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In vivo estimation of periapical bone reconstruction by chondroitin sulfate in calcium phosphate cement

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Abstract

Biphasic calcium phosphate cements using an equimolar mixture of tetracalcium phosphate and dicalcium phosphate dihydrate (TeDCPD) for the powder phase were experimentally developed for endodontic clinical use. The liquid for standard cement (CP-1) in this study was 2.1% citric acid solution. In the liquid for the modified cements, 2% (CP-2) or 6% (CP-3) chondroitin sulfate (CS) was added. The microstructure of the cements was observed by scanning electron microscope (SEM). X-ray diffraction analyses (XRD) were also performed. The cements were respectively placed in prepared mandibular first molar canals of rats. Results of SEM and XRD examinations of the cements indicated that the addition of CS accelerated the setting reaction of calcium phosphate cement. In experimental periapical lesion, alveolar bone could be significantly reconstructed with polarized cells along the margin using CP-3, but CP-1. It seems that CS in TeDCPD compound induce osteoblasts or osteoblast-like cells.

Keywords: Apatite; Biomedical applications; Bone reconstruction; Electron microscopy

1. Introduction

Calcium phosphate cement is reported to have excellent biocompatibility.[1–3] One type is a biphasic calcium phosphate cement developed by Chow et al.[4] The solid phase of the cement is an equimolar mixture of tetracalcium phosphate and dibasic calcium phosphate dihydrate (TeDCPD). The cement is kneaded with water or diluted acid solution.

Basis on this cement, a calcium phosphate cement was developed for use in dental clinics. The cement was composed of TeDCPD as the powder phase and a buffer solution containing a low concentration of citric acid as the liquid phase.[5] It was thought that the addition of citric acid should improve handling of the cement. Furthermore, a calcium phosphate cement including chondroitin sulfate sodium salt A (CS) was newly developed with an expectation of promoting tissue repair.[6–8]

This study confirmed the influence of CS on the physical properties of calcium phosphate cement. We also investigated the effect on alveolar bone reconstruction. The fine structure of this hardened cement was examined by a scanning electron microscope. Investigating by X-ray diffraction was also performed. This cement was applied to the mandibular first molar canals of rats, and subsequent periapical tissue responses were examined histologically.

2. Materials and methods

2.1. Preparation of experimental cements

Tetracalcium phosphate (TeCP) was prepared from a stoichiometric mixture of dicalcium phosphate dihydrate (DCPD) and calcium carbonate (Mol ratio of Ca/P = 2.0) by the dry synthesic method of sintering at 1400 °C for 8 h. DCPD was purchased from Wako Pure Chemical Inc. Co. (Tokyo, Japan) and passed through a 32 μm-sieve to standardize the particle sizes. The synthesized TeCP particles ranged in size from 0.6 to 44.0 μm with a 12–13 μm mean. In this study, three experimentally developed calcium phosphate cements were examined.

The powder phase (TeDCPD) of experimental cement was composed of an equimolar mixture of TeCP and DCPD. TeDCPD was kneaded with a two-fold concentration of McIlvain's buffer solution. This cement (CP-1) was the fundamental one which was examined in this study. The buffer solution contained citric acid (2.1%), dibasic sodium phosphate (7.2%) and sodium carboxy-

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methyl cellulose (CMC: 1.6%). CS purchased from Kanto Kagaku (Tokyo, Japan) was added in the liquid phase of the other experimentally developed cements instead of CMC. Concentration of CS in the liquid phase of the other cement (CP-2) was 2% (g/ml). CS was added in the liquid of another cement (CP-3) at a 6% (g/ml) concentration. All cements were respectively kneaded for 1 min with a powder/liquid ratio of 1.0 (g/ml) at room temperature.

2.2. Disintegration ratio of experimental cements

After kneading, each cement was stored to set in an incubator for 1 h at 37±1 °C in 99% relative humidity. The hardened cements were respectively immersed into distilled water in a glass vessel and kept for 23 h in the incubator. The cement was placed on an evaporating dish at 100 °C to dry, and then placed in an incubator at 150 °C. When decreasing of weight change of cement within 24 h was less than 0.5 mg, percentages of disintegration were calculated from the difference in the weight before immersion in distilled water and after complete drying. Three times of measurement were performed and differences were evaluated by Student *t*-test ($P<0.005$).

2.3. Scanning electron microscope (SEM) investigation of cement after setting

The surface and internal structures of experimental cements was observed by SEM investigation. Each cement just after kneading was poured into a 10 mm high teflon tube with a 10 mm internal diameter. They were hardened at 37±1 °C in 99% relative humidity for 1 h. The cement was removed from a tube, split and coated with Au–Pt ions. Then morphological characterization of the surface and internal structures was performed.

2.4. X-ray diffraction (XRD) analysis

The crystalline phases during the setting process of each cement from 1 h to 3 days after kneading were determined by X-ray diffract meter (XRD-6100; SHIMADZU, Kyoto, Japan) using Cu-K_α radiation generated at 40 kV and 30 mA. The divergence slit was 1°. Receiving slit width was 0.15 mm. The scanning range of the sample was from 10 to 60° with a scanning speed of 2°/min. The cements were stored at 37±0.5 °C with 99% relative humidity.

2.5. Histological examination of periapical area

This study was performed under the Guidelines for Animal Experimentation at Osaka Dental University. Access cavities in right and left manibular first molars of forty-eight 6-week-old SD male rats (Kurea Japan, Osaka, Japan) were opened using an engine-driven No. 1/2 round bur while dropping sterilized distilled water on the occlusal surface of the tooth. After pulp extirpation and root canal enlargement of the teeth with No. 15 to No.25 endodontic files, 1 mm of a No.25 file tip was extruded into the periapical area through the mesial and distal canal apical foramen to injure the periapical alveolar bone. Forty-eight rats were randomly divided into four groups of twelve animals. Using lenturo spirals, the prepared canals were filled with each experimental cements respectively; CP-1 (Group 1), CP-2 (Group 2) and CP-3 (Group 3). In Group 4, canals were kept hollow as a control group. All access cavities were sealed with a light-curing resin to prevent infection in the periapical area through the root canal. Oral cavities of rats were sterilized with a povidone iodine-soaked cotton pellet. Instruments and powder components of cements were sterilized with ethylene oxide gas. Liquid components were filtrated with a micro pore filter of 0.22 μm diameter. Both mandibles were resected from four rats in each group at 1, 2, 3 and 4 weeks postoperatively. They were fixed in 4% neutralized buffered formalin solution, decalcified in 10% EDTA solution (pH 7.2), dehydrated in ethanol and embedded in paraffin. Paraffin-embedded specimens were serially cut 6 μm thick, and the sections were stained with hematoxylin and eosin for optical microscopic examination.

