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Tissue reaction of hydroxyapatite sol to rat molar pulp

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Hydroxyapatite sol was prepared by dispersing unheated hydroxyapatite microcrystals into distilled water. A suspension of hydroxyapatite heated at 800 °C (HAp-800°C) in distilled water was used as a comparative material. These were characterized by X-ray diffractometry, SEM and electroconductivity measurements. 0.001 ml of each material was injected into the upper first molar pulp of rats. After 7–14 days post-operatively, the group with hydroxyapatite sol showed slight inflammation in the pulp cavities and a highly calcified tubular dentine bridge was observed. Osteodentine had developed and adhered to the surface of the hydroxyapatite microcrystals. Matrix vesicles were observed in the extracellular matrix between collagen fibrils and electron-dense granules. After 28 days, the inflammation completely disappeared and tubular dentine was observed below the osteodentine. Acid phosphates (ACPase) activity was detected along all biosynthesizing pathways in odontoblast-like cells. Localized dehydrogenate (LDH) activity indicated phagocytosis of hydroxyapatite microcrystals by odontoblasts. In the group with HAp-800°C after 7–14 days post-operatively, the inflammation was moderate. After 28 days, relatively fewer calcified dentine tubules were observed. ACPase activity was detected only along the membranes bounding cytoplasmatic vacuoles. It was concluded that hydroxyapatite sol is a safe biomaterial for use as dental pulp and induces early formation of dentine-bridge more so than HAp-800 °C. © *1998 Kluwer Academic Publishers*

1. Introduction

Since the invention of sintered hydroxyapatite (HAp) in the mid-1970s [1, 2], various biomaterials made of hydroxyapatite have been developed, including artificial bones and joints in the orthopaedic field [3], and dental cements [4, 5], dental implants [6, 7], and dental pulp capping materials, in dental fields. These materials were based on sintered or calcined hydroxyapatite heated at high temperatures of 650–1200°C [8-12]. The types were dense, porous, granular, powder, and composite bodies. On the other hand, hydroxyapatite sol, composed of hydroxyapatite microcrystals without heating, was developed as a new biomaterial [13], for uses including a drug carrier, an absorbent of virus and an accelerator of bone formation. Since then, many researchers have reported that hydroxyapatite sol can be used as an effective drug carrier [14], and it has an excellent biocompatibility with macrophage cells [15, 16], and absorbs HIV [17]. Recently, serological and haematological studies of HAp were presented [18]. Aoki and co-workers reported that bone formation in femur marrow is more accelerated by injection of the HAp-sol than by HAp-800 °C [19, 20], and the HAp-sol can be used as a hydroxyapatite coating agent on titanium for dental implants [21].

In the present study, the tissue reaction of the HAp-sol to rat molar pulp was examined histologically in order to investigate whether HAp-sol can be used as a successful medicament for direct pulp capping. To date, direct pulp capping agents made of calcium hydroxide, zinc oxide eugenol, α-tricalcium phosphate (α-TCP), and sintered or calcined hydroxyapatite have been employed as part of the treatment for mechanical and traumatic exposure of vital pulp [22, 23]. However, high alkalinity of calcium hydroxide and products decomposed from hydroxyapatite by a heating process at high temperatures, and cytotoxicity of zinc oxide eugenol, often produce a superficial necrotic layer at the exposure site. Komoriya *et al.* [24] concluded that α-TCP does not induce a dentine-bridge in 3 months. No application of HAp-sol to the pulp capping materials has been reported. The HAp-sol and HAp-800 °C suspension were injected into the dental pulp of rat molars. The tissue reaction in the pulp, was examined histologically using light and transmission electron microscopes, and enzyme cytochemistry, in order to ascertain the possibility of using HAp-sol as an injectable and effective direct pulp capping medicant.

2. Materials and methods

2.1. Preparation of materials

Calcium carbonate (99.99% purity, UBE Material Industries, Ltd, Japan) was calcined at 1050 °C for 3 h.

