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Use of a calcium phosphate matrix as a temporary cast for bony defect repair: a pilot study

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The bony repair effect of different Calcium Phosphate Bone Cements was tested in a dog model. Seven different formulations were synthesized and tested on their biocompatibility, osseoconduction and biodegradability. Three dogs were used in this pilot study, in each dog 4 cranial, circular defects were made with a critical size diameter of 12 mm. Autologous bone was used as a control. The dogs were sacrificed after 6 months. Mineral phase analysis showed a reaction of the cements to form a more or less crystalline calciumhydroxyapatite. Histologic evaluation revealed that the presence of the cements stimulated the formation of a thin bone layer on the cranial and caudal side of each defect. The cements did not evoke an inflammatory reaction. Two formulations showed extensive bone formation.

1. Introduction

In oncologic surgery, traumatology and for the treatment of congenital syndromes e.g. cleft palate, a bone graft or a bone substitute is often required. In cleft palate patients the processus alveolaris and/or the palate are unilaterally or bilaterally affected. An autologous bone graft is the ideal material of choice to repair the arch form. The graft stabilizes the segments, restores alveolar continuity, improves the periodontal condition of the neighbouring teeth and allows eruption of teeth through the graft e.g., the upper canine [1]. However, its use is problematic due to donor site morbidity, limited amounts and unpredictable resorption that inhibits proper eruption of the canine [2].

Extensive research is made to look for a suitable substitute for the autologous bone graft which could be used in cleft palate patients. The substitutes should be biocompatible, non toxic, osseo-conductive, available in large amounts, easy to manipulate and well adaptable to each defect. Moreover, they should have some strength to withstand the mastication forces and should be resorbed within some months to allow eruption of the upper canine. Possible substitutes are allografts (same species) e.g., demineralized bone powder, or alloplasts (synthetic material) e.g., Calcium Phosphate Bone Cements. Brown and Chow were the first to develop and patent a Calcium Orthophosphate Cement [3, 12]. Since then, several different formulations for Calcium Phosphate Bone Cements (CPBC's) have been proposed by different investigators [4–9]. CPBC's consist of a powder of (a) calcium phosphate(s), which is mixed with an aqueous solution to obtain a cement paste [10]. This mouldability is an important advantage as compared to hydroxyapatite granules or demineralized bone powder. The paste sets within 10 minutes with the formation of a new calcium phosphate (often hydroxyapatite), of which the entanglement of the crystals ensures the strength of the CPBC's. Moreover they

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are osseoconductive [11] and highly compatible with bone [12].

The aim of the present pilot study is to evaluate the bony repair effect of CPBC's in cranial defects in dogs in comparison with an autologous bone graft as a control.

2. Materials and methods

2.1. Calcium phosphate bone cements

As the major requirement for cleft palate repair is the material's resorbability, cement formulations were chosen which are expected to be resorbed within reasonable time. The compositions of the CPBC's used in this pilot study are summarized in Table I. These materials are compared with the healing capacity of autologous bone, used in 2 control defects.

2.2. The experimental set-up

A classic standardized experimental set-up was chosen: circular cranial defects which are not self healing within the experimental period [1, 2]. For the study two black Labradors and one mongrel dog were used weighing about 25 kg and between 12 and 18 months old.

All animal studies were performed according to the National Institute of Health guidelines for the care and use of laboratory animals (NIH 85-23 Rev. 1985). Preoperatively, the animals were sedated with an intravenous injection of Pentothal. Total inhalation anesthesia was performed during the surgical procedure. The surgical sites were shaved and cleaned with an antisepticum. Infiltration anesthesia was carried out using a 2% lidocaïne solution.

A midline incision was made through skin and periosteum on the calvarium followed by stripping of the periosteum and overlying musculature bilaterally to expose the frontal and parietal bones. Critical size defects of 12 mm were prepared using a drill and care was taken not to perforate the underlying dura mater. At each side of the midline, 2 defects were drilled: one anteriorly and one posteriorly, see Fig. 1. The 4 defects in dog 1 were filled with CPBC formulations 1, 2 and 3 and with autologous bone. In dog 2, formulations 1, 4, 5 and 6 were applied and in dog 3, formulations 1, 7 and autologous bone. The fourth defect in dog 3 was left

TABLE I Powder and aqueous solution components of the used CPBC formulation

Formulation	Powder	Liquid	Ref.
1	α-$Ca_3(PO_4)_2$ + apatite graft	4% Na_2HPO_4	16, 21
2	α-$Ca_3(PO_4)_2$ + $CaHPO_4$ + apatite graft	4% Na_2HPO_4	20, 21
3	α-$Ca_3(PO_4)_2$ + $CaHPO_4$ + $CaCO_3$ + apatite graft	4% Na_2HPO_4	21
4	$Ca_4(PO_4)_2O$ + $CaHPO_4$	0.25 M Na_2HPO_4 + 0.25 M NaH_2PO_4	10, 19
5	α-$Ca_3(PO_4)_2$ + apatite graft	8% Na_2HPO_4	16, 21
6	$Ca_8H_2(PO_4)_6 \cdot 5H_2O$ + $Ca_4(PO_4)_2O$ + $Ca(H_2PO_4)_2 \cdot H_2O$	H_2O	18
7	β-$Ca_3(PO_4)_2$	2M H_3PO_4 + 0.125 M H_2SO_4 + 0.1 M $Na_4P_2O_7$	17

