Tissue reaction against a self-setting calcium phosphate cement set in bone or outside the organism

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Calcium phosphate cements are able to set *in situ* when injected into bone tissue. We evaluated the tissue reaction occurring when a DCPD-based calcium phosphate cement was either set within the bone or implanted when already set. The samples were implanted in rabbit condyles and examined histologically after 8 and 16 weeks. The relative bone surface, the fibrous capsule around the implants and the implant section surface were measured. Solid material seemed to be better tolerated than paste implants. More bone was found at the solid implant contact whatever the implantation time and the solid material degraded much less rapidly. In conclusion, the physico-chemical modification of the biological environment occurring during setting increases the foreign body reaction against the material.

1. Introduction

The setting of calcium phosphate cements is obtained by an acid-base reaction between a powder and a liquid. Most of the end-products, and even the intermediary compounds obtained after setting are known to be biocompatible [1–3]. The setting process may however lead to a modification of the immediate physico-chemical environment. Setting due to an acid-base reaction may lead, in particular, to a dramatic decrease in pH at the implanted site.

A DCPD-based cement was used as a highly soluble, middle term carrier for active molecules [4, 5]. Histological examination of the integration of this material in rabbit condyles showed that although it was osteoconductive, a foreign body reaction could be observed during the early stages of implantation.

We compared the bone tissue reaction against material set *in situ* (pasty) or pre-set outside the organism (solid) to evaluate any differences in integration due to setting within the implanted site. Histological analyses were made of both implants at two different times.

2. Materials and methods

2.1. Animals

Ten female New Zealand white rabbits aged 6–7 months with body weights of 3700–5100 g and which had free access to food and water were used for the experiments. The animals were observed daily for one week after implantation and three times a week for the remaining implantation period. Observation of the skin in the implanted region, animal mobility and behavior were noted.

2.2. Material injected

Powder: 3 g (β-TCP: 2.954 g, anhydrous sodium pyrophosphate: 0.046 g). Liquid: 1.5 ml (phosphoric acid (4 M) and sulfuric acid (0.1 M) solution). The powder/liquid ratio was 1.2 and the conversion rate 60%. The resulting solid material consisted of 60% of dihydrated dicalcium phosphate, 39% of β-tricalcium phosphate, and 1% of various mineral phases (sodium-based and calcium sulfate). The density was 1.43, the porosity 45% and the mean pore diameter 7.1 μm.

2.3. Injection of bone cement

The cement was inserted under intramuscular ketamine anaesthetic, through a lateral longitudinal skin incision over the knee, into holes drilled in the external condyles of rabbits. The left hole was filled with the solid material and the right hole with the paste. Each hole was 4 mm in diameter and 10 mm in length. The paste was injected through a syringe as follows: the liquid phase was poured in a polyethylene bowl, while the solid phase was added

to the liquid at $t = 0$. The mixture was blended with a spatula for 30 s to obtain a homogeneous mixture. The cement was poured into a syringe and left to rest. It was then injected into the cavity after about 4 min, the cement was left to set for 10 min without any further manipulation. Five animals were sacrificed by nembutal injection after 8 and 16 weeks and the distal extremities of the femurs were collected.

The solid samples were set in a silicone mold at room temperature and were implanted a few weeks after setting. They were sterilized by γ irradiation.

2.4. Histological processing

The samples were fixed in a 4% formaldehyde solution for 48 h, dehydrated in increasing ethanol solution then embedded in polymethylmethacrylate (PMMA). Sections 5 μm thick were obtained by a hard tissue microtome (Reichert-Jung Type E). They were then stained with Giemsa solution and by the Von Kossa method.

Qualitative and quantitative studies were performed on longitudinal histological sections through the cylinder center. Three sections were taken very close to the highest cylinder diameter (less than 100 μm away). Three primary measurements were taken to characterize the structure of the newly formed bone at the implanted site. Mean surface of the fibrous capsule around the implant under a 2 mm length; trabecular bone surface in a 500 μm thick zone around the implant. The trabecular bone surface was defined as the bone surface to total tissue surface ratio (BS/TS). The bone surface comprised the mineralized and osteoid surfaces. The total tissue surface consisted of the bone surface, bone marrow, and stromal tissue. Finally, the mean surface of the implant section: only the surface of the bulk material was considered. The measurements were obtained using a computerized image analysis device coupled to a Reicher Polyvar microscope.