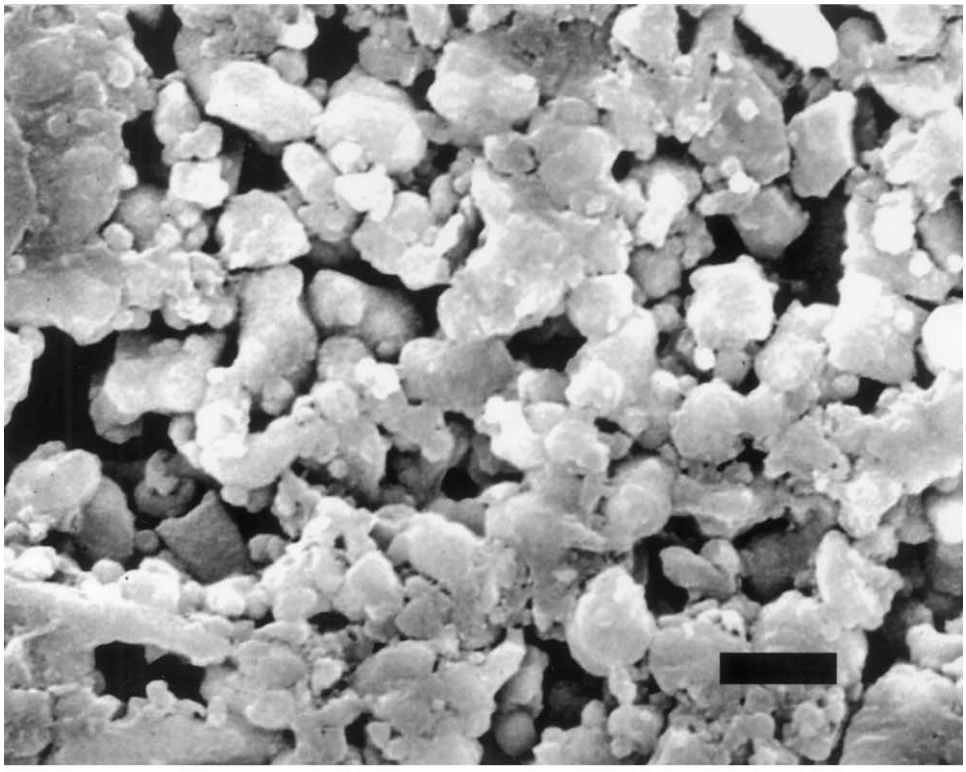
3. Results

3.1. Disintegration ratio of experimental cements

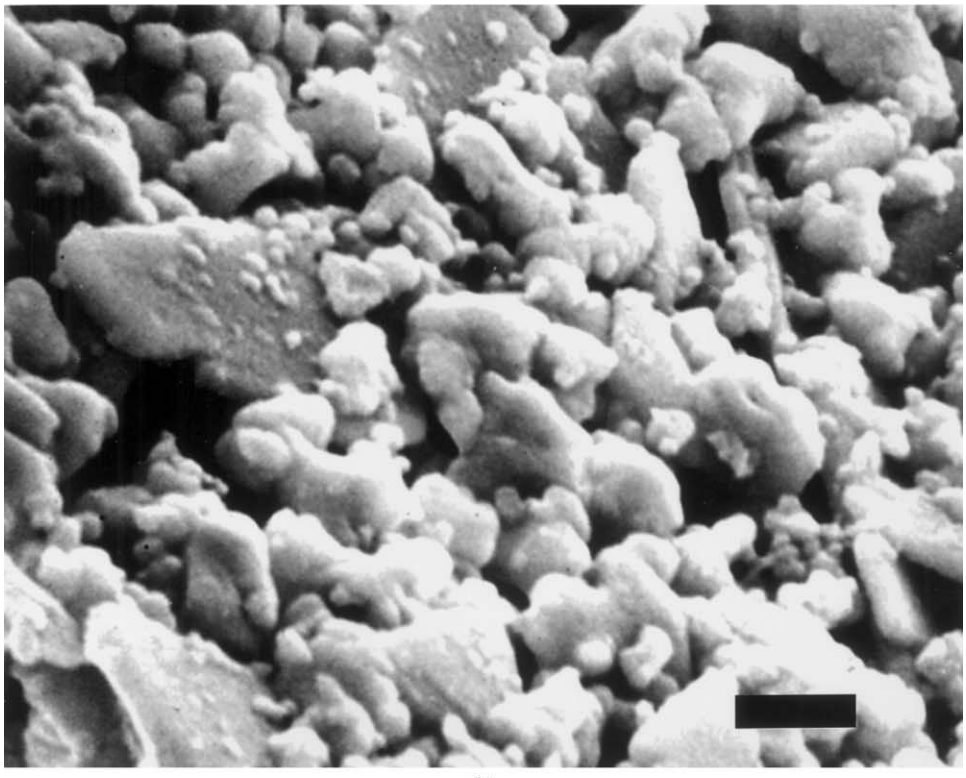
CP-1 showed a disintegration ratio of 0.89±0.07%. That of CP-2 was 1.21±0.05% and CP-3 was 2.12±0.04%. Significant difference in the disintegration ratio was recognized between CP-1 and CP-2. The ratio of CP-3 significantly differed from that of CP-2.

