Dental Materials Journal 2010; 29(1): 15–24

Observation of calcium phosphate powder mixed with an adhesive monomer experimentally developed for direct pulp capping and as a bonding agent

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In this study, morphological shape, elemental distribution and elution properties of Ca, P, Mg in four types of calcium phosphate powder were investigated using SEM, EPMA and ICP-AES. Calcium phosphate powder: OHAp, DCPD, β-TCP and OCP were observed in the white powder form and in the photopolymerized adhesive monomer they scattered like dispersed fillers in resin composite. In elemental analysis, CaKα showed a relatively high concentration in relation to PKα. In elution analysis, each calcium phosphate showed different elution of Ca and P. But Mg was almost equal to the detection limit of ICP-AES. Namely it was suggested that reparative dentin formation was effectively promoted under the following conditions: a calcification promoting effect by direct contact of the calcium phosphate powder, an ionic effect of Ca and P eluted from the powder located in the vicinity of the exposed pulp and environmental pH change of the surface in exposed pulp.

Keywords: Adhesive monomer, Calcium phosphate powder, SEM, EPMA, ICP-AES

Received Apr 2, 2009: Accepted Sep 3, 2009

INTRODUCTION

Vital pulp preservation is an important factor for the longevity of teeth. Many devoted investigators have focused on alternative materials for direct pulp capping agents. Adhesive resins have been reported to be one of the good alternatives to calcium hydroxide or its preparations because they require only simplified procedures, bond well and have a good sealing ability to the cavity. Our laboratory has conducted several studies by using human, monkey and rat teeth to investigate the pulpal response to adhesive resins[1-21]. Some investigators including ourselves have found that several adhesive resins were able to elicit a favorable pulpal response after direct capping of the exposed pulp[1-27]. The results of several long-term studies have suggested that adhesive resins were almost equal to calcium hydroxide or its preparations with regard to wound healing of exposed pulp[4,10]. However, dentin bridge formation was usually initiated earlier when using calcium hydroxide and its preparations in direct capping groups[1-3,5-9,11-15,17-21]. There have also been some reports which suggested negative biocompatibility of adhesive resins to pulp[28-33]. Currently, there is no widely accepted technique or consensus on the use of adhesive resins as direct pulp capping agents.

Nevertheless, we have developed experimental adhesive resin direct pulp capping and as a bonding agents containing reparative dentin-promoting agents and direct pulp capping effects have been examined histopathologically and immunohistochemically on exposed rat pulp[15,17-21]. As reparative dentin-promoting agents, four types of calcium phosphate: hydroxyl-calcium phosphate (hydroxyapatite: OHAp), dicalcium phosphate dihydrate (brushite: DCPD), beta-tricalcium phosphate (whitrokite: β-TCP) and octacalcium phosphate (OCT), were mixed with an experimental adhesive monomer to create materials for direct resin pulp capping and as a bonding agents of dental substrates. The four types of calcium phosphate were selected for the following reasons. In general, calcium phosphate itself is a chief component of dental hard tissue. In addition, crystallized deposition of the four types of calcium phosphate frequently appears at the area of remineralized caries lesions and sclerotic dental hard tissue[34-38]. It was considered that the four types of calcium phosphate mixed with an experimental adhesive monomer may play an active role to promote reparative dentin formation.

In this study, the shapes and elemental distributions of Ca, P and Mg in four types of calcium phosphate powder were investigated using scanning electron microscope (SEM) and electron probe microanalyser (EPMA). In addition, dispersion phases of calcium phosphate powder, elemental distribution and elution properties of Ca, P, Mg from photopolymerized samples of calcium phosphate powder and an experimental resin monomer were also investigated using EPMA and inductively coupled plasma atomic emission spectrometry (ICP-AES). Elemental analysis of Ca, P and Mg were selected for the following reasons. Namely, they are the chief components of dental hard tissue and Ca and P are present in the chemical formula of the calcium phosphate powder used. On the other hand, Mg is not present in the chemical formula, but traces of elemental Mg as an impurity in hydration cells of calcium phosphate are present. In addition, the Mg

concentration shows an interesting decrease and increase in specimens of developing, erupted and carious dental hard tissue[39].

The background of the reparative dentin-promoting effect in experimentally developed adhesive resin direct capping and as a bonding agents was carefully examined to discover the major points for wound healing effects of exposed pulp.

MATERIALS AND METHODS

Approval of the Ethics Committee

This study was approved by the Ethics Committee of the Nippon Dental University, School of Life Dentistry at Niigata (Receipt number: 24, July 5th, 2006)

Materials

The four types of calcium phosphate and the experimental adhesive monomer used in this experiment are listed in Tables 1 and 2. One type of calcium phosphate powder was mixed with an experimental adhesive monomer at the ratio of 5 wt% to create experimentally developed resin direct pulp capping and as a bonding agents for dental substrates. In total four kinds of experimentally developed direct pulp capping and as a bonding agents (MB1, MB3, MB5 and MB7) were prepared and the detailed specifications are listed in Table 3.