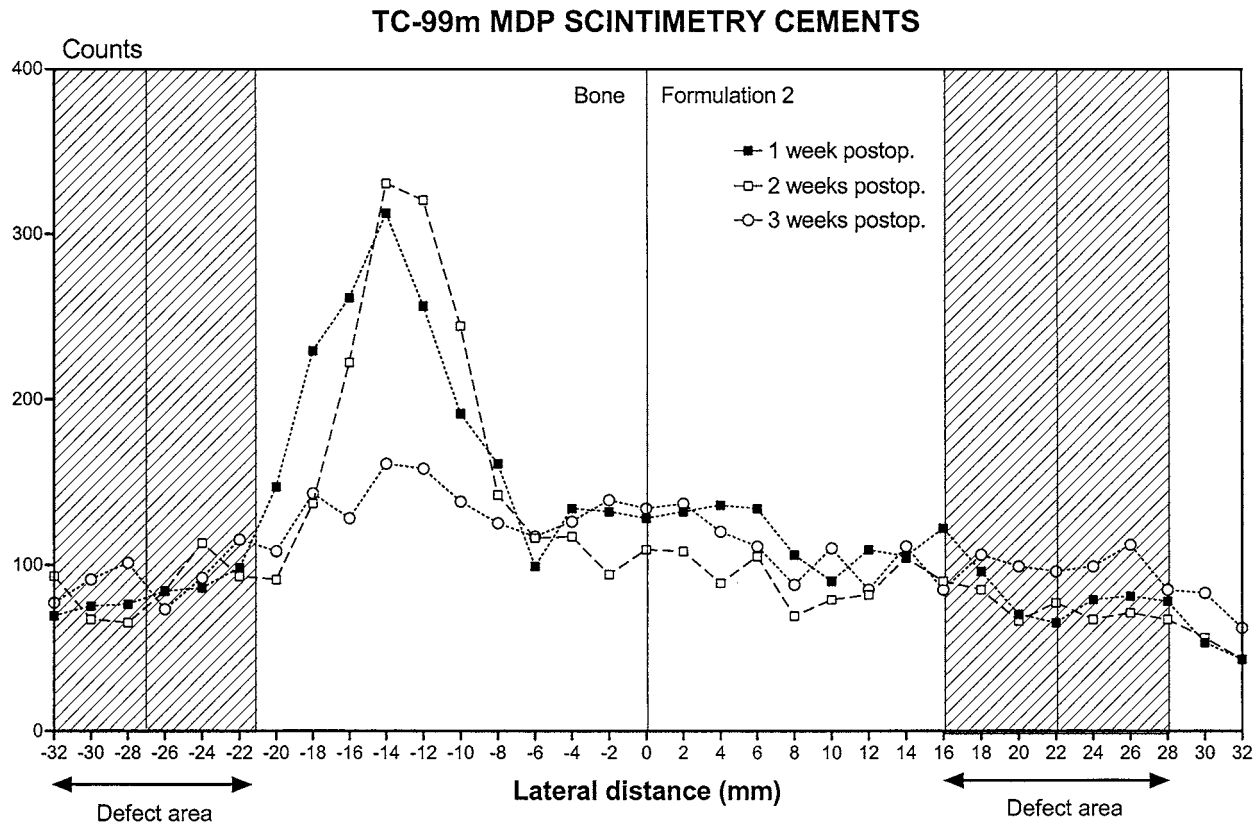


Figure 1 Schematic representation of the defects in the 3 dogs. The numbers refer to the applied CPBC formulations of Table 1.

empty. By filling one defect in each dog with formulation 1 this CPBC served as a reference. The autologous bone, used to fill two control defects in dog 1 and dog 3, was collected during the surgical procedure while creating the defects. A bone filter, mounted on the aspiration tube, kept bone particles apart from blood. The collected bone consisted of cortical bone particles and bone chips.

The overlying periosteum and musculature were carefully repositioned over the implantation sites and sutured. Postsurgically Amoxicilline (10 × 200 mg–Pfizer) was administered to ensure antibiotic coverage during 5 days. Postoperative pain was alleviated with an intramuscular injection of Temgesic (Buprenorphinum 0,3 mg/ml–Schering Plough). Six months after the creation of the artificial defects, the animals were sacrificed.

2.3. Evaluation methods

Qualitative and quantitative evaluation of bone metabolism were carried out in the first dog by means of repeated Technetium-99m methylene-diphosphate (Tc-99m-MDP) uptake measurements: *scintimetry*. Scintimetry is a standardized quantitative evaluation system by means of Tc-99m-MDP uptake measurements. This radionuclide counting method was based on the experiments described by Gepstein *et al*. [13], Aspenberg and Wittbjer [14], De Bie *et al.* [15] and Kuyl *et al.* [1]. On the day of measurement, approximately 555 MBq (15 m Ci) Tc-99m-MDP was administered intravenously, depending on the weight of the experimental animal. Thereafter, 3 till 4 hours were allowed to elaps until the radiopharmaceutical had disappeared from the blood pool and the soft tissues and had penetrated in the defect area. The activity in and around the defect was measured with a pinhole collimator and a Bicron 1,5 × M $^1/_2{}''$ Nal crystal, linked to a Canberra Series 20 multichannel analyzer. The collimator opening was 2 mm. The animals were positioned in a special standardized self-designed fixation apparatus, while the collimator was moved over the defect in 2 mm increments with a measuring time of 30 seconds for each measurement.