3.2. SEM investigation of cement after setting

SEM findings of the surface of CP-1 are shown in Fig. 1a. The major axes of the largest calcium phosphate crystal particles were about 20 μm, while these of the smallest globular crystals were less than 2 μm. Microstructure of internal portion of CP-1 is shown in Fig. 1b. Interconnected spherical and platy crystal particles ranging from less than 1 to 30 μm in major axes were observed. It was found that surface of CP-2 and CP-3 appeared different from that of CP-1. The crystal particles on the surface of CP-2 (Fig. 2a) and CP-3 (Fig. 3a) seemed to connect viscously with each other. Pleats were observed on the surfaces of platy crystal particles in the internal portion of CP-2 (Fig. 2b). The needle-shaped crystals might mature from the pleats on

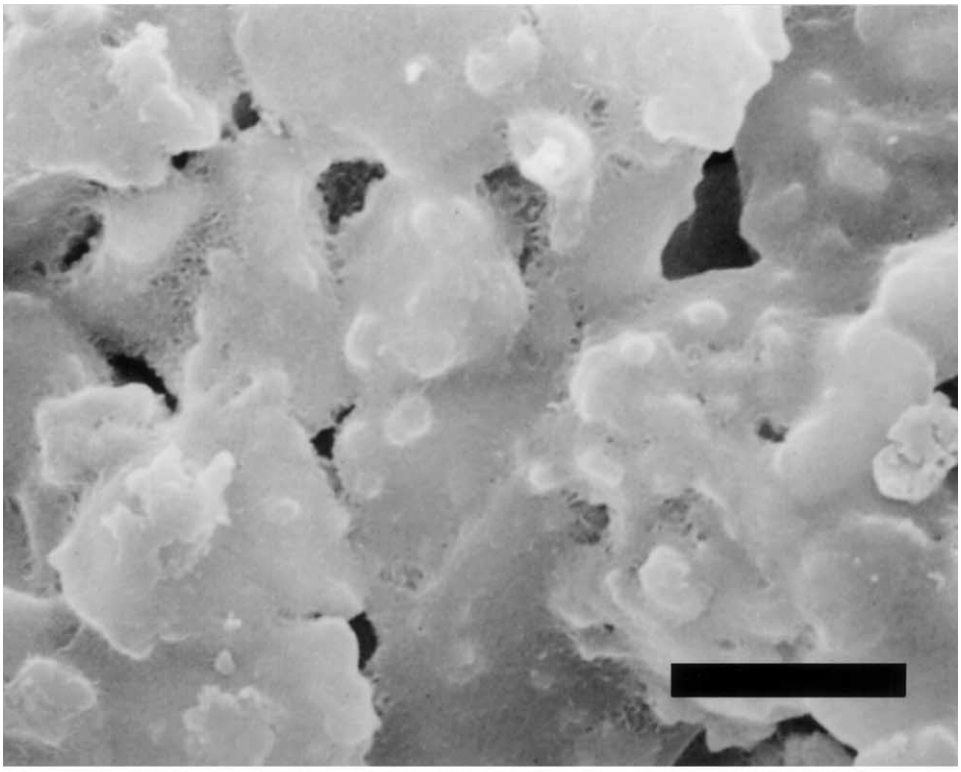


(a)



(b)

Fig. 1. (a) SEM photograph of the surface of CP-1: platy and globular crystals were seen (Bar: 10 μm). (b) SEM photograph of the internal structure of CP-1: interconnected spherical and platy crystal particles were seen (Bar: 10 μm).