2.5. Statistical analysis

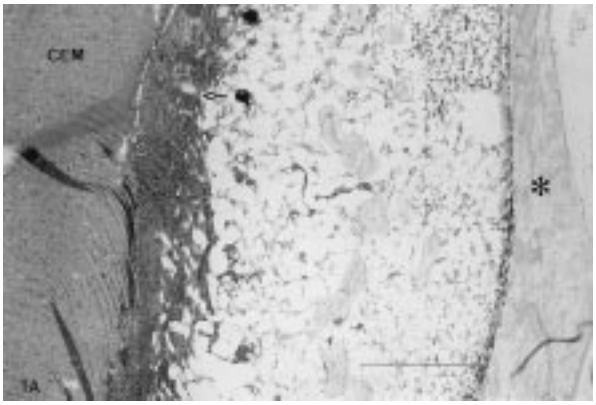
The measurements were obtained on three sections from each implanted site. Statistical analyses were carried out according to a two-way anova test.

3. Results

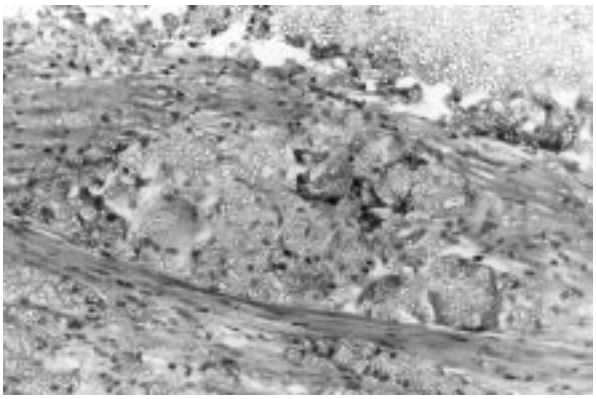
3.1. Samples implanted for eight weeks

Pasty samples. All the implant sections showed the same characteristics:

- a fibrous reaction had occurred around the material. The fibrous capsule consisted of mono and plurinucleate cells in a collagen network, the fibers of which were parallel to the material surface (Figs 1 and 2).
- a bone marrow tissue without any bone trabeculae was observed around the fibrous reaction.
- the implants showed numerous traces of degradation. The fibrous tissue contained many particles that had been phagocytosed by macrophages. The implant surface was highly irregular and rough. This roughness was probably due to degradation. In one implant, a vasculo-conjunctive tissue penetrated deeply into the material.



(a)



(b)

Figure 1 (a) Micrograph of an 8 week pasty implant. The cylinder (CEM) is surrounded by connective tissue containing numerous macrophages (→) and separated from bone (*) by bone marrow cavity. Giemsa staining. Bar: 400 μm. (b) Most of the macrophages contains calcium phosphate particles. Giemsa staining.

Figure 2 Micrograph of an 8 week pre-set sample. A thin bone layer (*) surrounds the cylinder (CEM). Giemsa staining. Bar 300 μm.

Pre-set samples. The aspect of the tissue at the contact of the different samples was homogeneous:

- almost all the surface of the implant was coated with a thin layer of bone.
- very few calcium phosphate particles were found in the bone marrow around the implant. The implants were not fragmented (Fig. 3).

3.2. Samples implanted for eighteen weeks

Pasty samples. The implants were coated with fibrous tissue. In three implants, a few trabeculae were forming in proximity to the material in the fibrous tissue (Figs. 4–6). These trabeculae were separated from the material by macrophages and were linked to the bone tissue in which the cylinder had been implanted. The forming bone tissue was not always totally mineralized. No osteoblast was observed at the material surface.

Pre-set samples. As for 8 week implantation time, the implants were surrounded by bone tissue (Figs. 3–7). Some implants had been partially resorbed, trabecular bone had formed in the degraded zone, and the pores of the bone in contact with the material were filled with fibrous tissue and macrophages. Fibrous tissue containing a few macrophages was often found between the bone and the material while in other regions the bone was in direct contact with the material. Some fragments of the material were dispersed within the bone matrix without any interposition tissue. Some osteoblasts were occasionally found at the material surface.