Table 1 Four types of calcium phosphate powder used in the experiment

Materials	Abbr.	Chemical Structures	Molar Ratio Ca / P	Lot #	Manufacturers	Remarks
Hydroxyl-calcium phosphate (Hydroxyapatite)	OHAp	$Ca_{10}(PO_4)_6(OH)_2$	1.67	030606	Ube Materials Industries Ltd (Ube-City, Japan)	Experimental Product (Sintering at 1200 °C
Dicalcium phosphate dihydrate (Brushite)	DCPD	$CaHPO_4 \cdot 2H_2O$	1.00	M7H6575	Nacalai Tesque Inc (Kyoto, Japan)	The First Class Reagent
Beta-tricalcium phosphate (Whitlockite)	β-TCP	$Ca_3(PO_4)_2$	1.50	04080401	Taihei Chemical Industrial Co Ltd (Osaka, Japan)	Experimental Product
Octacalcium phosphate	OCP	$Ca_8H_2(PO_4)_6 \cdot 5H_2O$	1.33	SA3131	Taihei Chemical Industrial Co Ltd (Osaka, Japan)	Experimental Product

Table 2 Composition of the experimental adhesive monomer used in the experiment

Composition	Lot #	Manufacturer
Bisphenol A Diglycidylmethacrylate 2-Hydroxyethyl Methacrylate Hydrophobic Aliphatic Dimethacrylate 10-Methacryloyloxydecyl Dihydrogen Phosphate (MDP) Amine dl-Camphorquinone	041110	Kuraray Medical Inc (Tokyo, Japan)

Table 3 Composition of each group experimentally developed for adhesive resin direct pulp capping and as a bonding agents containing calcium phosphate powder

Experimental Groups	Composition (wt%)			
	MB1	MB3	MB5	MB7
Experimental adhesive monomer	100	100	100	100
OHAp	5			
DCPD		5		
β-TCP			5	
OCP				5
Lot #	21008	21008	41006	41006

(n=5)

Sample preparation and characterization

Sample forms used for SEM, EPMA and ICP-AES observations are shown in Fig. 1. Calcium phosphate powder was spread on carbon double-sided tape (Nissin EM Co., Ltd., Tokyo, Japan) which was fixed onto aluminum stabs and then sputter coated with platinum/palladium using a vacuum coater (Hitachi E101, Hitachi Co, Tokyo, Japan). First, the morphological shapes of calcium phosphate powder were observed using SEM (Hitachi S-800, Hitachi Co, Tokyo, Japan) at an accelerating voltage of 15 kV. Second, other morphological shapes and elemental distributions of CaKα, PKα and MgKα of calcium phosphate powder were also observed using EPMA (EPMA-8705, Shimadzu, Kyoto, Japan) at an accelerating voltage of 20 kV, and a specimen current of 10 nA, to obtain back scattered electron (BSE) images and characteristic X-ray images, as well as secondary electron (SE) images using specimen current 0.5 nA.

Elemental distributions of CaKα, PKα, MgKα and dispersion phases of calcium phosphate powder in photopolymerized samples were observed using EPMA under the same observational conditions. The sample was finished and polished by dry grinding with 1000-grit waterproof abrasive paper (Kovax Corp, Tokyo, Japan). After that the specimens were fixed on to aluminum stabs using electroconductive paste (Graphite Paste, Okenshoji Co., Ltd., Tokyo, Japan), the specimens were treated with carbon vacuum evaporation using a carbon coater (Carbon Coating System CC-40F, Meiwafosis, Osaka, Japan). First surface analysis of CaKα, PKα and MgKα were made. Secondary an approximate distance of 500 μm of the line profile of the SE and BSE images was undertaken with a sample speed of 50 μm/min and chart speed of 20 mm/min, to investigate CaKα, PKα and MgKα concentrations.

Elution properties of Ca, P and Mg from photopolymerized disk plate were measured using ICP-AES (ICP-AES-IRIS AP, Thermo Fisher Scientific KK, Franklin, MA, USA). Experimental apparatus for ICP-AES measurement were fabricated as follows. Namely, a mixture of adhesive monomer and 5 wt% calcium phosphate powder of OHAp, DCPD, β-TCP or OCP was poured into the polyester plate which was first covered with polyestel film and secondary covered with micro slide glass (Matsunami Glass Ind., Ltd., Osaka, Japan)