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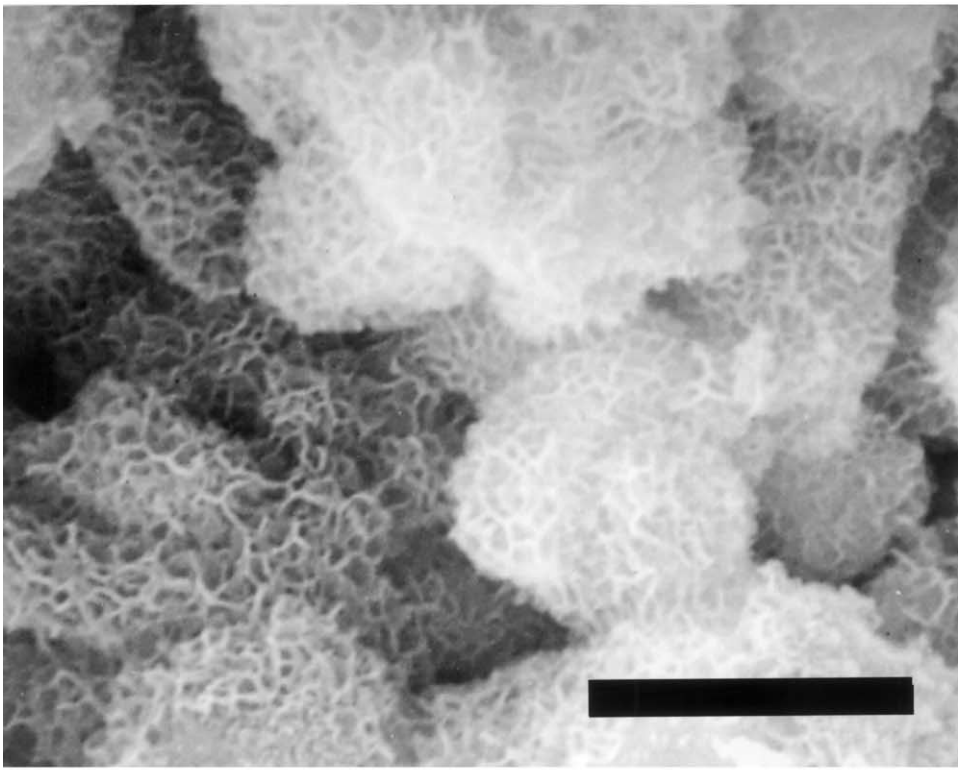
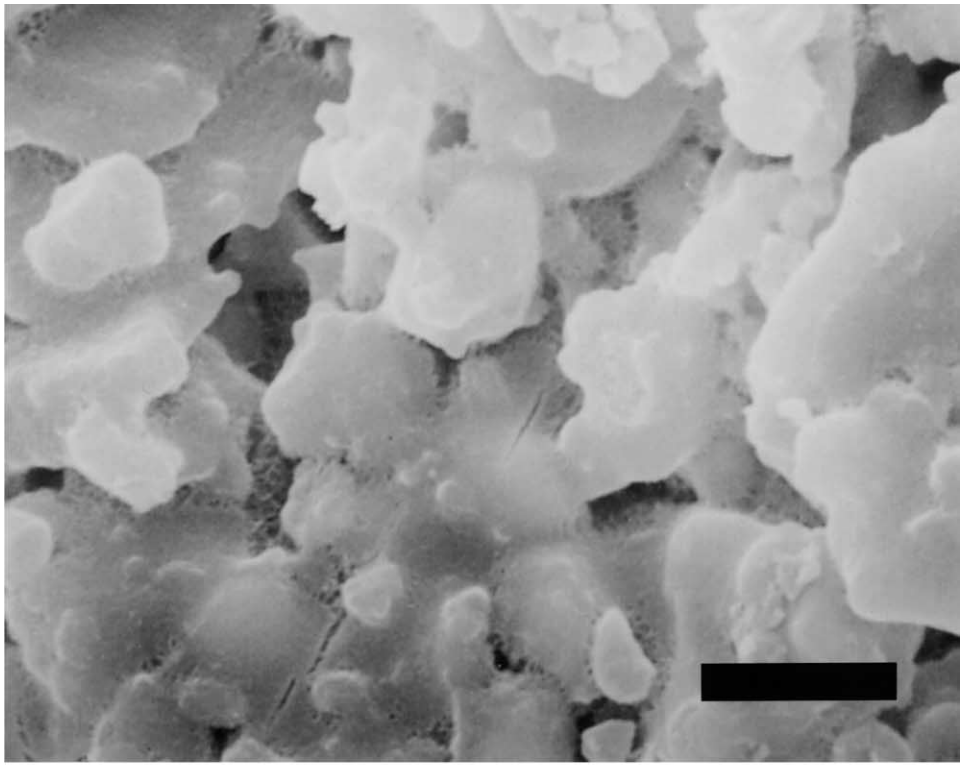
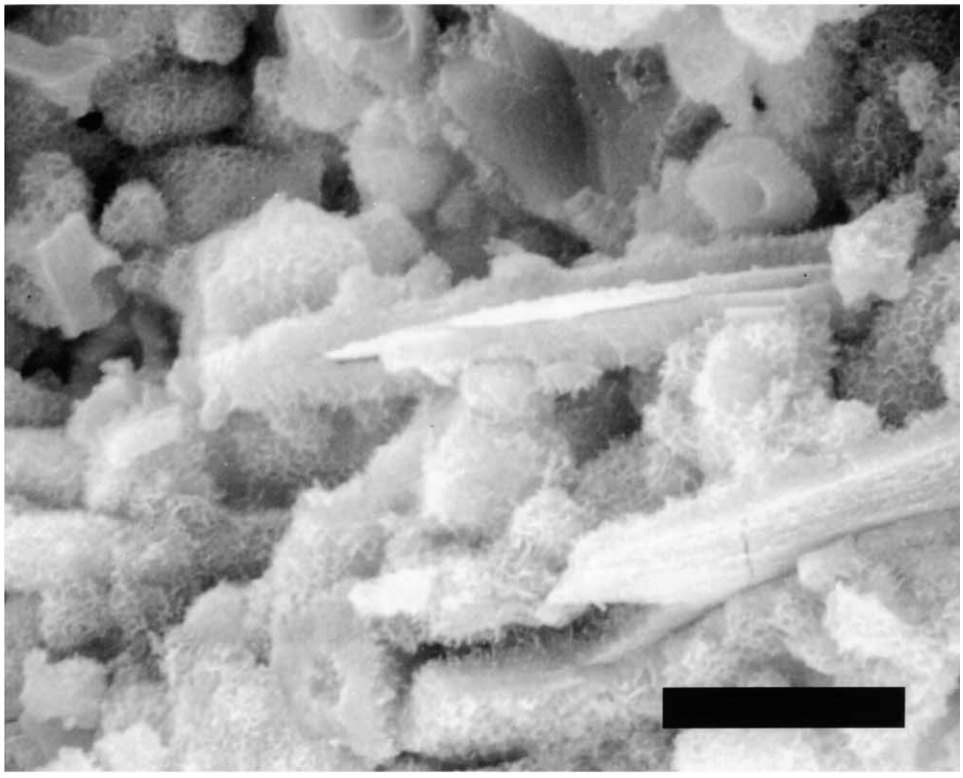


(b)

Fig. 2. (a) SEM photograph of the surface of CP-2: the surface of hardened CP-2 appeared viscous (Bar: 5 μm). (b) SEM photograph of the internal structure of CP-2: pleats were observed on the surface of platy crystal particles (Bar: 5 μm).



(a)



(b)

Fig. 3. (a) SEM photograph of the surface of CP-3: it seems that crystals were viscously connected with each other (Bar: 5 μm). (b) SEM photograph of the internal structure of CP-3: needle-shaped crystals were seen between platy crystals with pleats (Bar: 5 μm).

platy crystals. In the Internal portion of CP-3, needle-shaped crystals were found between platy ones which had pleats on the surface (Fig. 3b). Major axis of CP-3 crystals was 30 μm or more.

3.3. XRD analysis

A small amount of DCPD and TeCP were demonstrated in CP-1 (Fig. 4a), CP-2 (Fig. 5a) and CP-3 (Fig. 6 a), 1 h after kneading. Low crystalline hydroxyapatite (HAp) was detected in CP-1 (Fig. 4b), CP-2 (Fig. 5b) and CP-3 (Fig. 6b) 1 day after kneading. The DCPD phase was not traced in CP-2 and CP-3, 1 day after kneading. Three days after kneading, traced crystalline structures of CP-2 and CP-3 were scarcely in accord with the phase of CP-1. Only the HAp phase was demonstrated in each cement (Figs. 4c, 5c and 6c).

