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PULPAL RESPONSE TO CALCIUM PHOSPHATE MATERIALS. *IN VIVO* STUDY OF CALCIUM PHOSPHATE MATERIALS IN ENDODONTICS

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Abstract

The aim of this study was to determine if calcium phosphate (CaP) materials could be used to substitute for calcium hydroxide (CH) as a pulp capping agent. Especially prepared and characterized CaP materials with CH as the reference or control material were used for pulp-capping teeth of pigs, rats, and dogs. The CaP materials included: DCPD (dicalcium phosphate dihydrate), OCP (octacalcium phosphate), β-TCP (β-tricalcium phosphate), BCP (biphasic calcium phosphate mixture of 50/50 HA and β-TCP), and HA (hydroxyapatite) which were used in particle sizes of $< 5\ \mu m$ or $< 150\ \mu m$. The animals were sacrificed after 21 days to 4 months after pulp-capping. The extracted teeth were immediately prepared for the following analyses: light microscopy, scanning electron microscopy (SEM) using backscattered electrons (BSE), and energy dispersive X-ray (EDX) microanalysis. Three types of mineralizations were observed: dentin bridge formation, dystrophic calcification and mineralization. All the CaP materials showed biocompatibility. Based on these results, it is suggested that the CaP materials tested may be useful for specific clinical applications in endodontics, e.g., pulp capping (microparticles of HA, TCP, BCP), and pulpectomy (HA, OCP, DCPD).

Key Words: *in vivo*, calcium phosphate, calcium hydroxide, dental pulp capping, dental pulp wound healing, endodontics, X-ray microanalysis, backscattered electrons, X-ray microradiography.

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Introduction

Calcium phosphate (CaP) materials are largely used in orthopedic surgery and in periodontology owing to their bioactive properties (Hong *et al.*, 1990). In endodontics, calcium phosphate materials have been used successfully for mechanical perforation of the pulp chamber floor (Himel *et al.*, 1985), for root perforations (Sinai *et al.*, 1989), for apical closure of pulpless teeth (Roberts and Brilliant, 1975), and for apical wound healing (Sikri *et al.*, 1986).

The dentinogenic effects of CaP materials and the formation of dentin bridge were studied on pulp amputation (Heller *et al.*, 1975; Ikami *et al.*, 1990) and on pulp capping (Jean *et al.*, 1988; Noguchi, 1989; Chohayeb *et al.*, 1991; Furusawa *et al.*, 1991; Frank *et al.*, 1991b; Jaber *et al.*, 1992).

The aim of the present preliminary investigation was to study the pulpal response to CaP materials and compare that with classical data obtained with calcium hydroxide. There were two questions: first, pulpal biocompatibility and animal model significance; and second, the dentinogenic effects of various calcium phosphate powders. The original feature of this research was the use of teeth of different animal species and various analysis techniques of complementary interest to study the mineralization process of dental pulp. To our knowledge, no similar studies have been published so far.

Materials and Methods

The teeth of two 3 month-old male pigs, twelve 8 week-old Wistar rats, and six 2 year-old male beagle dogs were used in this study (76 observed teeth). Pigs and dogs were anesthetized by intravenous injection of sodium pentobarbital, rats by intraperitoneal injection. The teeth were cleaned and isolated with a rubber dam (pigs and dogs) and with a homemade plastic field (rats).

A labial class V cavity preparation was made on the primary incisors, cuspids and first molars of the pigs, on the cuspids and first molars of the dogs, with a round carbide bur at high speed under continuous irrigation by normal saline solution. An occlusal class I cavity was prepared on the first upper molars of the rats. A

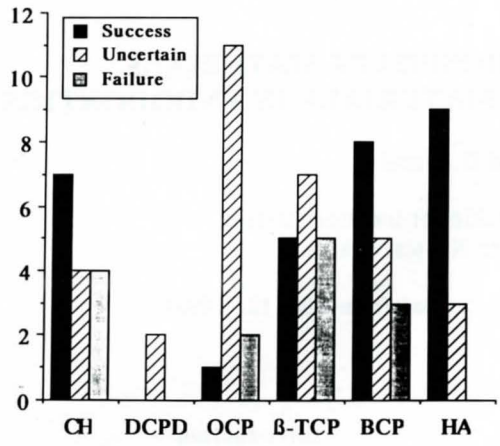

Figure 1. Distribution of the results (number of success, uncertain, or failure) obtained with the 76 observed teeth with different calcium phosphates.

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small pulpal exposure (about 1/2 mm) on the dentin wall of the cavity was made with a round carbide bur at slow speed (< 2000 rpm).