The product was hydrated to $Ca(OH)_2$ by adding distilled water and vigorously stirring, a solution of H_3PO_4 (0.6 mol) in 750 ml distilled water was added dropwise (100 ml/15 min) using an ultrasonic homogenizer to produce hydroxyapatite microcrystals at room temperature. The reaction mixture was aged for 3 days at room temperature. A little HAp-sol was filtered and dried at 60 °C for 24 h. The powder was heated at 800 °C for 2 h in air (HAp-800 °C). The resulting HAp-800 °C powder was suspended in distilled water. The concentration of HAp-sol and HAp-800 °C was 14.8 mg ml^{-1}.

2.2. Characterization of materials

HAp-sol and HAp-800 °C suspensions were identified by X-ray diffractometry with a horizontal goniometer (RINT-Ultima, Rigaku Co.). The crystals in HAp-sol and HAp-800 °C were observed by SEM (S-405, Hitachi Co.).

2.3. Solubility

Continuous electroconductivity in solutions of HAp-sol and HAp-800 °C was measured using an electroconductivity meter. The supernatants of the HAp-sol and HAp-800 °C after the solubility test were collected by centrifugation. The calcium and phosphorus contents in the supernatants were measured by the ortho-cresol phthalein complexone (OCPC) method [25]. The calcium and phosphorus contents were expressed as total calcium and phosphorus contents.

2.4. In vivo study

Wistar male rats, 24 in numbers, 6 weeks old each with body weight of about 150 g, were used *in vivo*. Anaesthesia was induced by intraperitoneal injection of sodium pentobarbital Nembutal (30 mg kg^{-1}). The rats were attached to a new experimental apparatus to regulate the pulp exposure of rat molars [26]. The operating field was disinfected with 3% diluted tincture of iodine and 70% ethyl alcohol. Of each sample, 0.001 ml was injected into the pulp of the upper first molar of rats that had been exposed by a no. 1/2 round bur at low speed through the mesial surface of the teeth using a microlites syringe. After injection, the pulp was covered by a temporary sealing material.

2.5. Light microscopy

At 7, 14 and 28 days post-operatively, 16 rats were perfused through the left ventricle with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.2 M buffer of pH 7.4 under anesthesia with intraperitoneal sodium pentobarbital. The teeth were carefully extracted; the parts containing the exposure site, 2 mm long, were excised with a diamond disc saw under a stream of Ringers solution. The specimens were immersed in the same fixative as was used for perfusion for 2 h and then post-fixed in 2% osmium tetroxide in 0.2 M buffer (pH 7.4) for 1 h. The blocks were then dehydrated in increasing concentrations of ethyl alcohol and embedded in Epon 812. Sections with a thickness of about 4 μm were stained with haematoxylin–eosin and toluidine blue for light microscopic orientation.

2.6. Transmission electron microscopy

Areas for ultrathin sectioning by a diamond knife were always chosen from surface regions of the exposed pulps. The ultrathin sections were mounted on Formbar-coated 150-mesh copper grids and examined with and without uranyl acetate and lead citrate (U-Pb) staining. A selected-area electron diffraction technique was used to identify newly formed mineral phase. *d*-spacings were calculated from the diffraction patterns calibrated by gold diffraction patterns with completely identical conditions.

2.7. Enzyme cytochemistry

Eight rats were fixed by perfusion with 1% glutaraldehyde–0.1 M cacodylate buffer of pH 7.4 for 20 min.