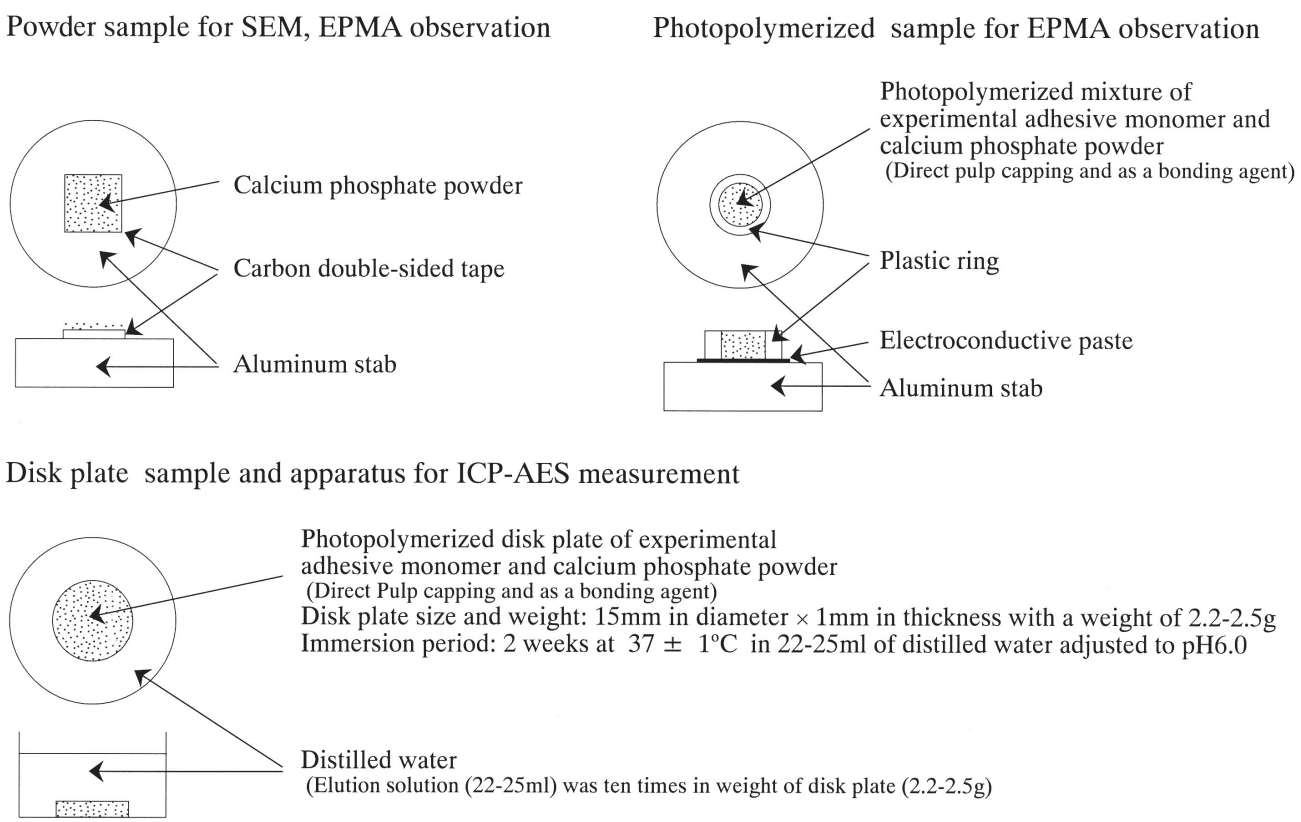

Fig. 1 Three kinds of sample forms used for the experiment,
upper left: powder sample for SEM, EPMA observation
upper right: photopolymerized sample for EPMA observation
lower left: disk plate sample and apparatus for ICP-AES measurement

and pressed gently by hand. The mixture was then photopolymerized. The sample was finished and polished with 1000-grit waterproof abrasive paper to remove the unphotopolymerized surface or low photopolymerized layer of the specimen. Finally, a disk plate 15 mm in diameter × 1 mm in thickness with a weight of 2.2-2.5 g was made. The disk plate was stored at 37 ± 1°C in 22-25 ml of distilled water adjusted to pH6.0 for 2 weeks. Elution amounts of Ca, P and Mg measured in μg/g in distilled water were evaluated using ICP-AES. Measuring conditions were as follows: RF forward power: 1150 W, auxilliary flow (Argon gas): 0.5 L/min, pump rate: 130 rpm, analysis limit: 0.01 μg/ml and measurement wavelength: Ca: 396.847 nm, P: 185.943 nm and Mg: 285.213 nm.

RESULTS

Morphological shapes of calcium phosphate powder

Representative SEM morphological images observed in four kinds of calcium phosphate powder are presented in Fig. 2.

The findings observed are summarized as follows: Four kinds of calcium phosphate powder were observed to be of a white powder form to the naked eye. OHAp showed spherical particles in many different sizes and their surfaces were comparatively smooth. DCPD showed cylindrical shapes in small gatherings and their surfaces were comparatively smooth. β-TCP showed small granular and uniform shapes and their surfaces were comparatively smooth. OCP showed needle-like shapes in small gatherings of cotton-like structures.

Elemental analysis of calcium phosphate powder and photopolymerized samples by EPMA

Representative EPMA morphological images and characteristic X-ray images of CaKα, PKα, MgKα observed in four kinds of calcium phosphate powder are presented in Fig. 3. In addition, representative EPMA surface images and line profiles of elemental distributions of CaKα, PKα, MgKα and dispersion phases of calcium phosphate powder in photopolymerized samples are presented in Fig. 4.

The findings observed are summarized as follows: CaKα showed a relatively high concentration in relation to PKα, but MgKα was found only in a trace amounts and was detected only when using line profiles. The calcium phosphate powder in the photopolymerized adhesive monomer scattered like dispersed fillers in resin composite.