Scintimetry was carried out at the following times:

(1) day of surgery
(2) 1 week postoperatively
(3) 3 weeks postoperatively
(4) 7 weeks postoperatively
(5) monthly, till the dog was euthanized.

During the experimental evaluation period *computal tomographic scans* (CT-scans) were taken on dogs 2 and 3. Therefore the animals were anaesthetized and positioned on a movable table. Transversal sections of 1 mm thickness were made along the defect.

Intravenous administration of different bone markers was carried out to evaluate the new bone ingrowth during the experiment. Two different *vital staining* procedures were followed: one dog received oxytetracycline (14 mg/kg) 3 weeks before euthanasia and calceine (7 mg/kg) 1 week before. Two dogs received 3 times monthly tetracycline (7 mg/kg), followed by calceine during one month and finally, oxytetracycline, 10 days before euthanasia.

After 6 months the dogs were euthanized for macroscopic, histologic and chemical evaluation, of the defects. The cement implant sites were bissected into two portions: one portion was used for the histologic evaluation, the other one for the mineral phase analysis. The samples for *histological evaluation* were fixed in 4% neutral buffered formalin (4°C).

From each sample, one part was decalcified with Ethylene Diamine Tetra-Acetic acid (EDTA) for a period of 5 weeks. After washing and dehydratation, the samples were embedded in paraffin and sliced as 5 μm thick sections. They were stained with haematoxylin and eosin to define the general morphology, with Masson's trichromatic staining for collagen demonstration and with toluïdine blue as a metachromatic stain for cartilage proteoglycans (ground substance). Histological evaluation was done by light microscopy. The other part was embedded in epoxy resin without prior decalcification. Sections were cut at 10 μm and evaluated under the fluorescence microscope. Fluorescent labels allowed a descriptive evaluation of bone remodeling/metabolism.

The *phase composition* of the cement after the in vivo experiment was characterized by physical analysis using X-ray diffraction and infrared spectroscopy. X-ray powder diffraction patterns were recorded using a microprocessor-controlled diffractometer system PW 1830 (Philips, NL-7602 EA Almelo, the Netherlands). Ni-filtered copper K_α radiation was used with an automatic divergence slit PW 1836 and a graphite monochromator. IR spectra of the cement samples dispersed in CsBr tablets were recorded using a Galaxy 6030 Fourier transform infrared spectrophotometer (Mattson, Madison, WI 53717, USA).

3. Results

3.1. Scintimetry

The scintimetric activity profiles of the readings at 1, 3 and 7 weeks postoperatively for dog 1 were superimposed in Figs 2 and 3. A remarkable activity profile can be seen at week 3 postoperatively along the defect filled with formulation 1. At 7 weeks postoperatively the profiles reached more or less the baseline. The defects filled with formulation 2 and 3 showed no activity at all in the scintimetric profiles. In the defect filled with bone, activity was found after the first week already and it reached a maximum level at week 3. As scintimetric measurements were a very time consuming method with limited information—there was no activity measured next to the defect area—they were not performed for the other dogs.

3.2. CT-scanning

Although the imaging of the CPBC's on CT-scans is radio-opaque, new bone ingrowth could be differentiated from the original implant material. There was no or little change in radiolucency in the defects implanted