3.3. Histomorphometry

Fibrous tissue (Table I). After 8 and 16 weeks continuous fibrous membrane had been interposed between the material set *in situ* and the bone marrow. No significant difference in membrane thickness was observed between these two periods. A very thin membrane was observed with a very high standard deviation indicating that the fibrous tissue was not continuous and was contained within the trabecular bone pores in the pre-set implanted materials. The thickness of this membrane was significantly lower $(p < 0.05)$ than that of the pasty material.

Trabecular bone surface (Table II and Fig. 8). The relative trabecular surface around the implants was very low for the pasty samples even after 16 weeks, although the amount was significantly higher than after 4 weeks. The TBS was significantly higher $(p < 0.05)$ at the contact of samples implanted when already set and did not differ between 4 and 16 weeks.

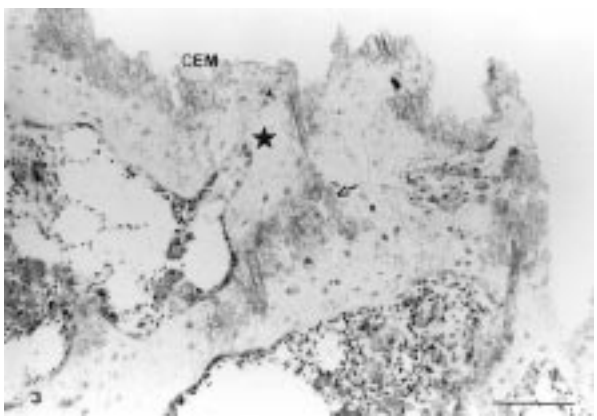


Figure 3 Micrograph of a 16 week pre-set sample. Cancellous bone had formed at the surface of the remaining implant. Some trabeculae (*) are growing into the cylinder (CEM). Calcium phosphate fragments are integrated within the bone (→). Giemsa staining. Bar: 200 µm.

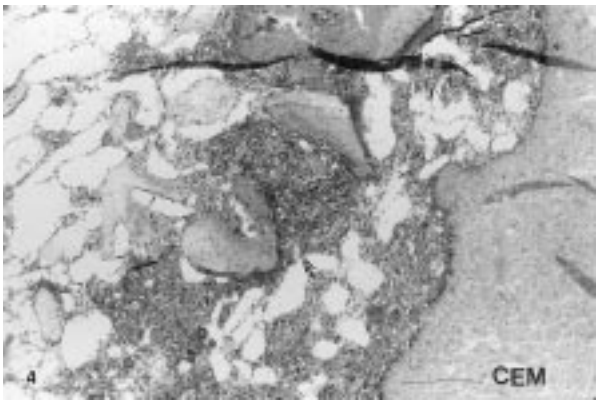


Figure 4 Micrograph of a 16 week pasty sample. Fragments of calcium phosphates released from the cement (CEM) have been phagocytosed by macrophages. Bone trabeculae are forming in a macrophage islet (→). Giemsa staining. Bar 300 µm.

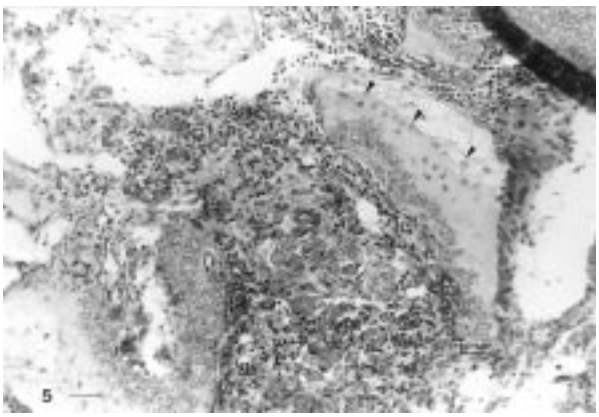


Figure 5 The volume of the trabeculae has not been entirely mineralized. A large zone is made of unmineralized tissue (>). Giemsa staining. Bar: 100 µm.