Pulp hemorrhage was controlled by light pressure with sterilized cotton pellets and paper points. The tested materials were directly applied to the dental pulp tissue. The following materials, synthesized by precipitation (by Dr. LeGeros, New York University, College of Dentistry), were tested: DCPD (dicalcium phosphate dihydrate) $CaHPO_4.2H_2O$; OCP (octacalcium phosphate) $Ca_8H_2(PO_4)_6.(OH)_2$; β-TCP (beta-tricalcium phosphate) $Ca_3(PO_4)_2$; BCP (biphasic phosphate mixture of 50/50 HA and β-TCP); HA (calcium hydroxyapatite) $Ca_{10}(PO_4)_6(OH)_2$.

The powders of the calcium phosphate materials (microparticles < 5 μm, or macroparticles < 150 μm) were sterilized (130°C) and inspected by X-ray diffraction (by Dr. LeGeros, NY). Calcium hydroxide, CH (Merck, Darmstadt, Germany) was used as reference (control).

Carboxylate cement was used to form a protective base before placing the final restoration with Concise (3M, St Paul, MN) in the pigs, and silver amalgam in the dogs and rats.

The animals were sacrificed by an overdose of anesthetic (pigs, dogs) or ascending aorta perfusion with fixative solution (rats) after 3 weeks to 4 months. The teeth were immediately extracted and fixed in a paraformaldehyde (2%) / glutaraldehyde (2.5%) mixture in sodium cacodylate buffer (pH 7.2) after the apical part of the root (dogs and pigs) had been removed. The teeth were embedded in methyl-methacrylate and cut into planoparallel sections (100 μm pigs and dogs, 50 μm rats) with a diamond saw (Leitz 1600). Microradiographs of these undecalcified sections were obtained using a Philips PW 1008 generator at 20 kV and 25 mA. Then, these sections were stained with Movat's Pentachrome modified for plastic embedded microtome sections or with Solochrome Cyanine R. For SEM, the sections were carbon coated and examined with either a Hitachi S450 or a JEOL 6400F SEM using backscattered electron (BSE) imaging. Energy dispersive X-ray (EDX) microanalysis was performed with an EDAX 9100-60. The remaining teeth were decalcified using 4% nitric acid in formalin solution, embedded in paraffin, serially sectioned at 10 μm, and stained with Masson's trichrome. Stained sections and microradiographs were observed in the light microscope.

Results obtained were grouped as success, uncertain or failure. Necrosed pulp or no appearance of mineralized bridge were considered as failures. Irregular calcification, diffuse mineralization, and mildly inflamed pulp were regarded as uncertain results. Regular calcification, regular collagen matrix, and absence of inflammatory cells were considered as favorable results, i.e., success (Stanford, 1980; Kirk and Meyer, 1992).

Figure 2. Dog, CH (c), 21 days, decalcified stained section (Masson's trichrome). We observe an heterogeneous barrier (g), continuous with regular dentin (r), and some regular cells in the border pulp (p). Bar = 100 μm.

Figure 3. Pig, CH (c), 10 weeks, microradiograph. The earlier dystrophic barrier (z) is hypermineralized, the regular dentin (r) shows X-ray density as the dentin wall. Bar = 100 μm.

Figure 4. Pig, OCP (o), 10 weeks, microradiograph. Lacunar and globular mineralized tissue filling pulp chamber. Bar = 100 μm.

Figure 5. Pig, β-TCP, 10 weeks, microradiograph. Pulp chamber dentin wall made of orthodentin (d); flexuous lacunar dentin (f) fills the pulp chamber and regular dentin (r) lines the radicular dentin wall. Bar = 100 μm.

Figure 6. Rat, β-TCP, 21 days, undecalcified Solochrome stained section. A dystrophic bridge (d) separates the pulp chamber, filled by material (m) from the radicular pulp (p). Bar = 100 μm.

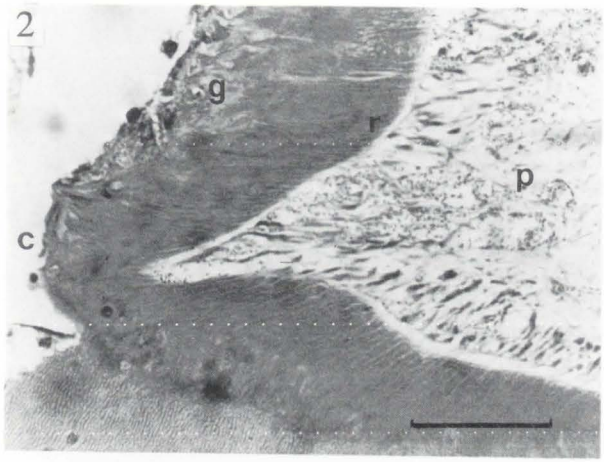
Figure 7. Dog, β-TCP, 10 weeks, microradiograph. The mineralization is continuous between the dentin of pulp chamber wall (w) and the macroparticles (t). Bar = 100 μm.