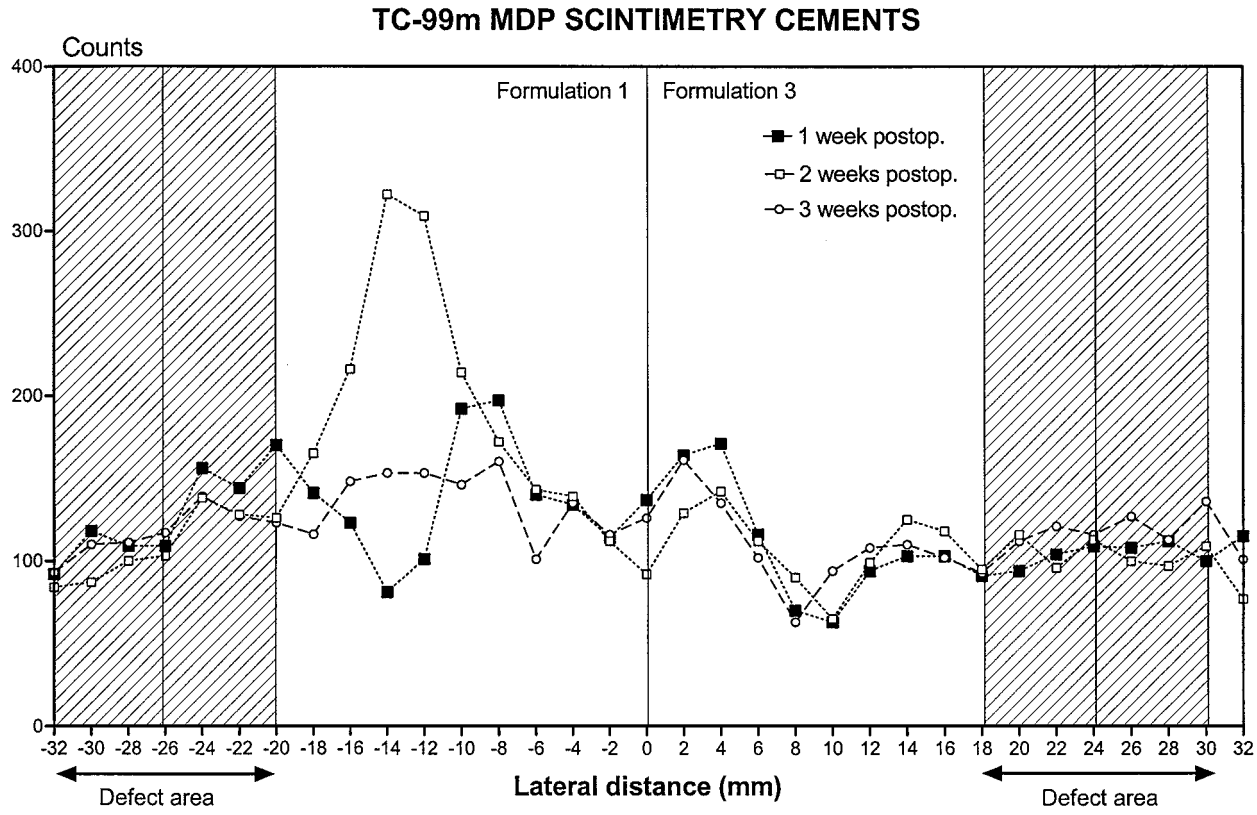


Figure 2 TC-99m MDP scintimetric findings of the anterior defects, filled with formulations 1 and 3 at different times, after implantation.

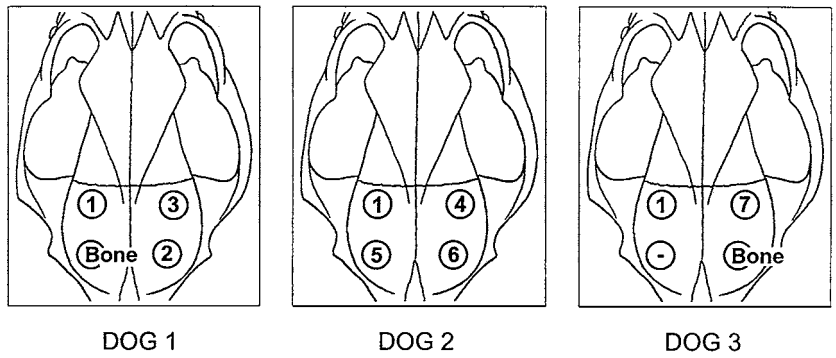


Figure 3 TC-99m MDP scintimetric findings of the posterior defects, filled with bone and formulation 2 at different times, after implantation.

with cement 1, 4, 5, and 7. Formulation 6 showed a remarkable decrease of the defect size in all dimensions and the cement had become less opaque at the end compared to the original scans. In the bone filled defect an enormous growth of new bone could be detected. The empty defect was filled only with fibrous tissue.

3.3. Macroscopy

It could be observed that the control defects were filled with clear and hard bone, the defect was completely closed, but a central dip was present. The empty defect was closed on a level far below the skull surface. Formulation 1 in dog 1 could hardly be detected, the newly formed bone had a good bond with the edges and the margins were hard to recognize. No macroscopic visible changes could be seen in the cement implants in dogs 2 and 3. The cements were clearly visible, hard and had minimal bond with the edges of the created defects.

3.4. Histology

The originally unfilled defect site was replaced by fibrous connective tissue with blood vessels at the caudal side of the defect and with striated muscle tissue and loose connective tissue at the cranial side. After 6 months there was no spontaneous bony closure of this 12 mm diameter defect. Indeed, the defects were not self-healing within the experimental period.

Fig. 4 illustrates that the edges of the original defect could be clearly recognized: they were sharply marginated without remarkable lateral ingrowth of newly formed bone. No osteo-necrotic areas, characterized by empty lacunae, were found. Fig. 5 shows that in the *bone filled* defects, the original margins were no longer detectable. The implanted bone, combined with fibrous connective tissue, was fully integrated in the original defect site. The light microscopic results of the implanted CPBC formulations 2, 3, 4, 5 and 7 were comparable. An example for formulation 2 is shown in Fig. 6.