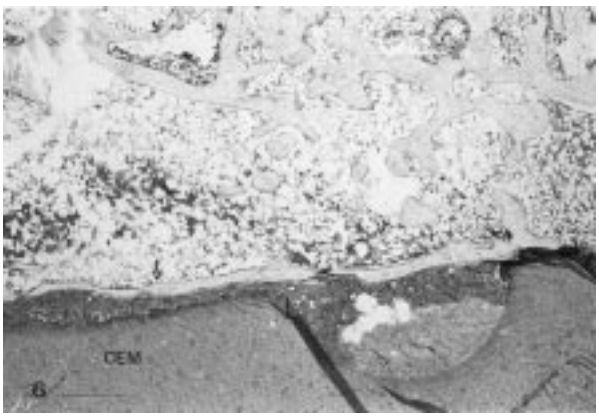


Figure 6 Bone formation (black trabeculae) occurred at the cement contact (CEM) at 16 weeks when implanted pasty. The cement disappeared from the section under the microtome blade. Von Kossa-Giemsa staining. Bar 250 µm.

Implant surface (Table III and Fig. 9). The implant surface was significantly higher $(p < 0.05)$ for the pre-set implanted material at both implantation times. The section surface did not decrease significantly between each period for both materials.

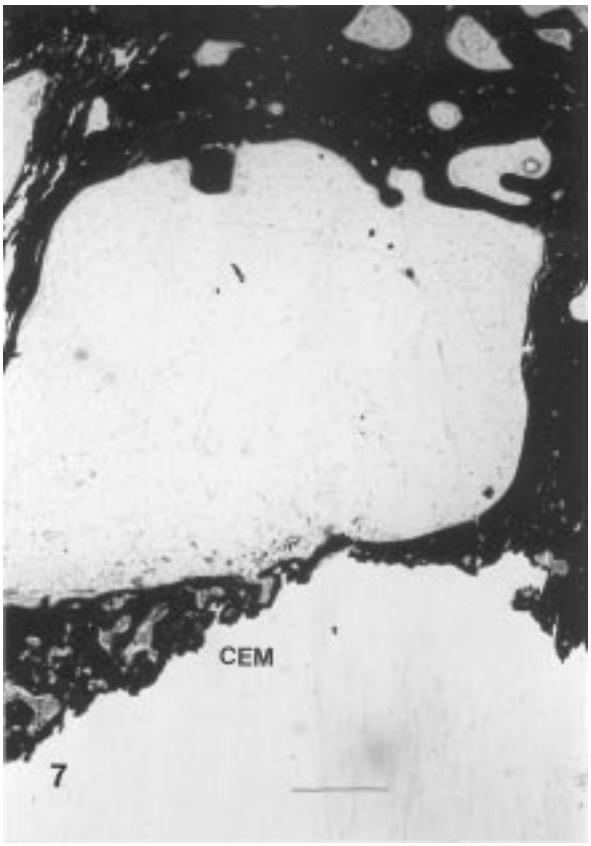


Figure 7 At 16 weeks, bone trabeculae were close to the surface of the pasty cement surface (CEM) from which they are separated by a thin foreign body reaction. Giemsa staining. Bar: 300 µm.

TABLE I Mean surface of the fibrous membrane (mm^2) around the implant under a length of 2 mm

Implant	Pasty	Solid
8 weeks	0.88 ± 0.21	0.1 ± 0.45
16 weeks	0.58 ± 0.29	0.08 ± 0.2

TABLE II Mean trabecular surface in a 500 µm thick zone around the implant (results are in % of the total surface observed)

Implant	Pasty	Solid
8 weeks	2 ± 3.4	31.7 ± 3.55
16 weeks	5.1 ± 2.69	32.2 ± 14.11

4. Discussion

The setting reaction had an effect on the biocompatibility and osteoconductivity of this material. Bone formation took place at the contact of samples implanted when already set although most of this bone formation occurred during the first eight weeks. Bone formation did not take place at the contact of the pasty implanted material during this period. After 16 weeks only a very small amount of bone had formed in the periphery of the pasty implants. The fibrous tissue encapsulating these implants persisted for 16 weeks. These results are not consistent with those found in a previous study in which the amount of bone was much greater at the implant contact even when implanted in a pasty state [4]. The higher degradation rate was probably due to less complete setting *in vivo* due to the dilution of the different compounds in the formulation.