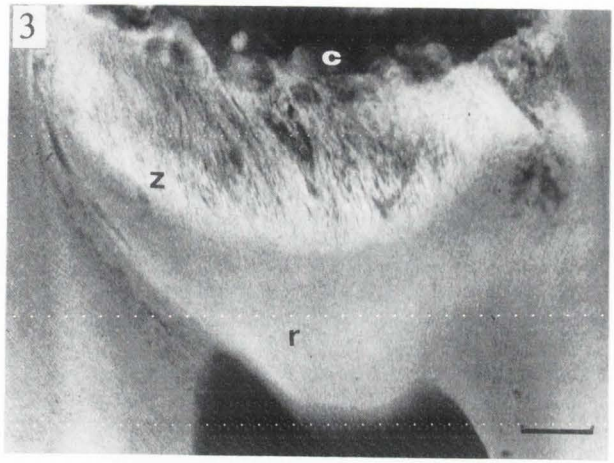
Results

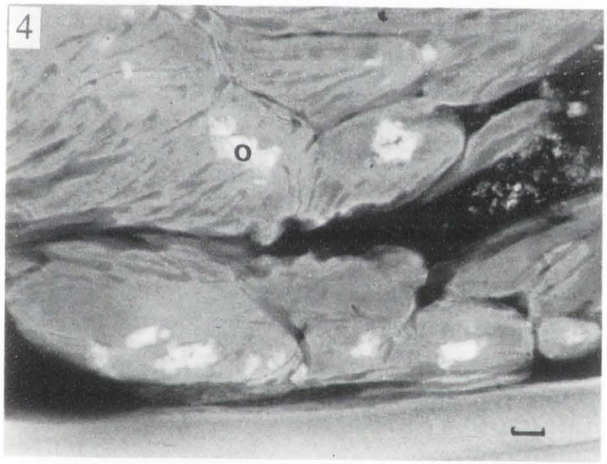
76 teeth were observed in all. The results obtained are shown on Figure 1.

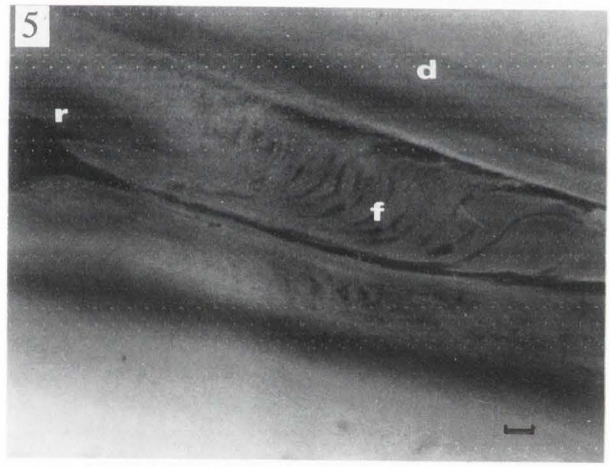
CH, control group

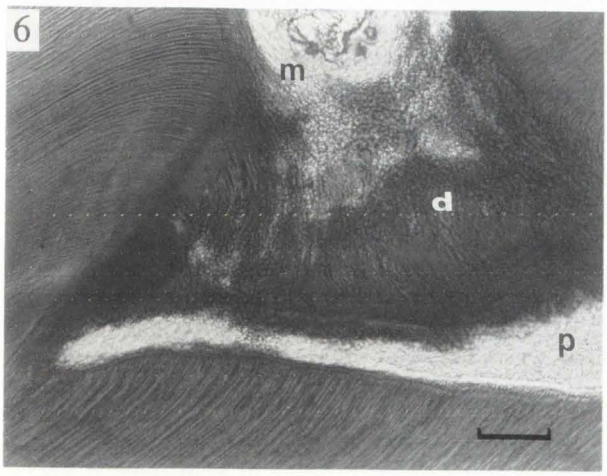
The decalcified Masson's trichrome stained sections showed a dentin bridge: the earlier reparative layer was irregular, and the pulpal zone was regular and tubular (Figure 2). Some pulpal cells were aligned along the dentin bridge wall.