3.4. Histological examination of periapical area

In each group, mild inflammatory reaction with alveolar bone absorption in the apical region was found after 1 week postoperatively. In Group 1, as shown in Fig. 7, inflammatory reaction was observed in the periapical area near the apical foramen at 2 weeks post operatively. Four weeks postoperatively, incomplete alveolar bone reconstruction was found. In Group 2, granulation tissue was seen with no inflammatory responses in the apical portion 3 weeks postoperatively

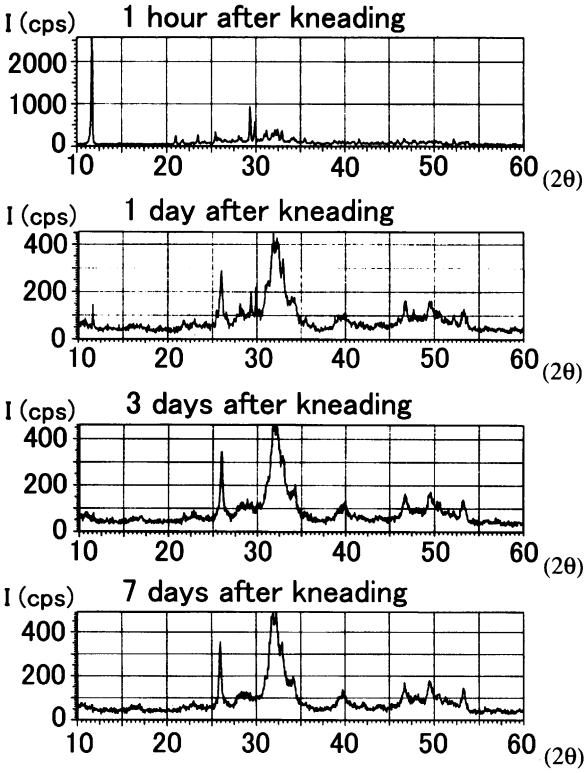


Fig. 4. X-ray diffraction pattern of CP-1.

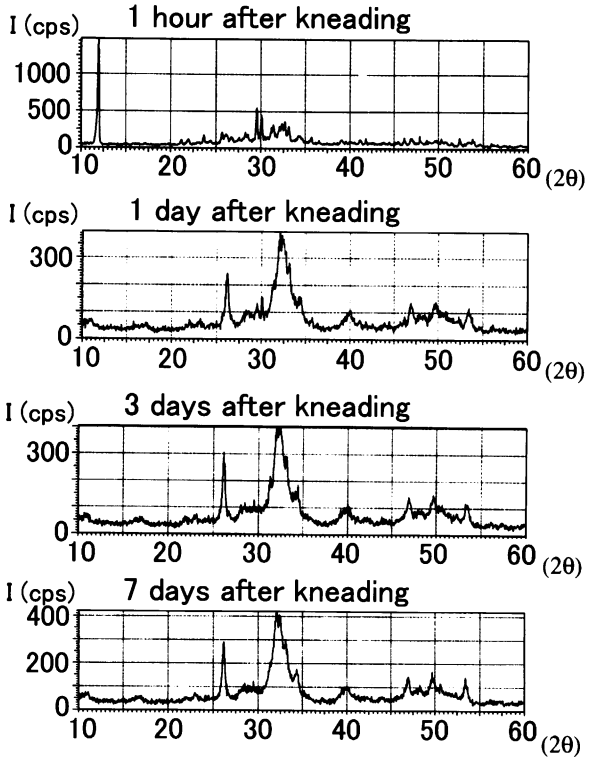


Fig. 5. X-ray diffraction pattern of CP-2.

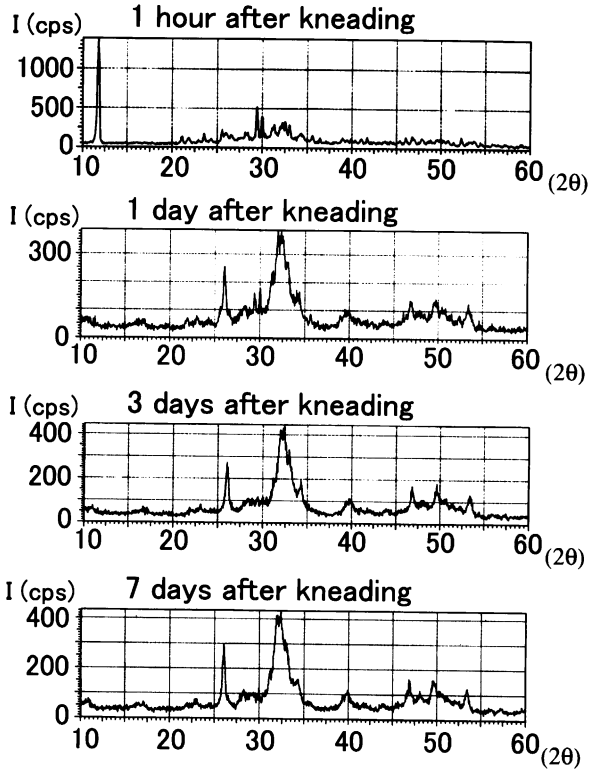


Fig. 6. X-ray diffraction pattern of CP-3.

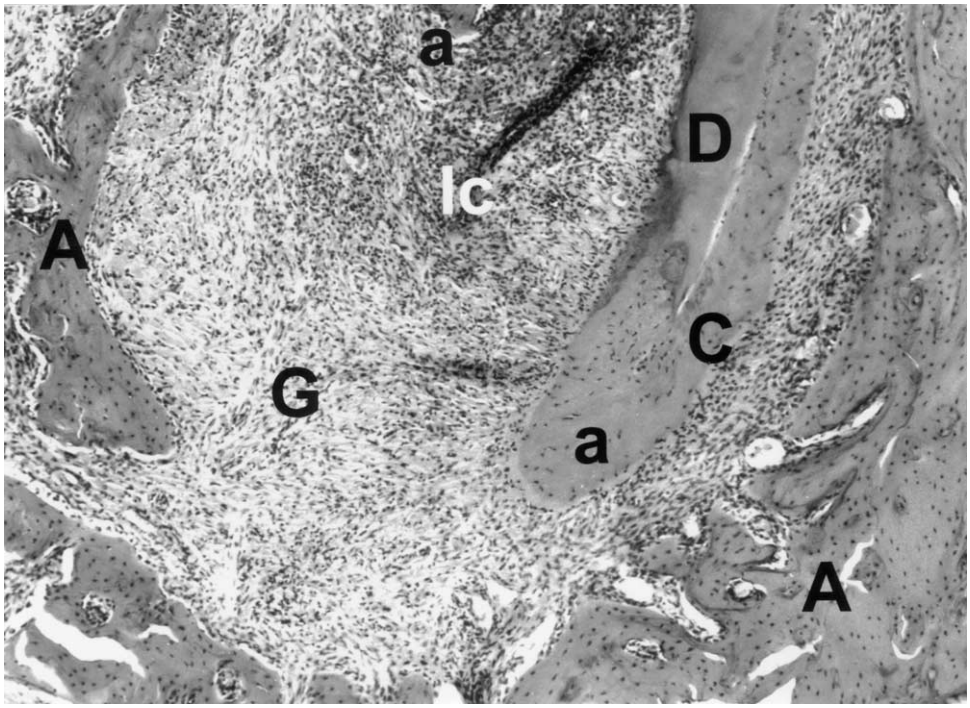


Fig. 7. Two weeks after placement of CP-1 in the apical root canal. Inflammatory reaction was observed in the periapical area near the apical foramen. Polymorpho-nuclear leucocytes were present. Ic: Inflammatory cells; G: Granulation tissue; D: Dentin; a: Apex; A: Alveolar bone; C: Cementum (Orig. Mag. ×16.0).