Elution property analysis of Ca, P and Mg from photopolymerized samples by ICP-AES

Elution amount of Ca, P and Mg from photopolymerized solid mixture are presented in Table 4.

The results observed are summarized as follows: The elution amount of Ca increased in the following order: MB1, MB5, MB(C)1, MB7 and MB3. On the other hand, the elution amount of P increased in the following order: MB(C)1, MB1, MB5, MB7 and MB3. It was found that the elution amount of Mg was almost equal to the detection limit of ICP-AES.

DISCUSSION

The four types of calcium phosphate powder were composed of elements such as Ca, P and Mg. The dispersion phases of calcium phosphate powder in the photopolymerized adhesive monomer, direct pulp capping and as a bonding agents, were highly influenced by vibration and trituration effects during the hand mixing of the materials. In well-mixed sample, the calcium phosphate powder in the adhesive monomer scattered uniformly in the photopolymerized

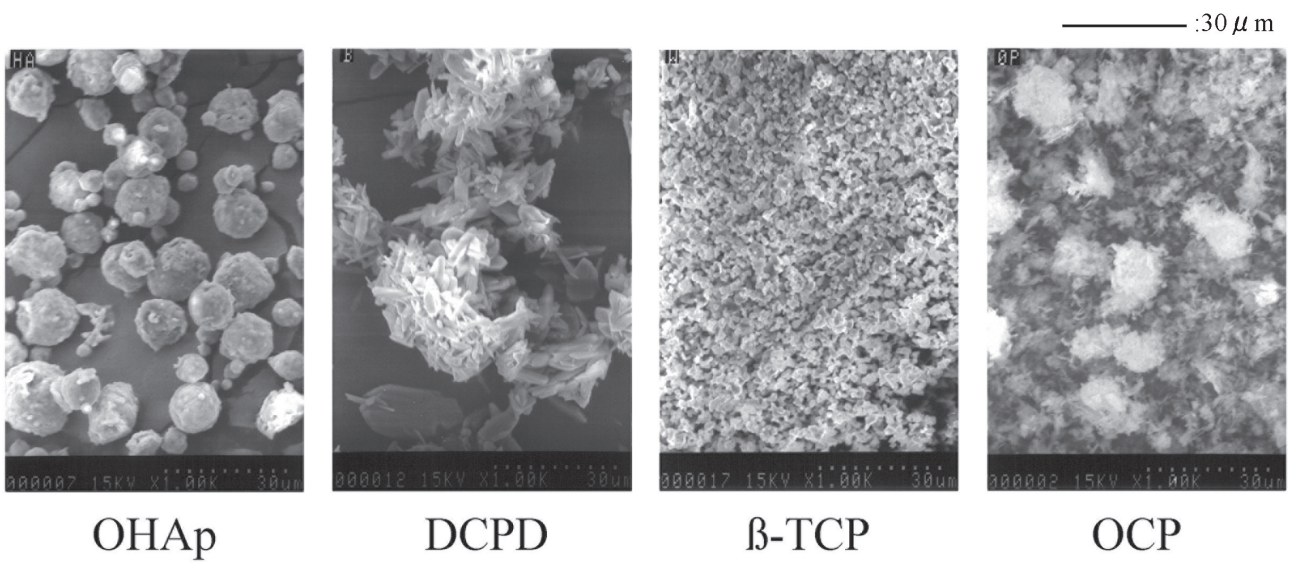

Fig. 2 Representative SEM morphological images observed in four kinds of calcium phosphate powder