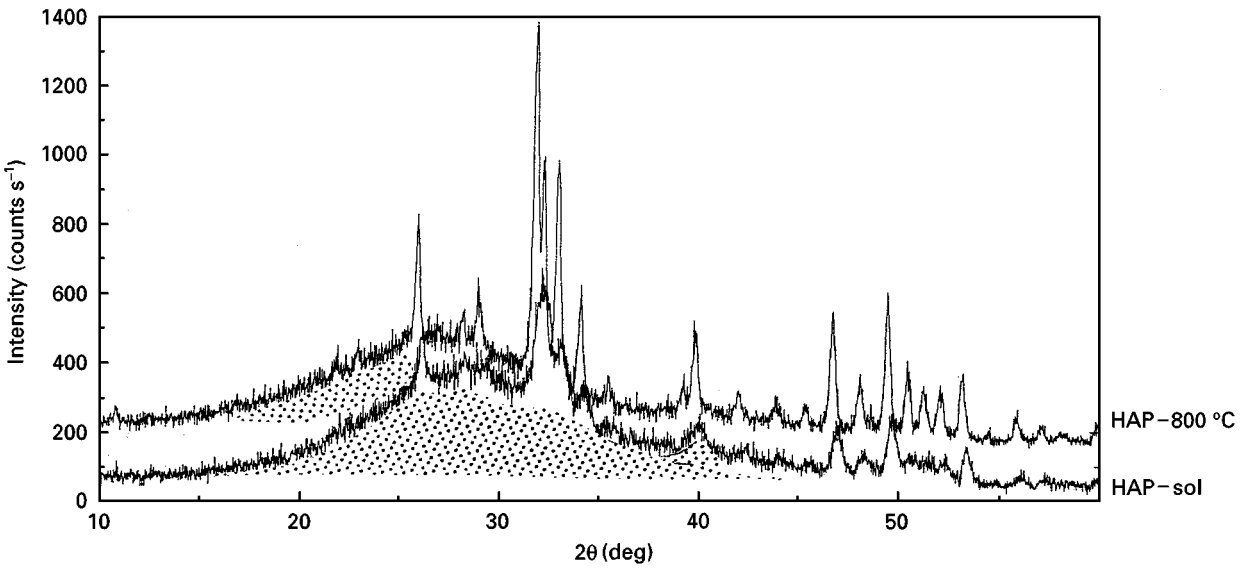

Figure 1 X-ray powder diffraction patterns of HAp-sol (below) and HAp-800°C suspension (upper).

After tissue dissection, the fresh teeth with pulp were again immersed at 4 °C in the same fixative for 1–2 h. After immersion for 1–2 h in 20% dimethyl sulphoxide solution of pH7.5, the samples were cut with a Vibratome (Oxford, UK) or freezing microtome at thickness of 20–60 μm. Acid phosphates (ACPase) and localized dehydrogenate (LDH) activities were determined cytochemically. After incubation for enzymatic activity, the sections were post-fixed, and embedded as mentioned above. Ultrathin sections were not stained or were doubly stained with both uranyl acetate and lead citrate and examined under a Hitachi H-600 electron microscope at 75 kV.

3. Results

3.1. Characterisation of materials

Fig. 1 shows X-ray powder diffraction patterns of the HAp-sol and HAp-800 °C suspension. The pattern of the HAp-sol showed line-broadening peaks of hydroxyapatite on a halo peak caused by water structure (dotted area). On the other hand, the pattern of the HAp-800 °C showed sharp peaks of hydroxyapatite on the same water halo peak. The former line-broadening peaks are due to small hydroxyapatite crystals of less than 0.1 μm.

Figs 2 and 3 show scanning electron micrographs of aggregate crystals in HAp-sol and HAp-800 °C, respectively. The primary particle sizes in HAp-sol and HAp-800 °C were approximately 0.1 and 0.5 μm, respectively. Fig. 4 shows changes in the electroconductivity of HAp-sol and HAp-800 °C in distilled water. The initial solubility and dissolution velocity of HAp-sol were much larger than those of HAp-800 °C at room temperature. Fig. 5 shows changes in the calcium and phosphorus contents dissolved from the HAp-sol and HAp-800 °C in distilled water. In the first

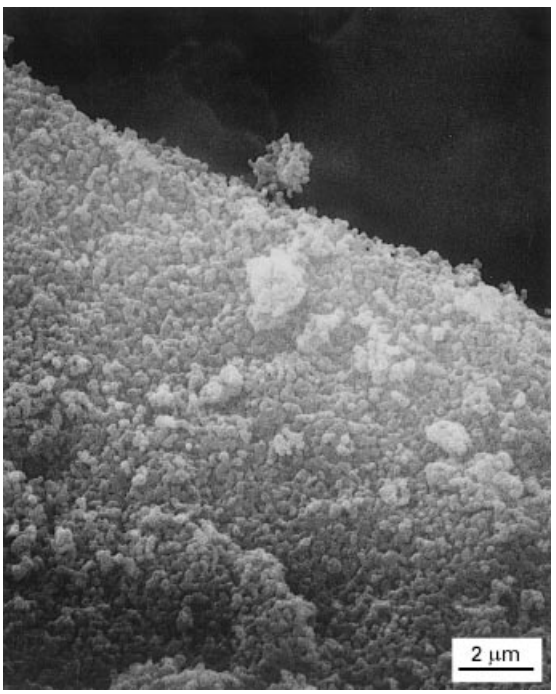

Figure 2 Scanning electron micrograph of aggregate crystals of HAp-sol.