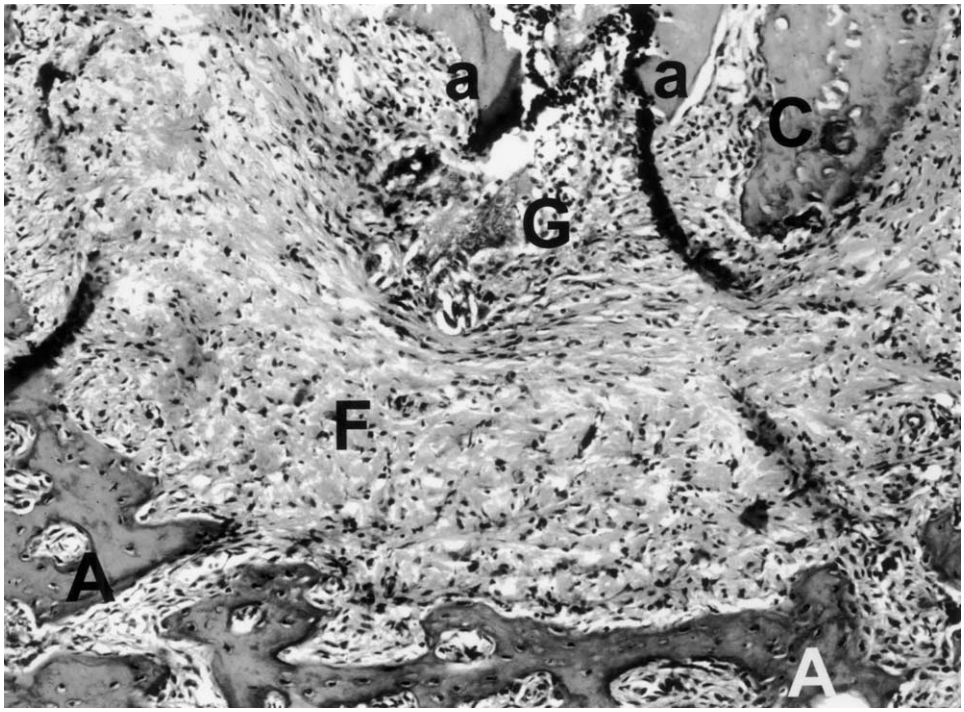


Fig. 8. Three weeks after placement of CP-2 in the apical root canal. At the apex, granulation tissue was present. There was no inflammatory response; G: Granulation tissue; a: Apex; A: Alveolar bone; C: Cementum (Orig. Mag. ×33.0).

(Fig. 8). Four weeks postoperatively, the alveolar bone was nearly reconstructed. In Group 3, polarized cells arranged on the margin of periapical alveolar bone were seen with no inflammatory response (Fig. 9 a). These cells might be osteoblasts (Fig. 9 b). Four weeks postoperatively, periapical alveolar bone appeared nearly normal (Fig. 9c). In Group 4, an inflammatory response occurred 1 and 2 weeks postoperatively, then dis-

appeared 3 weeks later. After 4 weeks, there was granulation tissue near the root apex adjacent to periapical fibrous scar, and alveolar bone reconstruction was not observed (Fig. 10).

4. Discussion

A tooth consists of dentine, enamel and cement. These hard tissues have apatitic construction. Tooth pulp is surround by dentine. Tooth pulp is connected to periapical tissue through the apical foramen. Injured tooth pulp is usually removed. When tooth pulp is removed, periapical tissue is certainly injured by mechanical irritation. As the result, bone defect ultimately occurs. In this histological study, alveolar bone defects was subsequently seen in all experimental groups. It is sure that the defects might be caused by pulp extirpation. It was also shown in this study that alveolar bone defect persisted when the root canal was kept hollow after root canal preparation. The tissue fluid which infiltrated into the canal from the apical foramen could be denatured by several factors in the hollow canal.[9] The denatured fluid acted as a new factor promoting bone defect.[10,11] Root canal should be hermetically obturated for reconstruction of the alveolar bone defect. A desirable material for the purpose must be developed. Because the final reactant of calcium phosphate cement is known to be hydroxyapatite, this cement may be a suitable root canal filling material.

It is known that TeDCPD, as the powder phase, has a buffering action.[12] When citric acid buffer solution was used for the liquid phase, the pH during the hardening process of the cement was about 8.6.[7] This pH value doesn't injure tissue. The fundamental cement in this study (CP-1) consisted of TeDCPD and a low concentration of citric acid buffer solution. It was thought that CS played an important role in tissue repair because CS is an intercellular substance in connective tissue.[13,14] Then, the addition of CS to CP-1 resulted in CP-2 and CP-3.

The shapes of the areas that require filling in an endodontic clinic are infinitely variable. Therefore, calcium phosphate cement after setting is not used. Cement just after kneading is poured into the root cavities. Cement before setting affects periapical tissue through the apical foramen. Yoshikawa et al.[15] and Miyamoto et al.[16] respectively reported that foreign-body giant cells were collected around a calcium phosphate cement implanted in subcutaneous tissue in rats. Fine particles in the powder phase of calcium phosphate cement or those remaining on the hardened cement surface should induce phagocytes in tissue by macrophages,[17] and more fine calcium phosphate particles may phagocytosed by polymorphonuclear leukocytes. Then, inflammation will occur in the region. This was proven from the finding that inflammatory responses in the periapical tissue were prolonged by root canal filling with CP-1. CP-1 shows no chemical irritation. Fine particles being released in periapical tissue from CP-1 might induce inflammatory responses.