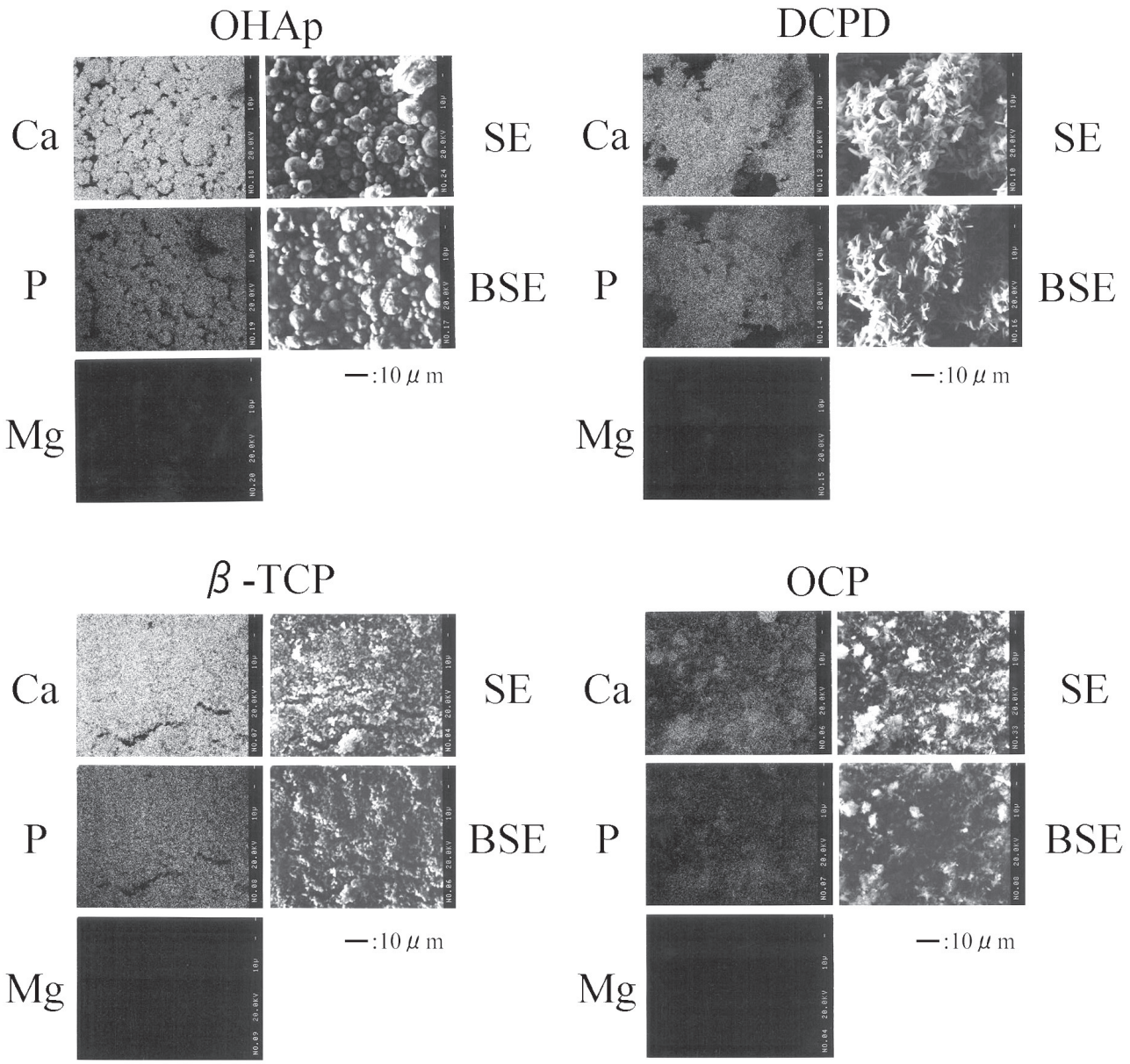

Fig. 3 Representative EPMA morphological images and characteristic X-ray images of CaKα, PKα and MgKα observed in four kinds of calcium phosphate powder

Table 4 Elution amount of Ca, P and Mg from photopolymerized disk plate evaluated using ICP-AES measurement

Experimental Groups	Weight of Sample Plate	Elution Solution	Ca		P		Mg	
	[g]	[ml]	[μg/ml]	[μg/g]	[μg/ml]	[μg/g]	[μg/ml]	[μg/g]
[MB1]	2.38	23.8	2.48	24.8	2.60	26.0	0.01	0.1
[MB(C)1]	2.32	23.2	4.41	44.1	1.92	19.2	0.01	0.1
[MB3]	2.23	22.3	17.4	174	19.0	190	0.01	0.1
[MB5]	2.20	22.0	2.60	26.0	3.31	33.1	0.01	0.1
[MB7]	2.47	24.7	4.97	49.7	4.64	46.4	0.02	0.2

(Detection limit; 0.01 μg/ml)

MB1: 5 wt% OHAp, MB3: 5 wt% DCPD, MB5: 5 wt% β-TCP, MB7: 5 wt% OCP
MB (C)1: The group which acidic monomer MDP was eliminated from the composition of the MB1 group.