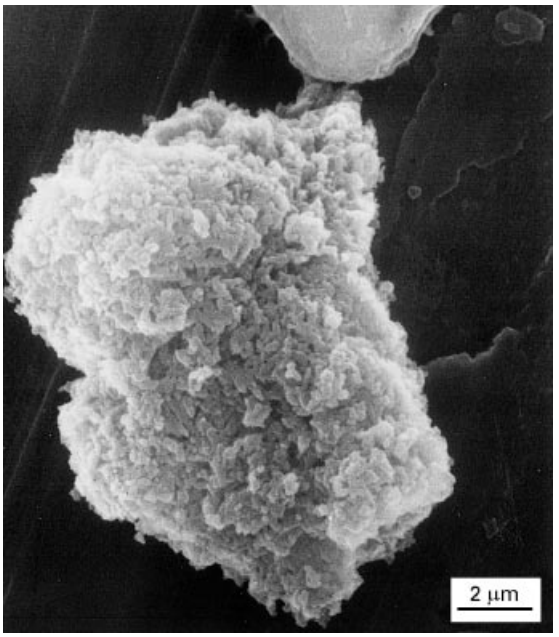

Figure 3 Scanning electron micrograph of aggregate crystals of HAp-800 °C.

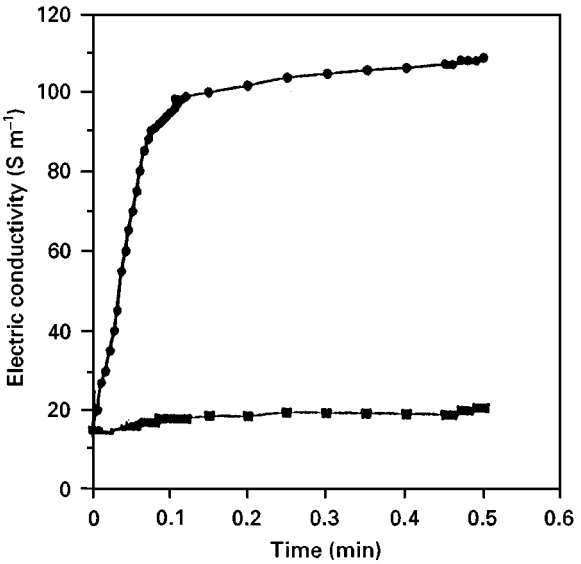

Figure 4 Changes in electroconductivity of (●) HAp-sol and (■) HAp-800 °C in distilled water.

1 min, calcium and phosphorus contents dissolved from the HAp-sol were 4.6 and 3.6 p.p.m, respectively. On the other hand, the calcium and phosphorus contents dissolved from the HAp-800 °C were 2.0 and 0.8 p.p.m., respectively. The dissolution velocities of the HAp-sol and HAp-800 °C decreased with time.

3.2. Light microscopy

After 7 days, in group A, the surface of the pulp became necrotic and a continuous layer of a conglomerate of substances was seen in the most superficial part of this necrotic layer. Inflammation was slight in the pulp cavities and degenerated cells were found at the border of the necrotic layer and the vital pulp tissue. Primary calcific bridges had formed below the