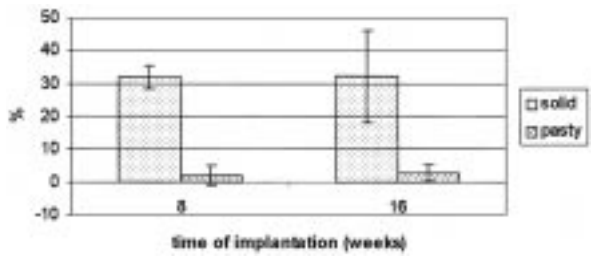


Figure 8 Mean relative surface of bone trabeculae in a thickness of 500 µm around implants.

TABLE III Mean implant surface (section performed in the cylinder diameter). Original surface 50 mm^2) in mm^2

Implant	Pasty	Solid
8 weeks	19.28 ± 13.14	39.6 ± 20.21
16 weeks	18.04 ± 4.61	32.1 ± 7.24

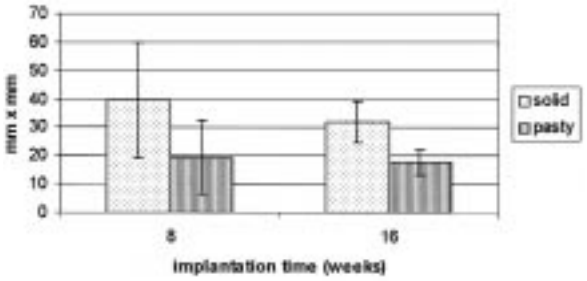


Figure 9 Surface of implant section.

The fibrous reaction was solely observed at the surface of the material set *in situ*. This reaction could indicate that either the high degradation rate or the high surface area of the material due to incomplete setting affects bone healing. High surface area may lead to modification of the ionic content of the extracellular fluids due to dissolution-precipitation processes that affect cell behavior [6]. The release of numerous particles into the surrounding tissues may produce an onslaught of macrophages in the vicinity of the implant. The delay in ossification at the implant surface could be caused by the production of factors by the macrophages that affect the osteoblasts. Horowitz and co-workers have shown that phagocytosis enhances the release of mediators of inflammation, particularly TNFα which then stimulate the synthesis of granulocyte macrophage colony stimulating factor (GM-CSF) and interleukins (IL-6) by the osteoblasts [7, 8]. Each of these molecules recruits new macrophages, osteoclasts and other inflammatory cells at the interface with the material. TNFα also stimulates PGE_2 production by the osteoblasts which may then decrease TNFα production by the macrophages and activate osteoclast activity [9]. In this study however, we could not evidence any osteoclast activation in the trabecular bone around the material. Furthermore, some newly forming trabeculae were apparent within the macrophage membranes.

The instability of the material surface may also explain the delayed bone formation at the surface of *in situ* set materials. No osteoblasts were apparently fixed to the

surface of the pasty implants although some could be observed at the surface of the solid material. This suggests that ossification may be centrifugal in the case of solid implanted samples and centripetal when the cement is injected in paste-form.

The low pH during setting could be another reason for the presence of a foreign body reaction against materials set *in situ*. Setting results from the mixing of a powder and a low pH liquid composed of phosphoric and sulfuric acids. The pH of the mixture alters from 2 to 4 during the early stages of setting. It should also be noted that the setting process continues for several weeks since the strength of the material increases over the same period. This indicates that ionic modifications may continue to occur in the microenvironment around the implant for some time.

The difference in integration compared with previous reports suggests that the method used is critical as regards the subsequent osteoconductivity. It is very probable that the timing of the injection during the setting reaction is crucial to the success of the osteoconductivity. If the material is injected into the implantation site too soon, the stoechiometry of the reaction is upset by the extracellular fluids.

5. Conclusions

A difference is observed in the biocompatibility of this self-setting cement depending on whether it is set in the bone tissue or completely pre-set outside the body and then implanted. The best osteoconductivity and biocompatibility was demonstrated in pre-set materials. This means that the implantation testing of this class of material must be carried out using *in situ* set samples to avoid any overestimation of their tolerance.

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