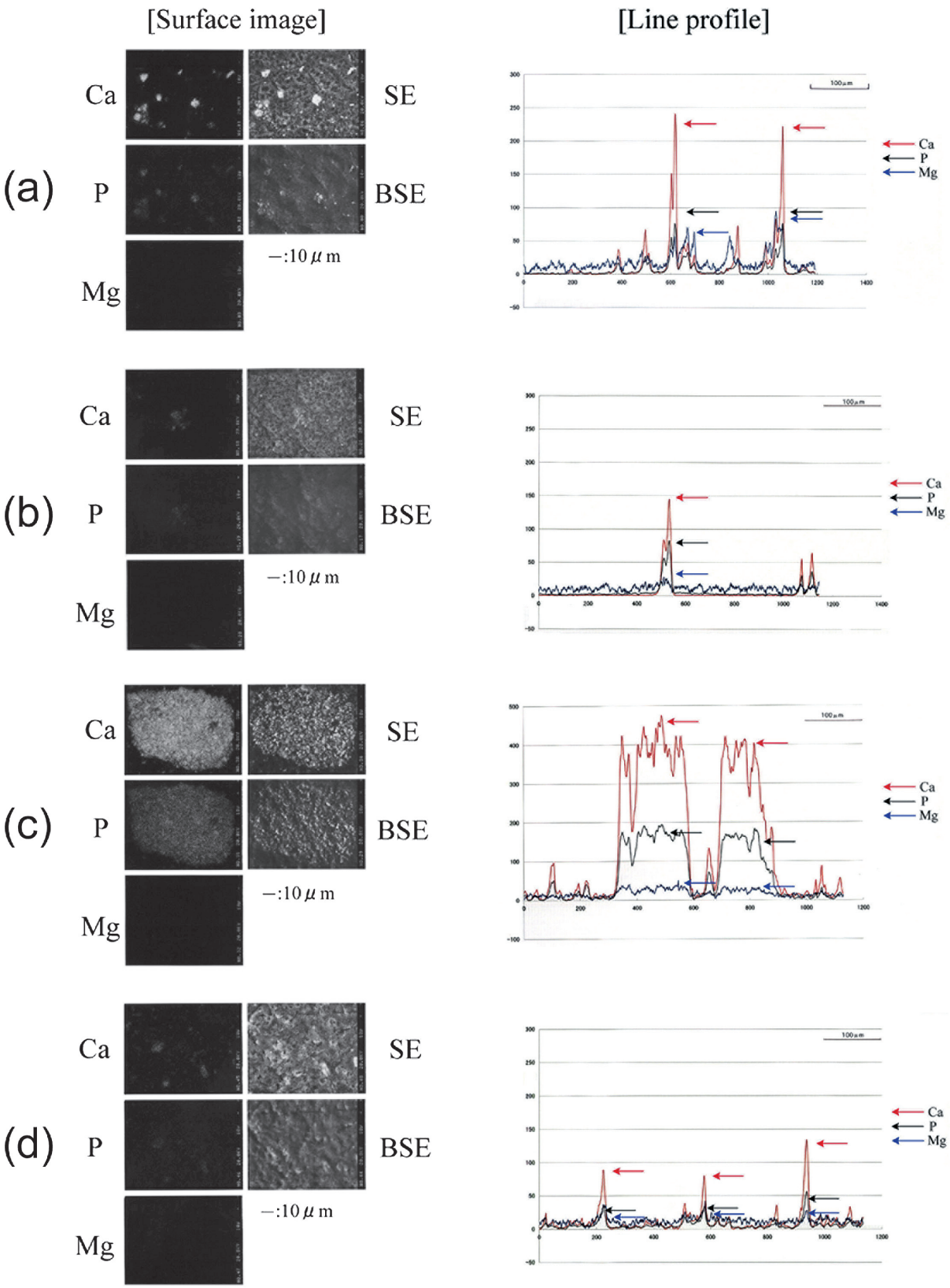

Fig. 4 Representative EPMA surface images and line profiles of CaKα, PKα and MgKα during the dispersion phases of calcium phosphate powder in photopolymerized samples,
(a): MB1: 5 wt% OHAp, (b): MB3: 5 wt% DCPD, (c): MB5: 5 wt% β-TCP, (d): MB7: 5 wt% OCP

sample. But due to deficiency of the mixing resulted in the calcium phosphate powder was inhomogeneous in the photopolymerized sample.

The elution amount of Ca increased in the following order: MB1, MB5, MB(C)1, MB7 and MB3. On the other hand, the elution amount of P increased in the following order: MB(C)1, MB1, MB5, MB7 and MB3. Each calcium phosphate showed different elution of Ca and P. The amount of elution was affected by the solubility product (*K*sp) of each type of calcium phosphate. The solubility product (*K*sp)[35,36] of each calcium phosphate powder at 37°C distilled water adjusted pH6.0 was reported as follows: DCPD; 1.87×10^{-7}, OCP; 1.25×10^{-47}, β-TCP; 1.37×10^{-20} and OHAp; 9.18×10^{-60}. The elution amount of P totally included the following: P from the calcium phosphate, the unpolymerized MDP monomer and the photopolymerized sample. MB(C)1, the group which contained no acidic monomer MDP, had a tendency to increase the elution amount of Ca while the elution amount of P decreased. This result was caused by the effect of MDP absorption with calcium phosphate which resulted in the elution amount of P from the photopolymerized sample to decrease. It was found that the elution amount of Mg was almost equal to the detection limit of ICP-AES. Fundamentally, Mg was not present in the chemical formula in the calcium phosphate powder used, but traces of elemental Mg as an impurity in hydration cells of the calcium phosphate was detected when line profiles were investigated.

