabeculae were observed in the periphery of the defect in continuity with the progenitor bone. Bone tissue surrounding the granules was observed and macrophages were also present. Sometimes multinucleated cells were observed (Fig. 7).

By week 12, it was difficult to distinguish the new trabeculae in the periphery from the osteoprogenitor bone. In the central zone, where the osteogenesis process was more active, abundant blood vessels and osteoid were observed, with cubic osteoblasts. Nevertheless, no complete resorption of the cement was achieved, mainly due to the dense zones present.

4 Discussion

This study, to our knowledge, is the first that reports about the *in vivo* performance of a Macroporous Cement prepared and injected *in situ*. In other works the cement used was not prepared *in situ*, but implanted as an already set sample [23, 24].

For this purpose, the cohesion behavior and the setting reaction were studied, to ensure the performance of the cement once *in vivo*. One of the major risks of CPC is its disintegration or its inability to set once in contact with body fluids, that could elicit an inflammatory response [25, 26]. The

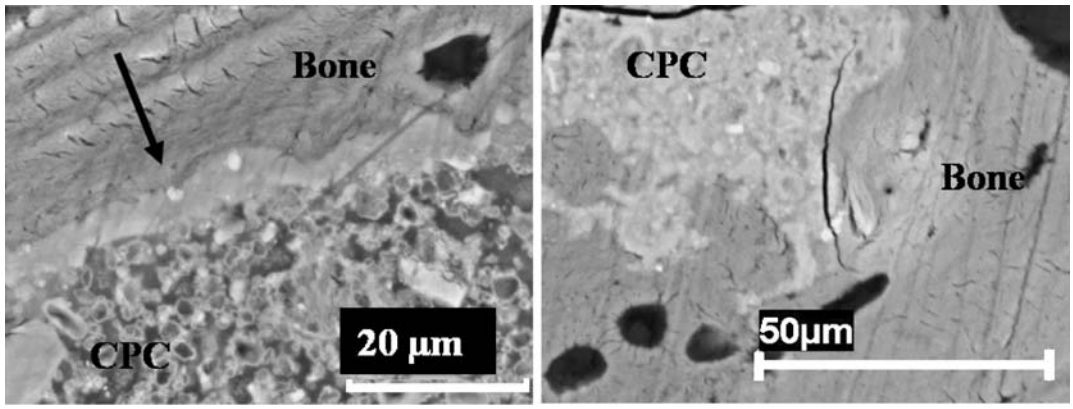

Fig. 6 (a) Interface of the Dense Cement and the Bone tissue. Arrow indicates the apatite layer formed *in vivo* (week 12). (b) Interface of the Macroporous Cement and the bone tissue

Macroporous Cement used in this experiment has to withstand the blood pressure to avoid pore collapsing and also needs a high resistance to water penetration and disintegration before the setting reaction is complete. The addition of Sodium Alginate improved the cohesion behavior and thus it could be injected directly after preparation. None of the additives used, Sodium Alginate or Albumen, produced a significant delay in the setting reaction *in vitro*.

The study also shows the good osteoconductivity of CPC materials. The 6 mm defect proved to be critical in size, as the control did not heal spontaneously. Formation of fibrous tissue and inflammatory response, which have been reported in apatitic cements in some subcutaneous or intramuscular implantation [27] and also in brushite cements [15, 28], were not observed in this experiment. None of the additives used elicited a negative response. Even if the cement contained Albumen, a mixture of exogenous proteins, no immunogenic response was detected.

Osteoid tissue was found to grow in close contact with both cements at week 4. In the case of the Dense Cement, bone growth was observed only on the surface of the implant. CPC microporosity was too small to allow cell penetration [29]. In the case of the Macroporous Cement, bone growth and neovascularisation was observed also inside the pores of the material as evidenced by a higher Proportion of Tissue Penetration value, as shown in Fig. 4. Bone growth was mainly in the peripheral pores, but in fact it did not occur only at the margins of the cement. It grew also in some central pores. The lack of a more extensive bone tissue penetration in the central part of the implant was due mainly to the limited interconnectivity between adjacent pores in the range of 50–150 μm, as observed by mercury porosimetry (Fig. 3) [30], although the pore size was between 100–300 μm. Indeed, a minimum pore size of 100 μm has been reported to allow bone tissue formation inside the pores of calcium phosphate ceramics [31], being the optimum pore size in the 200–400 μm range [32–34]. But the most important factor is pore interconnectivity. Extensive bone growth was observed when pore interconnections were in the range of 60–100 μm, being the optimal interconnection size 130 μm [33]. It has also been reported that additional smaller pores are beneficial, since they allow for body fluid circulation [35]. In our case this is guaranteed by the intrinsic microporosity of CPCs.