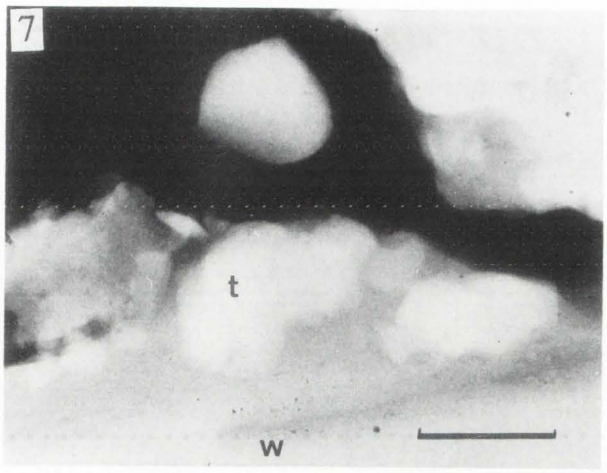












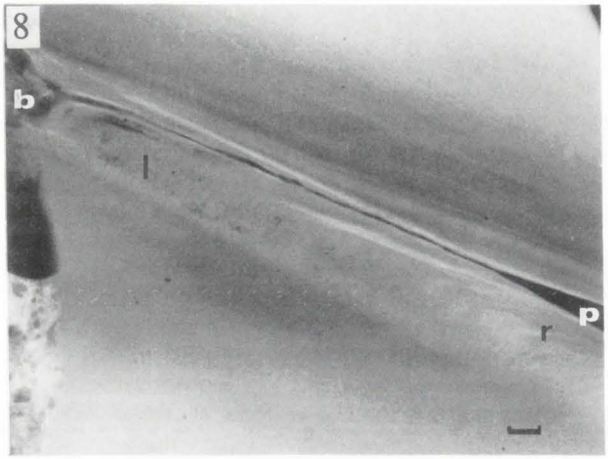
On the microradiograph (Figure 3), the earlier reparative layer in contact with CH was hypermineralized. It was a dystrophic heterogeneous reparative mineralization. The next layers were regular and continuous with the neo-dentin of the pulp chamber wall.

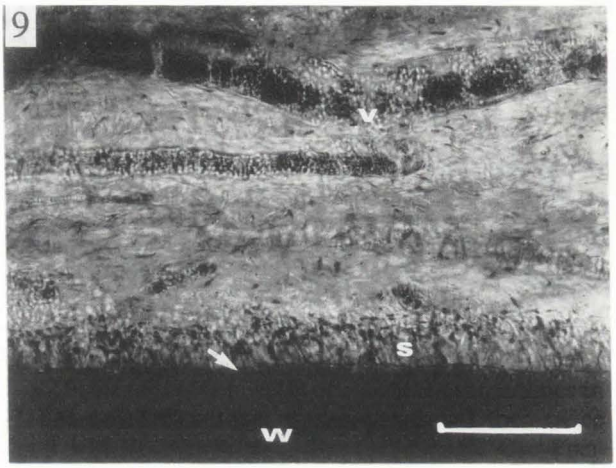
DCPD and OCP

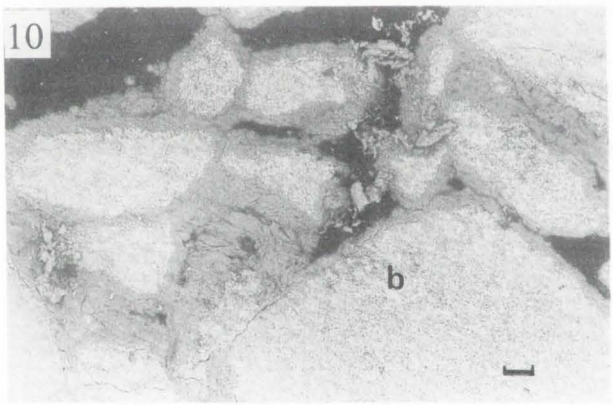
The pulp tissue responses were similar for DCPD and OCP: abundant calcified tissue filled the endodontic cavity (pig, Figure 4). The microradiograph showed that the mineralized tissue consisted of highly dystrophic globular blocks, sometimes around hypermineralized foci.