Suzuki M *et al.*[15] studied the wound healing process of rat pulp directly capped with experimentally developed adhesive resin systems including Clearfil Megabond®. In total twelve types of bonding agents were prepared at fixed amounts of calcium phosphate. The exposed pulp of rats were directly capped with combined use of experimental primers and twelve types of bonding agents which were as follows: MB1: 5 wt% OHAp, MB2: 10 wt% OHAp, MB3: 5 wt% DCPD, MB4: 3 wt% DCPD, MB5: 5 wt% β-TCP, MB6: 10 wt% β-TCP, MB7: 5 wt% OCP, MB8: 5 wt% OHAp + 5 wt% DCPD, MB9: 5 wt% OHAp + 5 wt% β-TCP, MB10: 5 wt% DCPD + 5 wt% β-TCP, MB11: 4 wt% OHAp + 3 wt% DCPD + 3 wt% β-TCP and MB12: 4 wt% OHAp + 2 wt% DCPD + 2 wt% β-TCP + 2 wt% OCP. The control group was capped with Dycal®: the preparations of calcium hydroxide and MBP and MBB: Clearfil Megabond® primer and bonding agent. After direct capping and bonding procedures of the exposed pulp, all the cavities were restored with a resin composite. The rats were sacrificed at the experimental periods of 1, 3, 7, 14 and 28 days and then pulp tissue were examined histopathologically and immunohistochemically. Representative results obtained on the 14th day and the 28th day are shown Figs. 5 and 6. In the groups which used calcium phosphate powder, reparative dentin formation was already recognized on the 14th day (Fig. 5-a and b) and increased over time up to the

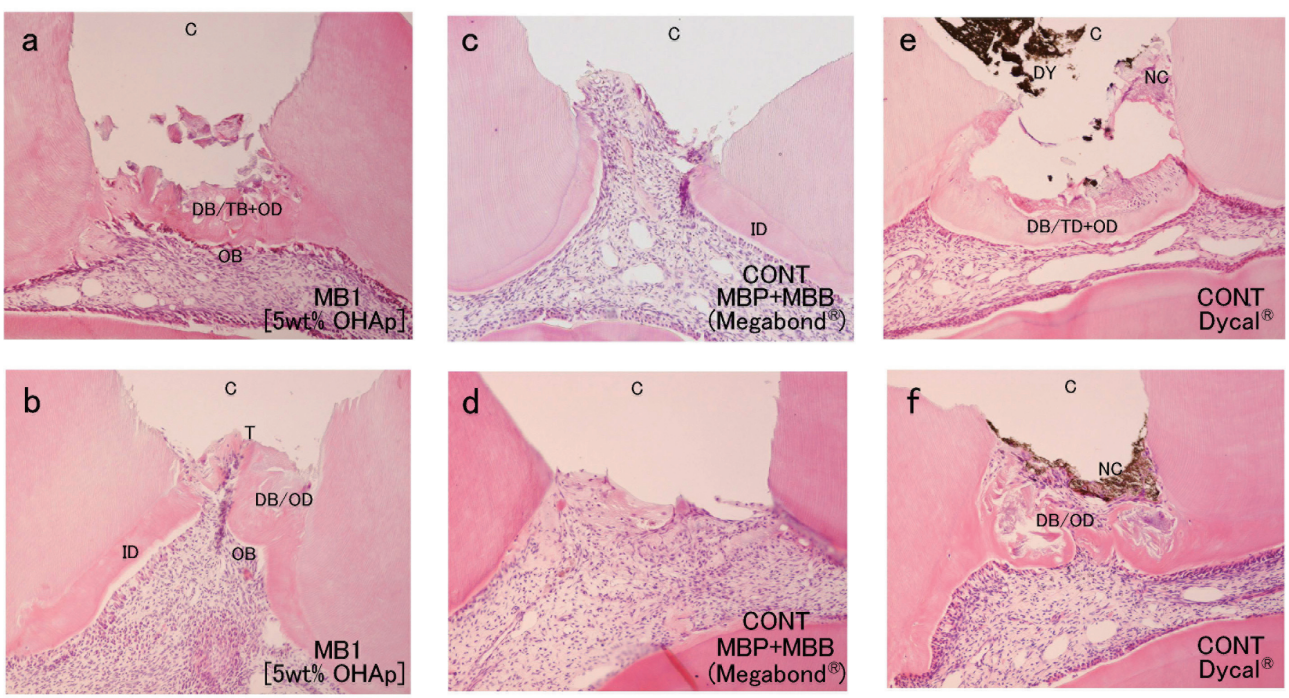

Fig. 5 Representative histological micrographs on the 14th day after direct pulp capping and restoration (H-E Stain, ×100),
C: Cavity, DB: Dentin bridge, TB: Tubular dentin, OD: Osteodentin, OB: Odontblast-like cells, ID: Irritation dentin, T: Tunneling, NC: Necrosis and DY: Dycal®