The growth in the peripheral pores was be enhanced by the resorption experienced by the Macroporous Cement, increasing the interconnectivity size. The Residual Material Volume value decreased already at week 4, and more strongly at week 12. In contrast, the Dense Cement did not show any detectable resorption during the implantation period, although the surface was completely surrounded by new bone. In both cements, bone was formed and started to grow from the material surface, indicating an osteostimulative behaviour.

At week 12, the Macroporous Cement was almost reabsorbed. Other authors have also found an increase in the resorption speed in the case of Macroporous Cements [23] and also in granules [36] made of calcium deficient apatite. Only the central part of the material was left, mainly due to its lack of macroporosity. However, bone formation seemed to take place at a lower rate than material resorption, and this could give rise to a lack of biomechanical stability.

Two resoprtion mechanisms take place in calcium deficient apatites: active resorption, mediated by the cellular activity of macrophages and osteoclasts, and passive resorption due to dissolution [36]. In the Macroporous Cement both mechanisms were probably occurring. The higher contact area with body fluids could enhance the dissolution of the calcium deficient apatite and promote cement disintegration. In fact, Cement Degradation particles were observed at week 4, surrounded by bone tissue. Macrophages, together with osteoclasts and multinucleated cells were present in the degradation region (Fig. 7). The presence of macrophages should not be interpreted in this case as an inflammatory

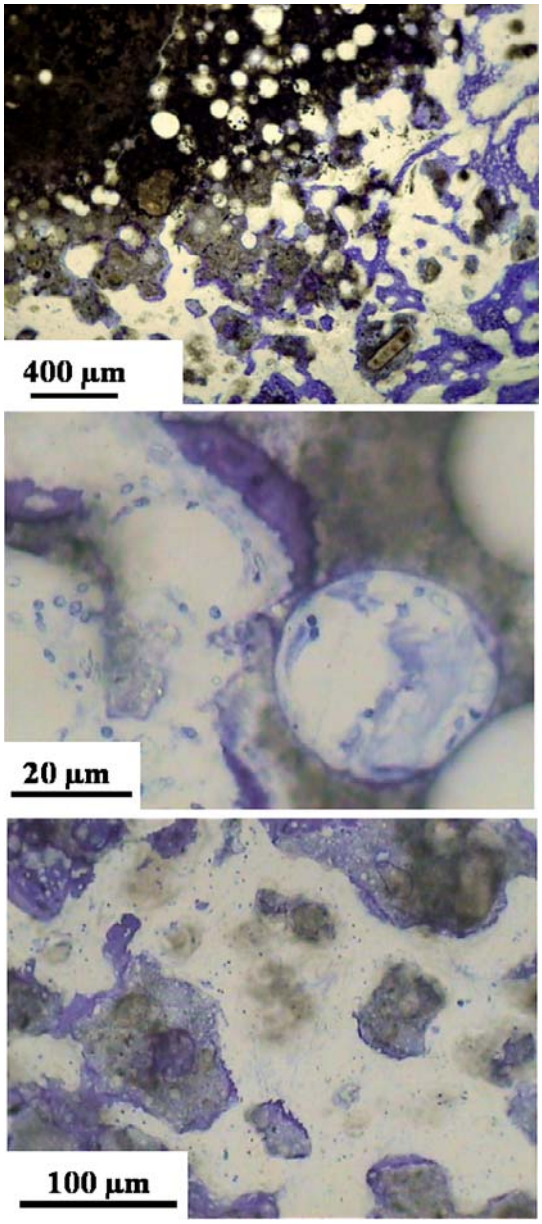

Fig. 7 Macroporous Cement at week 4 (4×). (b) Blood vessel formed inside a pore at week 4. (40×). (c) Degradation zone of the Macroporous Cement at week 4, where fragments of the implants are surrounded by bone tissue. (10×)

response. Actually, they have been observed in other *in vivo* studies with highly resorbable Calcium Phosphate materials [37, 38], whereas in the interfaces of slow rate resorption materials it is more common to find osteoclasts. It is believed that its presence play an important role in bone remodeling homeostasis.

5 Conclusion

In this study the *in vivo* performance of an Injectable Macroporous Bone Cement is presented. The Albumen protein solution was an effective foaming agent and the cement was able to keep its macroporous structure through implantation without disintegration or pore collapsing.

The presence of the macropores in the cement increased the bone ingrowth and tissue prenetration, although bone growth was mainly observed in the peripheral pores through all the implantation times. A higher resorption rate was obtained due to both a higher surface contact with body fluids increasing the dissolution and enhancing cellular activity due to particle degradation. The resorption speed was slightly higher than the bone ingrowth. In future works this could be tailored either by a more specific control of the macroporosity, or by stimulating bone formation.

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