β-TCP

X-rays (Figure 5, pig) showed a large layer of heterogeneous mineralized tissue. Flexuous longitudinal and lacunar zones filling the pulp chamber were







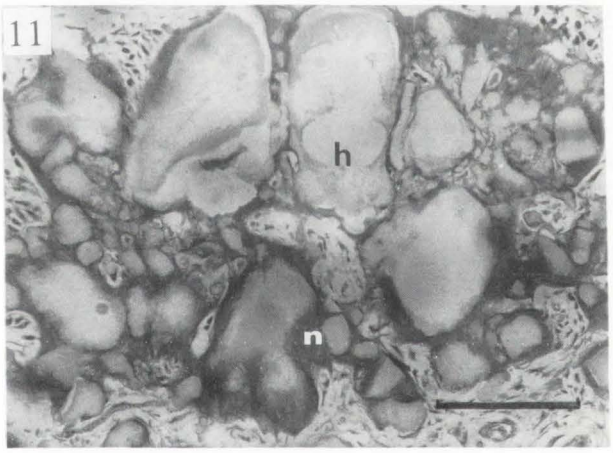


Figure 8. Pig, BCP (b), 10 weeks, microradiograph. An irregular earlier layer (l) fills the pulp chamber, continuous with a regular dentin layer (r) and the pulp (p). Bar = 100 μm.

Figure 9. Pig, BCP, 10 weeks, undecalcified stained section (Movat's pentachrome). The dentin bridge (w) is lined by a predentin layer (arrow). A regular odontoblast layer (s) proves that the pulp is functional with blood vessel (v). Bar = 100 μm.

Figure 10. Dog, BCP, 6 weeks, BSE image. The mineralization is homogeneous around large macroparticles (b). Bar = 100 μm.

Figure 11. Dog, HA, 21 days, decalcified stained section (Masson's trichrome). A fibrillar network (n) surrounds the macroparticles (h). Bar = 100 μm.

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observed. Mineralization similar to root wall neo-dentin was more regular, in the pulpal part.

Solochrome staining of undecalcified sections (Figure 6) showed, between the top and bottom of the pulpal chamber dentin walls, that the tissue, which was more regular than the tissues mentioned above, was in contact with the β-TCP particles and extended along the radicular walls (rat, 21 days).

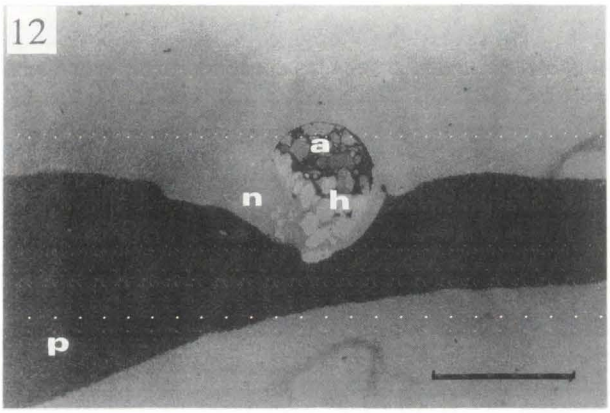
On the microradiograph (Figure 7, dog), the macroparticles close to the dentin wall were partially surrounded by radio-dense tissue after 10 weeks of implantation. However, some isolated particles, not embedded in radio-dense tissue, were also observed.

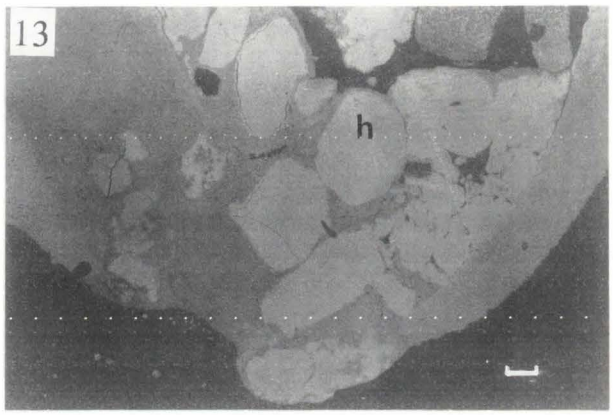
BCP

Microradiographs of the capping zone (Figure 8, pig) showed extensive mineralization, consisting of a thick two-layer dentin bridge, filling the pulpal cavity. The first layer was irregular. The second, or pulpal layer, was made up of well-homogenized tubular dentin. Movat's pentachrome staining of undecalcified sections (Figure 9) showed a predentin layer, normal pulp tissue with a regular odontoblast layer, mesenchymal cells and blood vessels. The BSE image in SEM (dog, 6 weeks, Figure 10) showed a homogeneous level of mineralization between and around the macroparticles and in close contact with them.