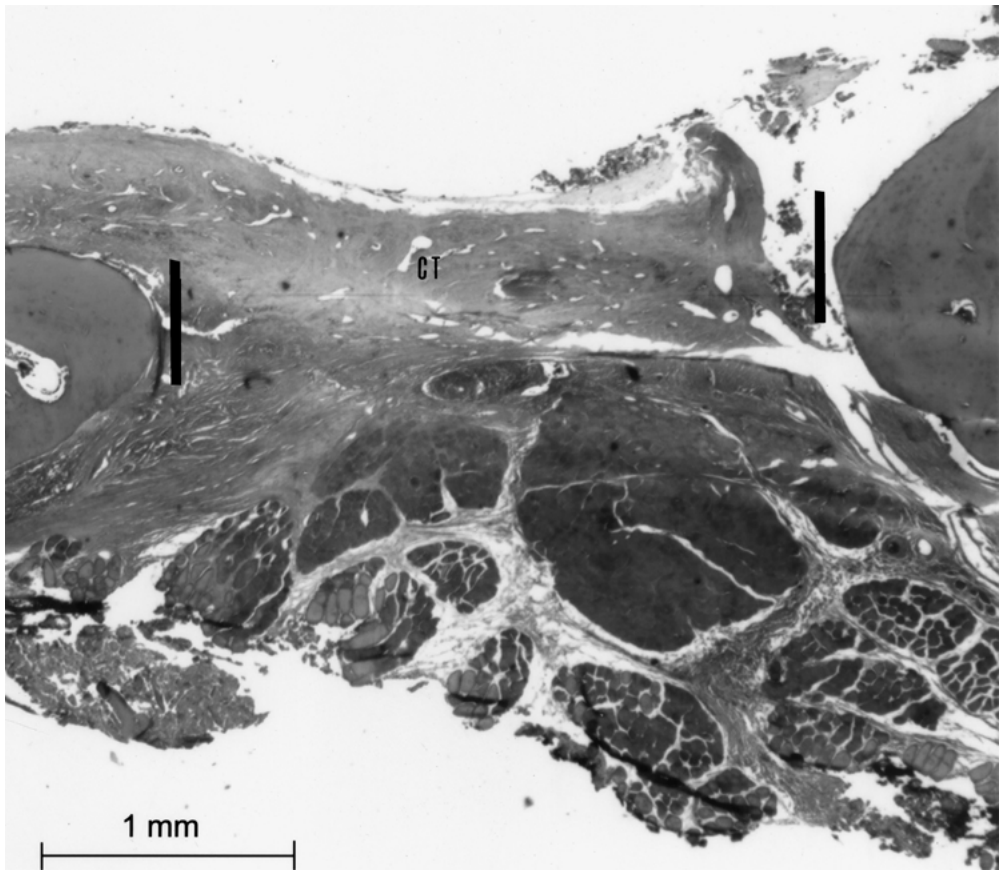


Figure 4 Light micrograph (magnification 30×) showing the untreated defect site after 6 months (vertical lines = defect margins). No bone formation was observed. The defect was mainly filled with connective tissue (CT).

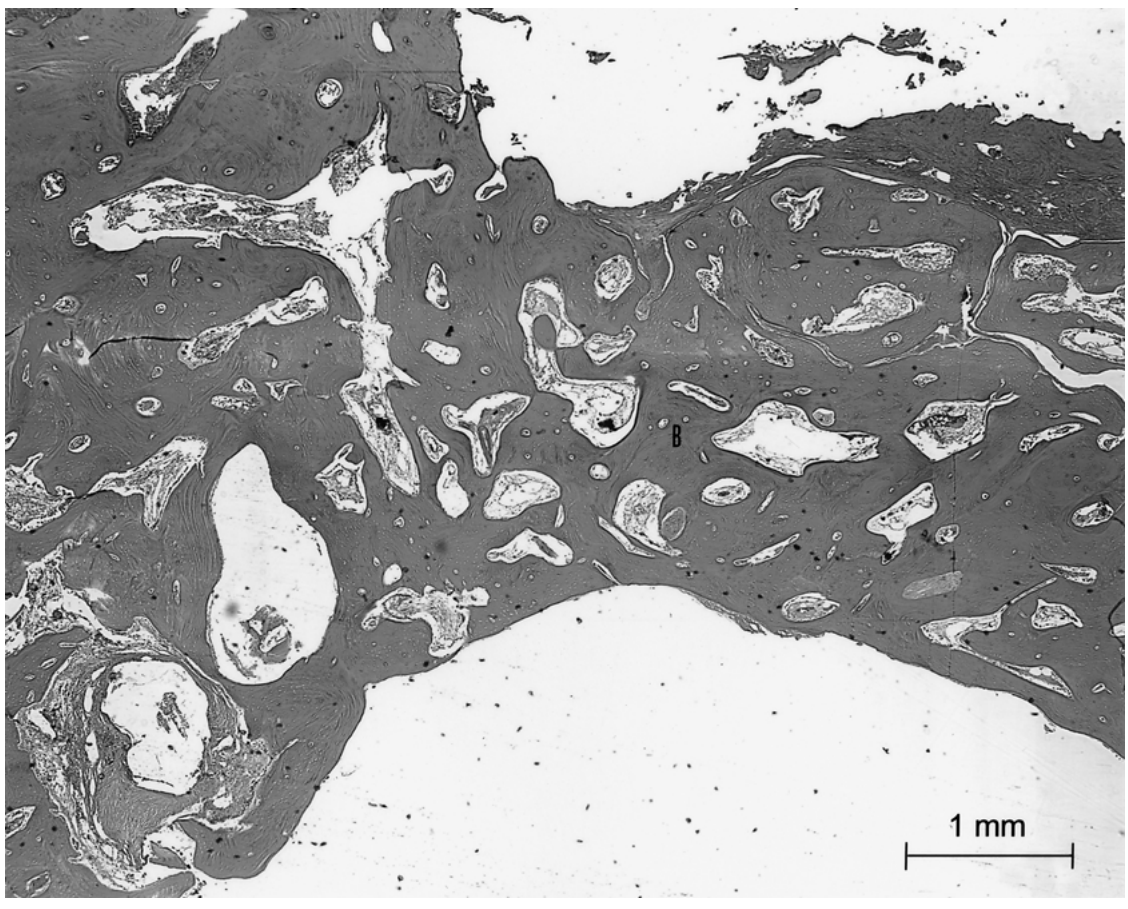


Figure 5 Light micrograph (magnification 20×) of the defects filled with bone particles (B). The original defect could no longer be detected.