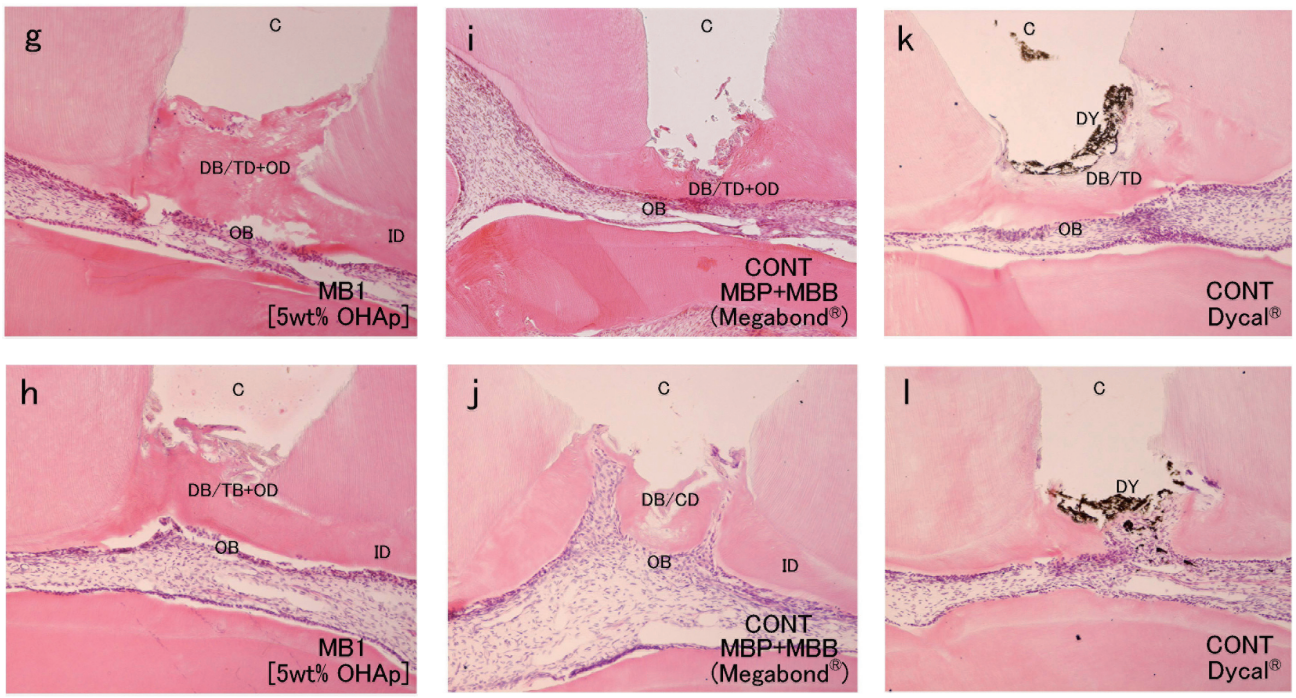

Fig. 6 Representative histological micrographs on the 28th day after direct pulp capping and restoration (H-E Stain, ×100),
C: Cavity, DB: Dentin bridge, TB: Tubular dentin, OD: Osteodentin, OB: Odontblast-like cells, ID: Irritation dentin, T: Tunneling, NC: Necrosis and DY: Dycal®

28th day (Fig. 6-g and h). Reparative dentin formation was clearly promoted by the types and concentrations of calcium phosphate powder.

This experiment employed 4 kinds of groups: MB1, MB3, MB5 and MB7 for SEM and EPMA observations. ICP-AES measurement employed 5 kinds of groups: MB1, MB3, MB5, MB7 and MB(C)1. These groups except MB(C)1 contained the same amount of calcium phosphate in wt% which was employed in the study conducted with Suzuki M *et al*. MB(C)1 was the group which acidic monomer MDP was eliminated from MB1 and the other compositions were identical to MB1.

To summarize the results of this experiment, it was suggested that reparative dentin formation was effectively promoted under the following conditions: a calcification promoting effect by direct contact of calcium phosphate powder or that found in the vicinity of exposed pulp, an ionic effect of Ca and P eluted from calcium phosphate powder and environmental change of the surface of the exposed pulp through the effect of pH change.

Also it was suggested that reparative dentin formation was particularly promoted and was confirmed the effectiveness of wound healing in the early stage after direct capping of exposed pulp.

CONCLUSIONS

Four types of calcium phosphate: OHAp, DCPD, β-TCP and OCP were observed in white powder form, but their morphological shapes differed as could be seen in SEM and EPMA images. The calcium phosphate powder in photopolymerized samples were scattered like the fillers in resin composite.

1. In elemental analysis of EPMA, CaKα showed a relatively high concentration in relation to PKα in both surface analysis and line profile. But MgKα was recognized in a trace amount and was detected only in the line profile.
2. In elution properties analysis by ICP-AES, Ca increased in the following order: MB1, MB5, MB(C)1, MB7 and MB3 and P increased in the following order: MB(C)1, MB1, MB5, MB7 and MB3. But as Mg was almost equal to the detection limit of the measuring apparatus. The amount of elution was affected by the solubility product (*K*sp) of each calcium phosphate powder.
3. It was suggested that when the experimental developed adhesive resin materials containing calcium phosphate powder were used as direct pulp capping and as a bonding agents, this led to reparative dentin formation and promoted the reliability of wound healing, especially improvement of wound healing in the early stage after direct capping of exposed pulp.

ACKNOWLEDGMENTS

The authors thank Kuraray Medical Inc. for the experimental adhesive resin systems and other materials they generously provided.

SOURCE OF FUNDING

This study was supported in part by grants-in-aid for Scientific Research (A)(1): project numbers: 14207082, 14207083 and (B)(2), project number: 15390578 from the Japan Society for the Promotion of Science.

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