HA

The decalcified stained sections (dog, 21 days, Figure 11) showed an extensive collagen fibrillar network around the macroparticles of HA powder. After 10 weeks, the BSE images (Figures 12 and 13) showed mineralization around the macroparticles characterized by a





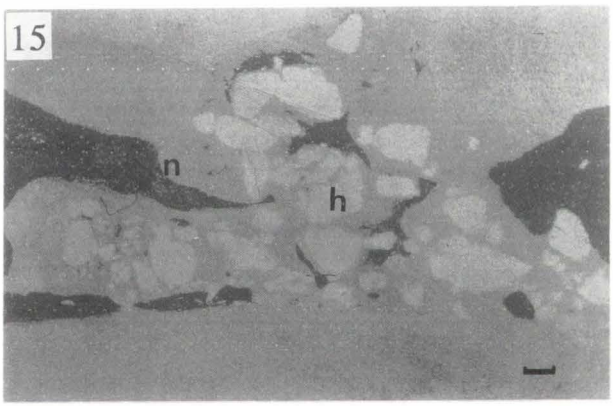


Figure 12. Dog, HA, 10 weeks, BSE. The site of pulpal exposure (a) shows the pulp chamber (p). It is sealed by the mineralization as neo-dentin (n) around the macroparticles (h). Bar = 1 mm.

Figure 13. Dog, HA, 10 weeks, BSE. Detail of Figure 12 showing that the level of mineralization is homogeneous around the macroparticles (h). Bar = 100 μm.

Figure 14. Dog, HA, 10 weeks, EDX Ca and P maps of Figure 13: Ca (left). P (right). Bar = 100 μm.

Figure 15. Dog, HA, 4 months, BSE. An homogeneous mineralization fills the pulp chamber, with a normal orthodentin (n) continuous with dental wall of the pulp chamber. Bar = 100 μm.

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very homogeneous mineral density in close contact with the particles. Its density was the same as that of the dentin walls. Ca and P mapping of the same zone (Figure 14), did not reveal any differences. After 4 months (Figure 15), all the macroparticles of HA were included in the newly-formed mineralized tissue. In the peripheral zones, the mineralized tissue was similar to the dentin walls.

The EDX microanalysis of various calcium phosphate powders and calcium hydroxide, showed that in all cases, the newly-formed mineralized tissue was made up essentially of Ca and P. We also noted small amounts of Mg (less than 0.5%).

The EDX results on the tested powders, before and after capping, were compared with the theoretical Ca/P molar ratio (Figure 16). Few variations appeared with β-TCP, HA, and BCP. Semi-quantitative microanalysis (Ca, P, Mg) of newly-formed mineralized tissue showed variations in the Ca/P molar ratio related to the tested materials (Figure 17). These results were compared with the dentinal Ca/P molar ratio.

Discussion

The biocompatibility of calcium phosphate powders observed by Alliot-Licht *et al.* (1991) and LeGeros *et al.* (1991) in human bone cell culture, and in pulpal cell culture (Alliot-Licht *et al.*, in progress) was confirmed in this *in vivo* study by the presence of predentin and odontoblasts along the bridges and the pulpal walls, in agreement with Frank *et al.* (1991b). But to our knowledge, no study has compared the results obtained, *in vivo*, with HA, β-TCP, BCP, OCP, DCPD and CH as pulp capping materials.

In the present preliminary study we used complementary methods of observation. Microradiography and light microscopy of undecalcified tissues, in combination with decalcified sections, permitted the comparison with reference (control) results obtained during pulp capping (Schröder, 1985). BSE imaging used for observation of minor changes in mineral density (Boyde and Jones,

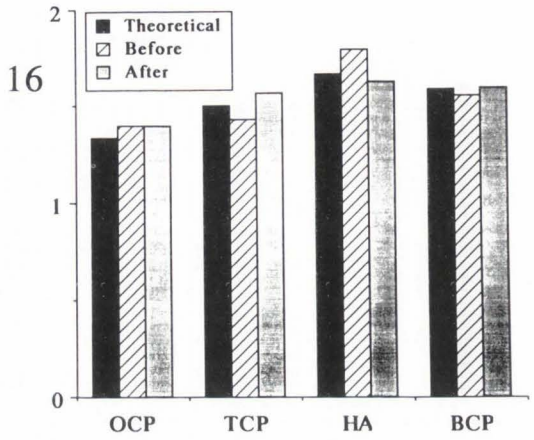

Figure 16. Semiquantitative EDX microanalysis: Ca/P molar ratio of various tested calcium phosphate materials. Comparison between theoretical and before/after pulp capping ratios.