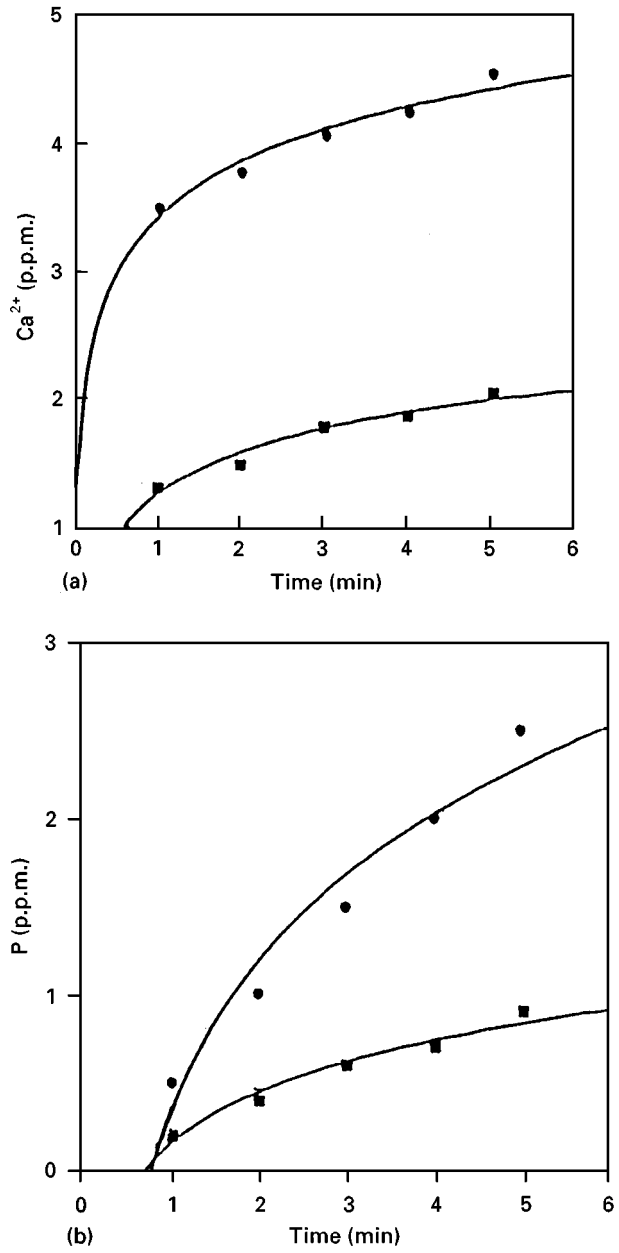

Figure 5(a) Changes in the calcium content dissolved from (●) HAp-sol and (■) HAp-800 °C into distilled water. In the first minute, the calcium contents in HAp-sol and HAp-800 °C were 4.6 p.p.m. and 2.0 p.p.m., respectively. (b) Changes in phosphorus content dissolved from (●) HAp-sol and (■) HAp-800°C. The phosphorus content in HAp-sol and HAp-800°C were 3.6 and 0.8 p.p.m., respectively.

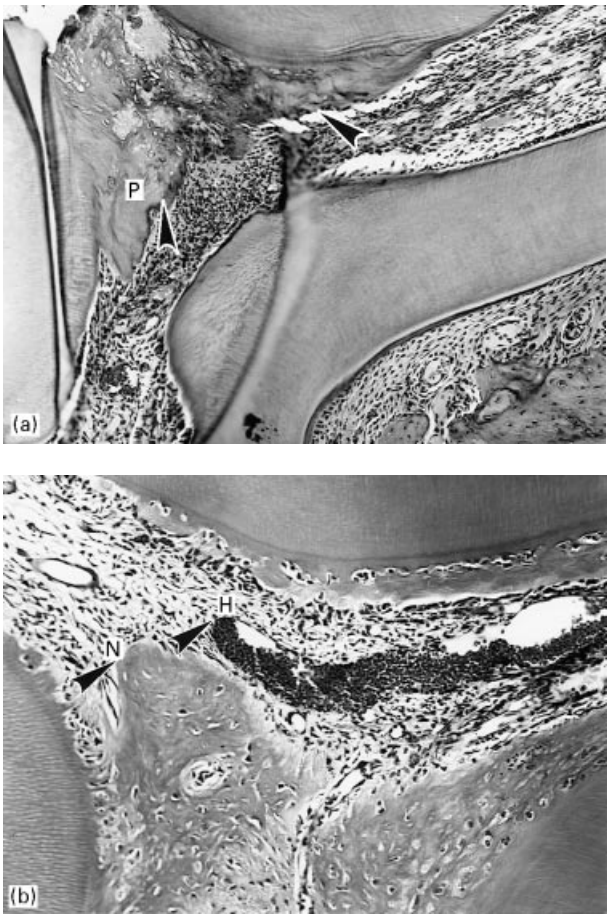

Figure 6(a) A light micrograph of pulp tissue 7 days after HAp-sol injection. Inflammation was slight in the pulp cavity and degenerated cells were found at the border of the necrotic layer and the vital pulp tissue. Primary calcific bridges (P) had formed below the applied HAp particles. (HE, X170). (b) Light micrograph of pulp tissue 7 days after HAp-800 °C suspension injection. The inflammation was moderate in pulp cavities and no extension of the necrotic areas was noted. However, large neutrophils were observed adjacent to the haem (H) or necrosis (N). (HE, X170).

applied HAp-sol (Fig. 6a). In group B, the inflammation was moderate in the pulp cavities and no extension of the necrotic areas was noted. However, large neutrophils were observed adjacent to the haem or necrosis (Fig. 6b).