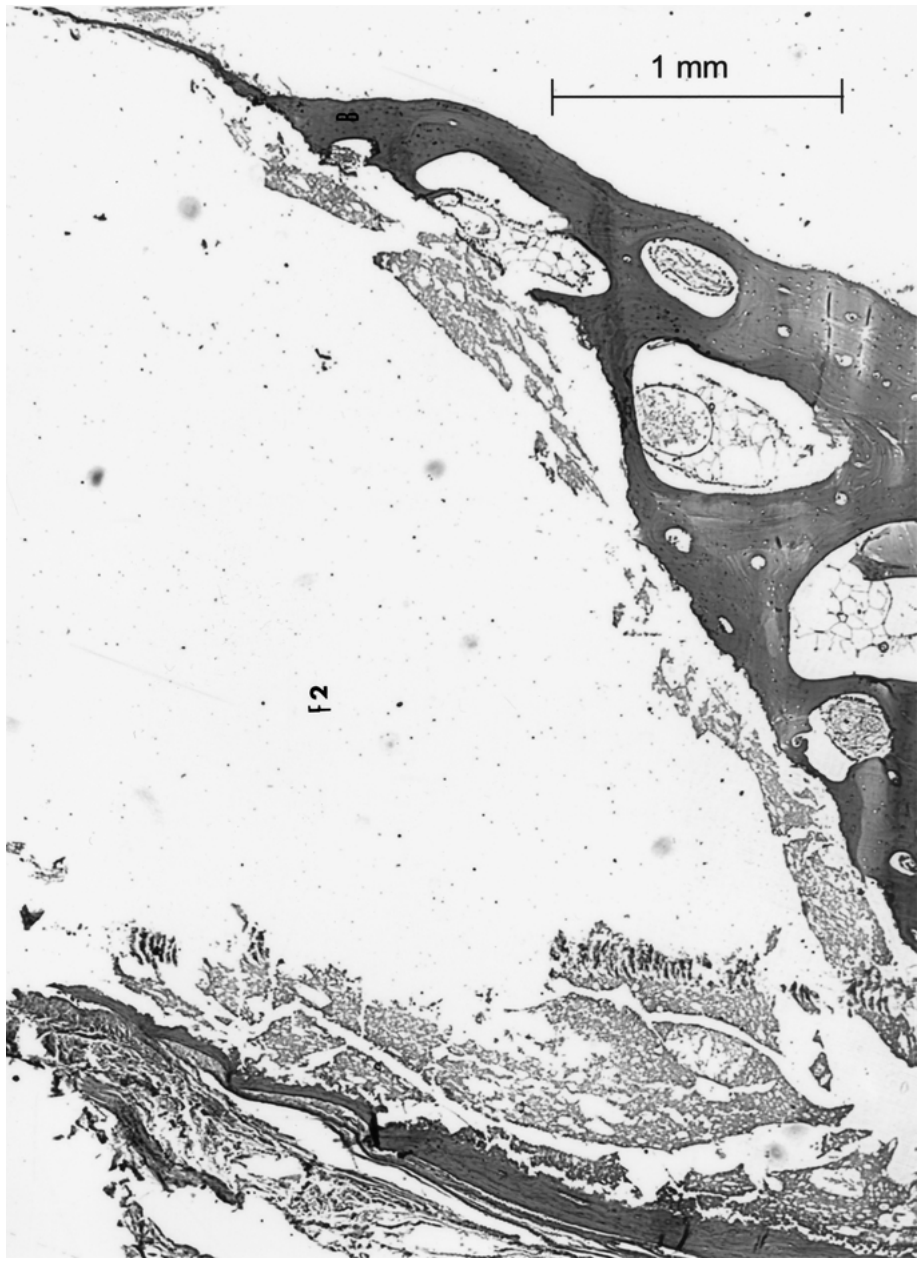


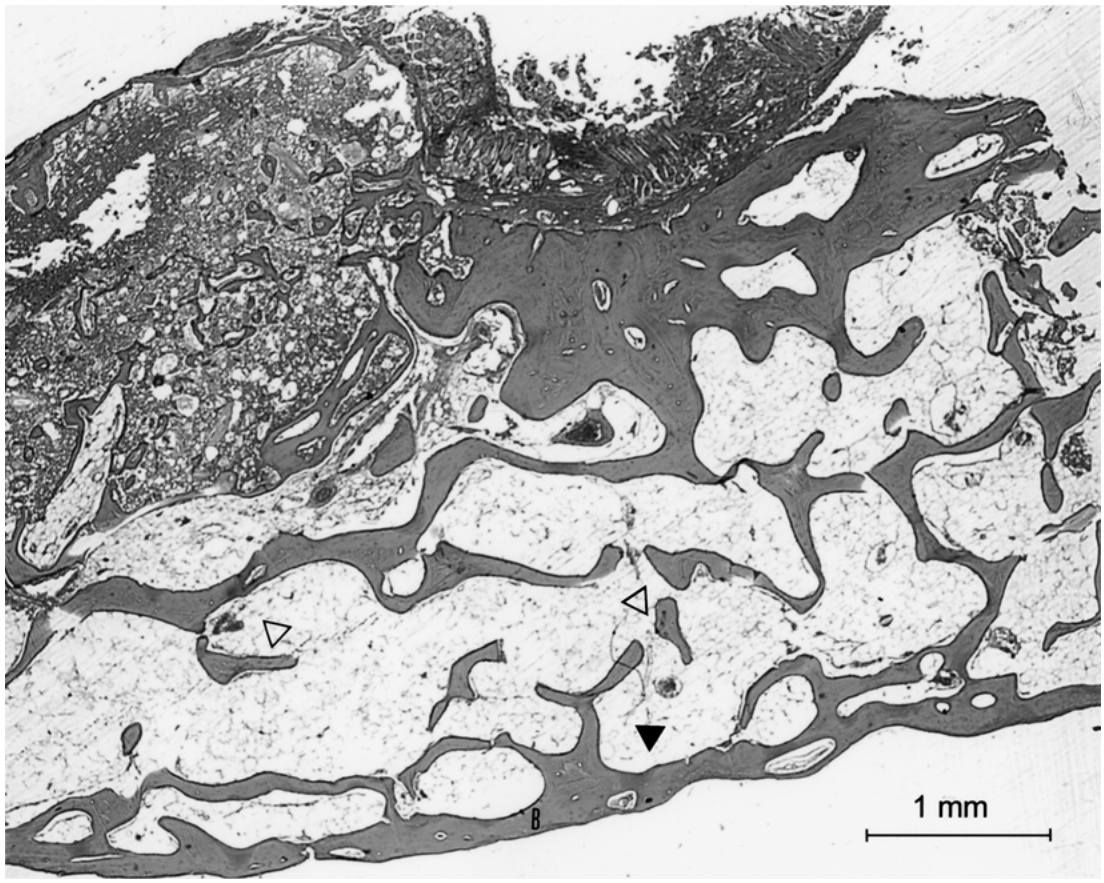
Figure 6 Light micrograph (magnification 40×) of a representative CPBC. Newly formed bone (B) was only observed as a thin layer at the caudal and cranial side (closed arrow) of the defect. The remaining part of the defect was still filled with formulation 2 (F2).

Two small newly formed bone layers, interconnecting the lateral defect sides and laying along the unresorbed implant material, were detectable at the cranial as well as the caudal side of the defect. Bone formation was always more pronounced at the cranial side. No bony ingrowth from the lateral side was observed. Between the unresorbed bone substitute and the lateral edges of the defect as well as between the unresorbed bone substitute and the newly formed bridges, remnants of a haematom were visible.