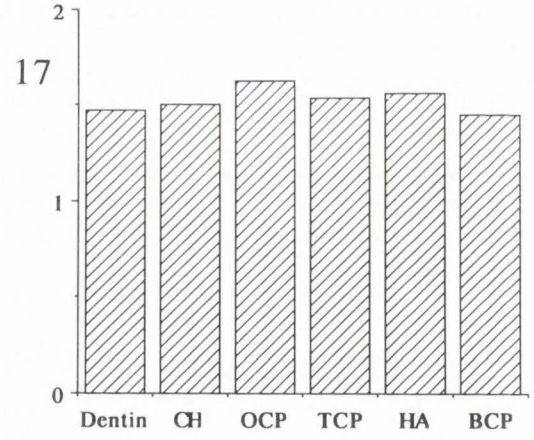

Figure 17. Semiquantitative EDX microanalysis: Ca/P molar ratio of reaction zone mineralization for different capping material.

1983) has demonstrated the homogeneity of dentin formation at the surface of CaP. EDX microanalysis indicated that the newly-formed mineralized tissue contained essentially Ca, P, with small amounts of Mg, as in normal dentin (LeGeros *et al.*, 1988).

Some failures (necrosed pulp, absence of mineralized bridge) can be attributed to leakage and bacterial infection (Boone and Kafrawi, 1979).

We observed three categories of mineralized reactions in this study.

i) Mineralization obtained with microparticles of BCP, β-TCP, and HA, was compared with reference bridges obtained with CH (Schröder, 1985). The double barrier, which closed the exposure site, consisted either of a coronal irregular mineralized layer described in the literature as bone-like tissue, or of osteodentin, or irregular dentin which also has been described in the literature. However, the elongated, crossed and flexuous canals observed also suggested a comparison with "vasodentine" (Baume, 1980; Jean *et al.*, 1988).

The second layer appeared as a regular dentin-like tissue: this normal orthodentin was synthesized by odontoblasts located in normal, functional, and healthy pulp (Watts and Paterson, 1981; Schröder, 1985).

In the present investigation, the results obtained with CH as a reference, were in agreement with data from other studies (Hermann, 1930; Schröder, 1985; Stanley, 1989). However, with all calcium phosphate materials, a thicker mineralized reaction tissue without necrotic layer was obtained compared with the control material.

ii) The second category, obtained with OCP and DCPD was an extensive dystrophic mineralized tissue located in the pulp chamber and along the root canal walls. OCP and DCPD are the most soluble calcium phosphate materials (De Groot, 1983; LeGeros, 1988), which perhaps accounts for this abundant mineralization. We consider these results as uncertain because, according to Baume (1980) and Jaber *et al.* (1992), abundant and heterogeneous calcification can jeopardize future dental pulp health and could interfere with future endodontic therapy. The use of OCP and DCPD for pulp capping has also not been reported by others.

iii) The third category was represented by mineralized areas around the macroparticles of TCP and BCP. With HA macroparticles the homogeneous mineralized tissue was closely associated with the dentinal walls. These results are in agreement with Noguchi (1989) and Frank *et al.* (1991b), in human dental pulp, and can be compared with the osseous results obtained by Bagambisa *et al.* (1990) and De Lange *et al.* (1990). These authors showed direct and intimate bone contact with the HA granule surface. Our results confirm the biocompatibility and dentinogenic effects of HA. This mineralized tissue can be called "fibrodentin" as described by Baume (1980), because decalcified sections revealed the presence of a network of collagen fibers, and microradiographs showed the secondary mineralization of this collagen network. This mineralization consisted essentially of Ca and P and the density was similar to dentin of the original walls. Unlike Jaber *et al.* (1992), we did not note any calcification or inflammation with HA in the rest of the pulp.

We cannot explain why the mineralization mechanisms were not similar when the pulp was capped with microparticles instead of macroparticles of calcium phosphate materials. The bridge obtained with microparticles of HA, TCP and BCP was like a classical dentin bridge obtained with CH. Perhaps the microparticles are more easily absorbed by multinuclear giant cells (Noguchi, 1989; Alliot-Licht *et al.*, 1991), or the macroparticles could promote the formation of a collagen fiber network. It is possible that microparticles are more irritative for pulpal tissue than the large particles, and promote dystrophic mineralization.

However, we noticed that the microparticles formed a dense package in contact with the pulp. The bridge extended beyond the capping material with no necrotic layer using calcium phosphate materials. On the other hand, the macroparticles appeared to push in pulpal tissue, and the mineralization was formed around and in close contact with them. Therefore, the principle of primary mineralization is different: the size of the particles is probably an important factor, as suggested by Chohayeb *et al.* (1991) and Frank *et al.* (1991a).

We observed, in agreement with Heller *et al.* (1975) and Ikami *et al.* (1990), that TCP enhances reparative dentin formation: good results were observed in pigs and rats. However, our findings, and those of Chohayeb *et al.* (1991) show that TCP cannot be successfully used in dogs.