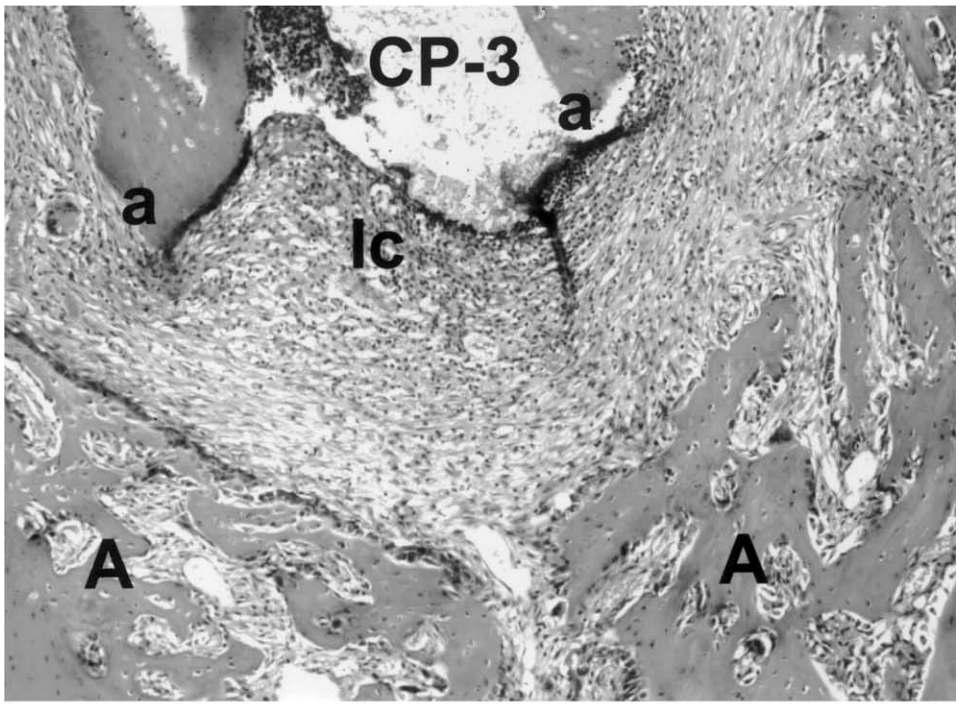
When calcium phosphate cement including CS was filled in the root canal after pulp extirpation, inflammatory responses in the periapical lesion might be inhibited in this study. There was no inflammatory response due to CP-2 or CP-3, because the number of particles released from each of these cements was less than that from CP-1. The SEM photograph showed that CS coated calcium phosphate crystals during the hardening process. It was considered that this coating prevented the release of crystal particles. Coating due to the addition of CS to TeDCPD cement might increase the disintegration ratio. It was thought that disintegration of CP-2 or CP-3 was only the result of solving the CS over the crystal surface.

Because an immediate response of calcium phosphate after kneading the cement causes the growth of crystals, reaction velocity is important for decreasing fine particle. The result of XRD showed that CP-1, CP-2 and CP-3 respectively converted to low crystalline hydroxyapatite. However, the DCPD phase was not traced in CP-2 and CP-3, 1 day after kneading. These results indicate the possibility of CS to promote the hardening reaction of TeDCPD cement. In SEM photographs, it seemed that the needle-shaped crystals observed in CP-2 and CP-3 were more mature than platy crystals in CP-1.

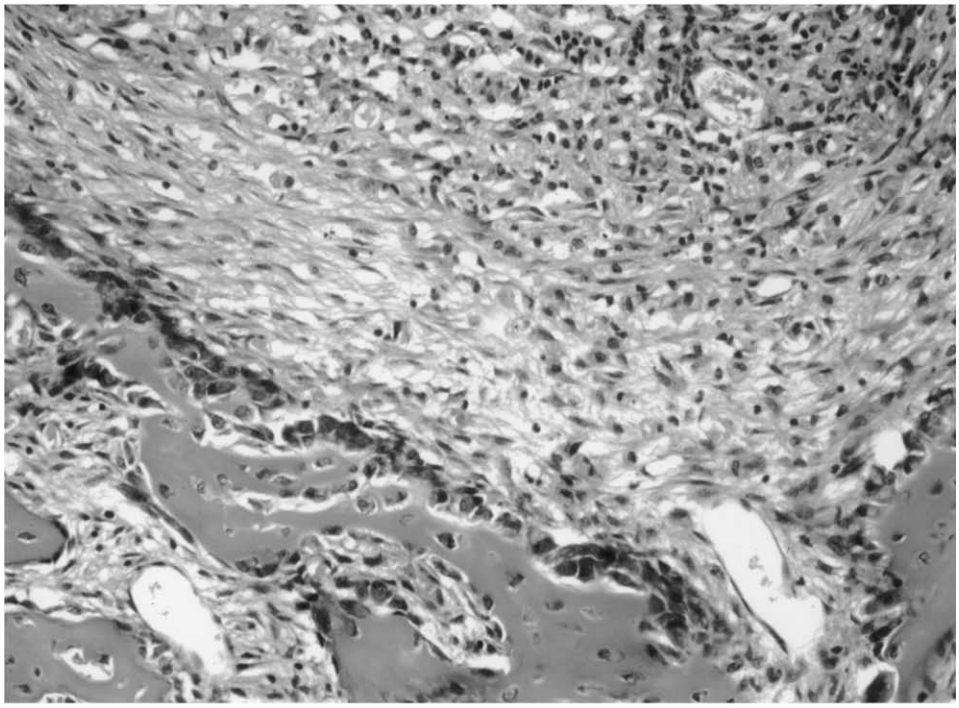
Concerning the possibility that CS plays an important role in reconstructing periapical alveolar bone defect, CP-2 and CP-3 were examined histologically in comparison with CP-1. In this study, adhesion of polarized osteoblasts on the margin of the periapical alveolar bone was recognized. Activated osteoblast polarizes. CS participates in maintaining the polarity of activated osteoblasts.[18] There are many reports concerning the role of CS in formation and maintaining bone.[19–21] It was reported that CS is related to cell adhesion.[19] It was proven from the results of this study that CS in a calcium phosphate cement might affect to periapical tissue and play a role in reconstructing periapical alveolar bone defect. It is certain that the effect depended on the CS concentration in the cement. In endodontic clinics, the addition of CS to calcium phosphate cement used for root canal filling is effective for reconstruction of periapical alveolar bone defect.