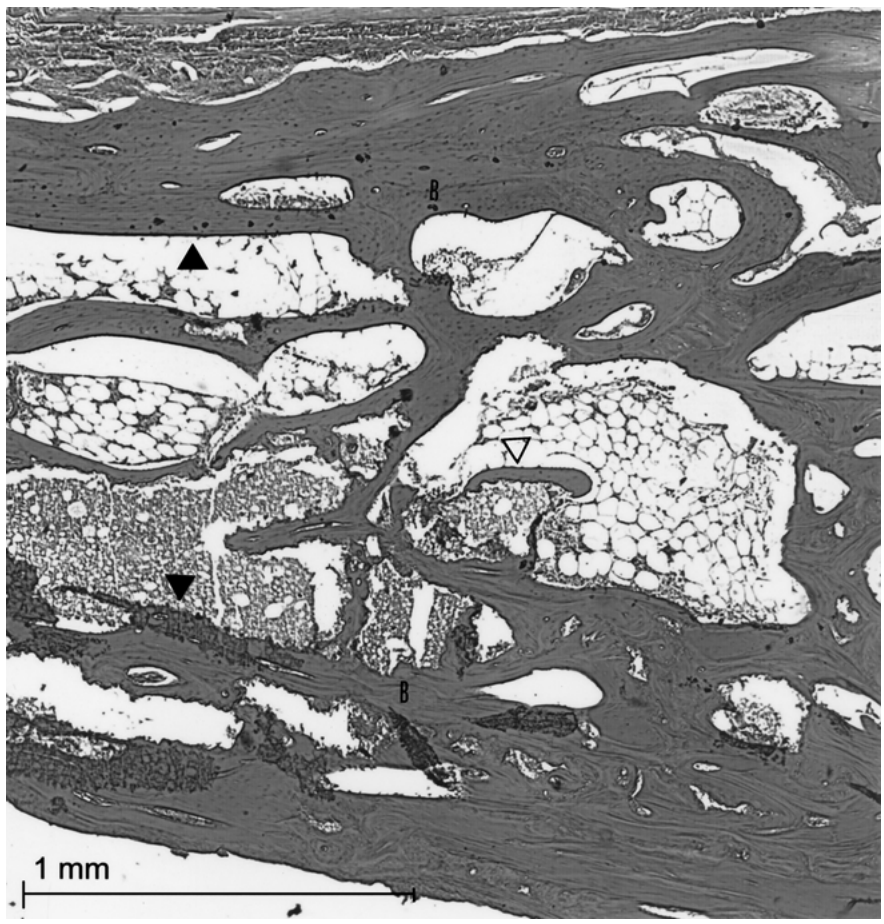
In contrast to the above mentioned formulations, bone formation was seen with the formulations 1 and 6 in Fig. 7a–b. Although remnants of a haematom and fibrous connective tissue were still present, islets of newly formed bone, delineated with osteoblasts, were observed. Islets were often situated around blood vessels and were distributed randomly all over the entire defect. At the cranial and caudal side, bone layers were bridging the defect. These layers were more pronounced as compared with the layers formed using formulations 2, 3, 4, 5 and 7. However, although formulations 1 and 6 were found to be the best substitute for autologous bone grafting the bony repair was not as obvious as in the defect filled with bone chips.