Moreover, BCP has the capability to enhance the formation of mineralized reparative dentin. Our results show that BCP is a biocompatible material, but it has not yet been used for pulp capping.

The qualitative EDX microanalysis showed that Ca and P were the major mineral elements of newly-formed tissue. Semi-quantitative analysis showed that the concentration and molar ratio of the dentin, in this study, were comparable with literature data (LeGeros *et al.*, 1988). The slight differences that appeared were likely to be related to C and Mg substitution. Further investigations will be needed to confirm this.

The amount of mineralized tissue differed between the animal species (pigs, dogs, rats), because the size and the shape of the teeth were different. However, the quality was comparable: for example, for OCP, the irregular globular aspect of mineralization was the same in pigs, dogs and rats.

All of the calcium phosphate powders used in this investigation proved to be biocompatible capping materials on pig, dog and rat pulps, and displayed dentinogenic effects, but further studies are necessary to account for the different pulpal reactions observed.

The possible clinical applications of the dentinogenic effects of these materials in endodontics are: pulp capping (microparticles of HA, β-TCP, BCP), pulpotomy (large particles of HA), and biopulpectomy (HA, OCP, DCPD).

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Discussion with Reviewers

Reviewer IV: One of the most important problems in this work relates to the lack of appropriate controls. Although the authors have used calcium hydroxide as a "reference material" (positive control?), there were, as far as I can tell, no negative controls. I recognize that calcium hydroxide has been compared to negative controls in the past, but nonetheless, such controls (e.g., sham operated, or operated-no liner) are essential in this study as well.

Authors: We are very surprised by this question, because an abundant literature on pulp capping makes a comparison between "sham operated or operated-no liner" and pulp capped with CH. The protocols and the results obtained when pulp is capped with CH are well known: they are identical with ours. In agreement with other authors, we think that CH can be the reference material. We do not think that it was necessary to redo all the protocols.

Reviewer IV: Interpretation of the study is made rather difficult due to the use of teeth derived from a number of different species. It is not clear to me why different species were used. For example, there is little information regarding the results within the different species and therefore, one would question the use of different species from that perspective. Certainly, the demonstration of biological phenomena in different species and/or systems can strengthen certain findings, but I cannot tell if this rationale was used here.

The authors have not endeavored to quantify their findings. Had they utilized teeth from one species, they could have produced enough samples so that morphometric assessments could be done. By the use of quantitative histomorphometry the authors might have been able to measure dentine bridge formation as well as cell population changes. This would have been far more useful and convincing than the above-described use of "success, failure, and uncertain" categories, when attempting to determine whether one lining agent is more effective or biocompatible than another.

Authors: One interest of this study was to compare the results obtained with different animal species. It showed that the same materials, used with the same protocol, on different animal species, gave different results. This fact raises questions about extrapolation of the data to humans. After an *in vitro* study (Alliot-Licht *et al.*, 1991), we tested calcium phosphate materials "*in vivo*". We used rats because they are small animals; young pigs, because the size of their teeth are comparable with human teeth; and dogs, because there are the reference to work on dogs in the literature.

The "success, failure and uncertain" categories were chosen because of the original methods (undecalcified and microradiographed sections, ...) used to observe the mineralized reaction of the pulp. Still, we agree with the Reviewer: further studies are necessary to confirm these notions.

J. Appleton: In the calcium hydroxide (CH) control group, how many controls were involved?

Authors: We observed 15 control teeth (rats, dogs and pigs) capped with this control material.

J. Appleton: How were planoparallel sections prepared for contact microradiography and how was their thickness accurately and reproducibly determined.

Authors: The precision of the Leitz 1600 diamond saw permits us to obtain, with accuracy, planoparallel sections of 50 $\pm$ 5 μm and 100 $\pm$ 5 μm. The thickness was controlled using a mechanical micrometer (TesaTronic).

Reviewer III: Your work shows the limited relevance of animal experiments with respect to human use. TCP is obviously not suited for dogs but in pigs and rats good results are found, hence the question: if tissue reactions are species dependent, what is the relevance of this animal study for human application?

Authors: The regulations in the U.S.A. (Food and Drug Administration, American Dental Association, etc.) and in Europe (I.S.O.) impose restrictions on the use of small and large animals. This study demonstrates that the results could be different in the same clinical situation. Consequently, at this stage, all extrapolations to human applications are speculative. There are no other possibilities to demonstrate the efficiency of a particular calcium phosphate (other than HA and TCP) prior to human use.