5. Conclusion

Chondroitin sulfate sodium salt A (CS) was added in liquid phase of biphasic calcium phosphate cement composed of an equimolar mixture of tetracalcium phosphate/dibasic calcium phosphate dihydrate. In

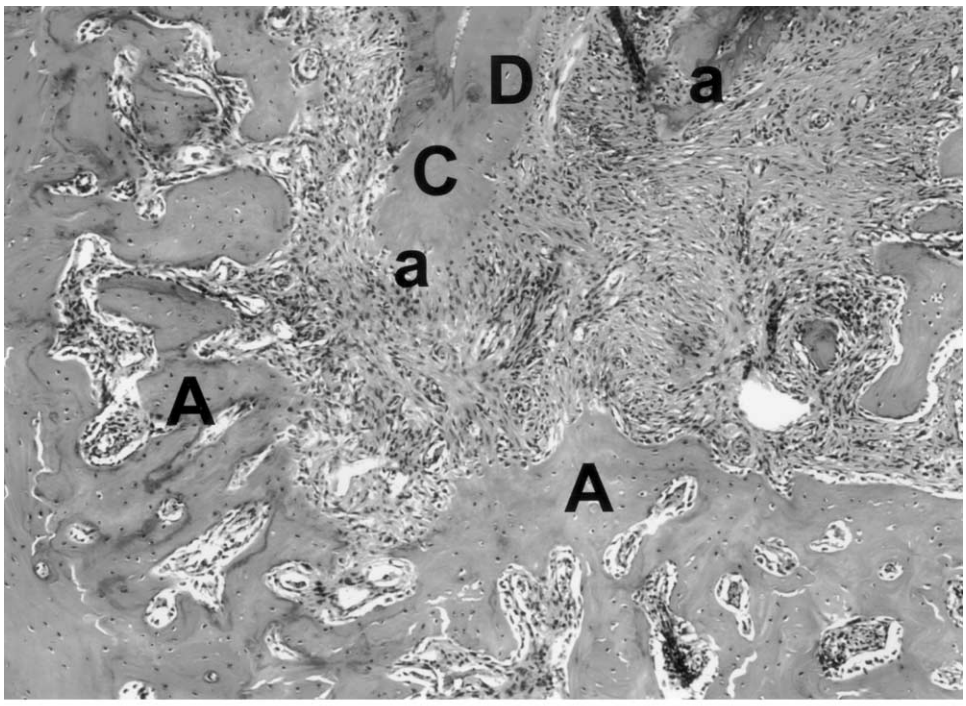


(a)



(b)

Fig. 9. (a) Two weeks after placement of CP-3 in the apical root canal. There was no inflammatory response in the periapical area. Many cells were arranged on the margin of periapical alveolar bone. CP-3: Calcium phosphate cement filled in the root canal; Ic: Inflammatory cells; a: Apex; A: Alveolar bone (Orig. Mag. ×20.0). (b) Higher magnification of (a). Polaraized cells arranged on the margin of periapical alveolar bone were observed. (Orig. Mag. ×50.0). (c) Four weeks after placement of CP-3 in the apical root canal. Periapical alveolar bone appeared nearly normal. D: Dentin; a: Apex; A: Alveolar bone; C: Cementum (Orig. Mag. ×20.0).



(c)

Fig. 9. (*continued*).

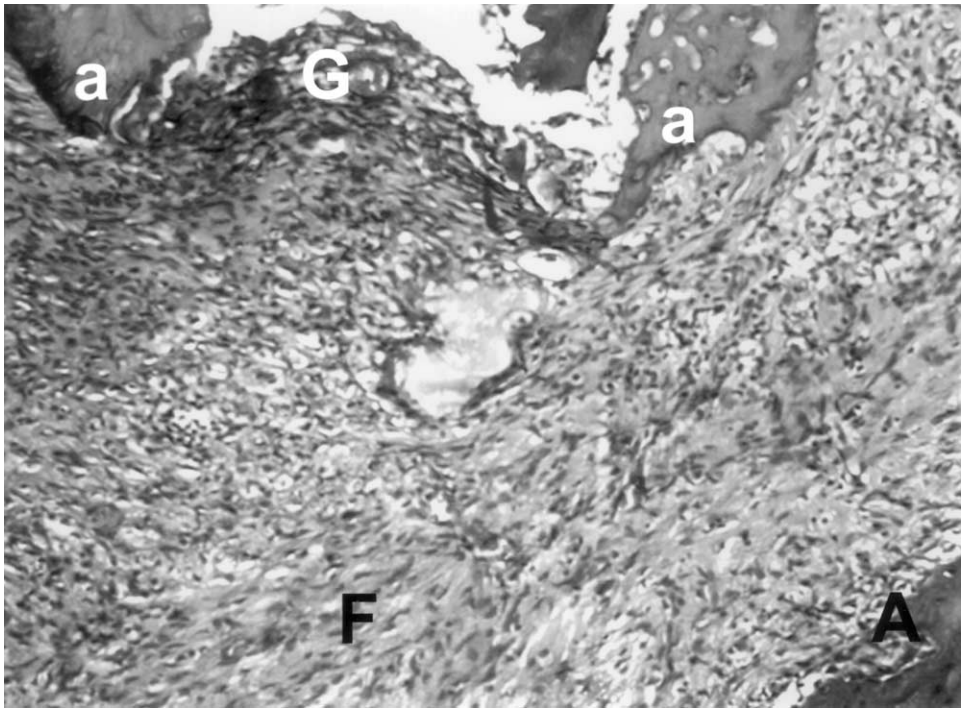


Fig. 10. Four weeks postoperatively in the control. Granulation tissue (G) were seen near the apical foramen. Alveolar bone reconstruction was scarcely observed. a: Apex; G: Granulation tissue; F: Fibrous scar; A: Alveolar bone (Orig. Mag. ×27.0).

biphasic cement, CS may promote the cement setting reaction. The cement, which was used to fill a root canal after pulp extirpation, inhibited inflammatory responses in the periapical tissue. Root canal filling with calcium phosphate cement that included CS promoted reconstruction of the alveolar bone defect in a CS concentration-dependent manner. It is concluded from the results of these experiments that the addition of CS to

calcium phosphate cement is effective for reconstruction of periapical alveolar bone defect.

Acknowledgements

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