In spite of all the above mentioned differences there was one common characteristic: there was always a small layer of bone present on the caudal and the cranial side of the defects implanted with a cement and there were no inflammation cells present. As far as the biodegradability is concerned, resorption of the implanted material was noticed for formulations 1 and 6.

The *fluorescence* results were not conclusive: in some areas there was a well marked labeling, in others a diffuse one. The labeling was randomly spread in those areas where new bone was present.



(a)



(b)

Figure 7 Light micrograph of CPBC formulation 1 (Figure 7a—magnification 20×) and 6 (Figure 7b—magnification 45×). Dense layers of bone (B) at the caudal and cranial side (closed arrow) were found. Randomly distributed over the remaining part of the defect, islets of newly formed bone (open arrow) were observed.

TABLE II Qualitative evaluation of bony repair

	Dog 1				Dog 2				Dog 3			
Formulation	1	2	3	bone	1	4	5	6	1	7	bone	–
Scintimetry	2	1	1	2								
Ct scanning					1	1	1	2	1	1	3	2
Macroscopy	3	1	1	3	1	1	1	1	1	1	3	2
Microscopy	3	2	2	4	3	2	2	3	3	2	4	1

Scintimetry: 1 = no bone activity, 2 = bone activity; Ct scanning: 1 = no calcification, 2 = limited calcification, 3 = much calcification; Macroscopy: 1 = unchanged visual aspect, 2 = defect difficult to define, 3 = defect margins not to define; Microscopy: 1 = fibrous tissue, 2 = compact bone on top and on the buttom of the defect, 3 = bone formation + capillaries, 4 = mainly bone formation.

3.5. Mineral phase analysis

X-ray diffractometry and IR spectrometry of the CPBC's samples indicated that a chemical reaction occurred upon implantation. From the position and the resolution of the diffraction peaks and the absorption bands, it could be seen that, with the exception of formulation 7, where a considerable amount of ß-$Ca_3(PO)_4$ was still detected, the cements have reacted to form a more or less crystalline calciumhydroxyapatite. For these cements, little or none of the starting reagents (see Table I) could be detected.

4. Discussion

For several years extensive research has been done for bone substitutes as autologous bone is only available in sparse amounts. Since the introduction of Calcium Phosphate Cements by Gruninger [12], CPBC's appear to be very promising materials for the replacement of bone.

In this study an evaluation was made of several CPBC's in critical size defects. The result of this present pilot study concerning the empty defect is in agreement with the findings of Kuyl *et al.* [1] indicating that the 12 mm defects were not self healing during the experimental period and corroborates the relevancy of the experimental design.

In our pilot study, as well as in the experimental study of Kuyl *et al*. [1] the scintimetric results are more pronounced at the left side of the skull. This can be coincidence: due to the implanted material at the left side resulting in higher Tc-99m-MDP uptake values or due to anatomical variation in the blood supply of the skull between the used animals resulting in higher Technetium omission at one side.

Table II summarizes the results of the different evaluation methods in a qualitative way. Although the defect filled with bone gave the best result, formulations 1 and 6 also seem to be very promising.

All the tested CPBC's showed a very *biocompatible* bone behaviour. Histologic evaluation of the cranial defects showed a good healing, without foreign body reactions. Remnants of haematom and fibrous connective tissue were present but no inflammatory or foreign body reactions were seen. This observation corresponds with findings of Koshino *et al.* [22] and Jansen *et al*. [11]. Moreover all formulations were *osseoconductive* and acted as a "guideline" for the formation of new bone. At least a small bone layer was seen on the apical and cranial side of each defect which was absent in the empty cranial defect according to the microscopic evaluation. This new bone formation was probably induced by both the dura mater and the periosteum.

Some formulations exhibited to some extent *biodegradability*. In this respect, formulation 6 is very promising: the corresponding defect was difficult to define after the implantation, extensive bone formation was seen and CT-scanning showed calcification. The biodegradability of this formulation could be explained by the fact that one of the starting reagents in formulation 6 was octacalciumphosphate, $Ca_8H_2(PO_4)_6 \cdot 5H_2O$, which is known to be a precursor of the mineral phase of bone [18]. Formulation 1 was also found to be degraded in dog 1 and bone formation was observed. Analogous results were found in the *in vivo* study on goats by Jansen *et al.* [11] where defects in the tibia were filled with a CPBC comparable to formulation 1. However, the present study showed that the performance of this formulation may be somewhat variable as there are distinct differences between the results with dog 1 on the one hand and dog 2 and 3 on the other hand (Table II). This can be due to a difference in batches of the starting reagents as well as by racial and/or age differences between the dogs. In view of this biocompatible, osseoconductive and biodegradable behaviour, it can be concluded that formulation 1 and 6 could be promising alternatives for autologous bone grafts to be applied in cleft palate patients allowing the eruption of the upper canine.

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