After 14 days, in group A, no inflammation was observed. A deeply stained region appeared just under the necrotic area. The newly formed secondary dentine bridges had developed below the primary calcific bridges, and the pulp was normal and covered with complete secondary dentine bridges (Fig. 7a). In group B, degenerated cells were found at the border of the necrotic layer and primary calcific bridges were seen. However, a large defect in the pulpal tissue was found in the exposed area (Fig. 7b).

After 28 days, in group A, a higher magnified view of the osteodentine-like structure (OsD) which appeared at the surface of the excised pulp, was taken. A more highly calcified tubular dentine bridge was observed (Fig. 8a). In group B, a relatively poorly calcified dentine bridge without the dentine tubules was observed (Fig. 8b).

3.3. Electron microscopy

After 7 days, in group A, the HAp particles were surrounded by degeneration cells and inflammatory cells. Cell debris was observed around the HAp particles. Macrophages containing phagosomes and phagocytosis of HAp particles were observed (Fig. 9). Electron-dense granular structures and mantle dentine around collagen fibrils were observed (Fig. 10a). Matrix vesicles were seen in the extracellular matrix between these macrophage cells and collagen fibrils, and had diameters of 50–300 nm. At a higher magnification, the matrix vesicles 1 and 2 were seen to contain HAp crystals (Fig. 10b). In group B, the HAp particles were not observed around the macrophages cells (Fig. 11).

After 14 days, in group A, a number of electron-dense granular structures developed between the

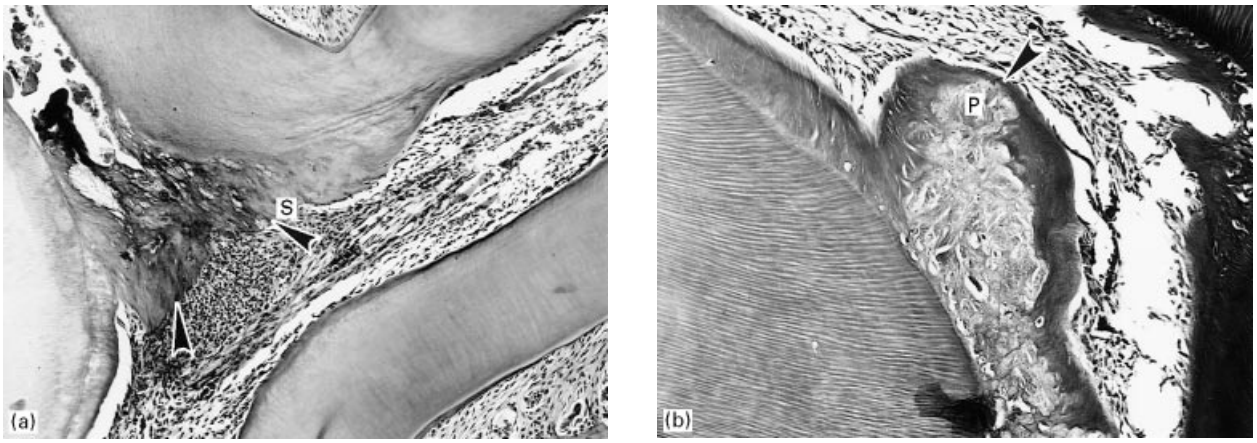

Figure 7(a) Light micrograph of pulp tissue 14 days after HAp-sol injection. Odontoblasts could be clearly observed and similar osteodentine (OSD) including pulp tissue had developed below the hard tissue. (HE, ×170). (b) Light micrograph of pulp tissue 14 days after HAp-800 °C suspension injection. Primary calcific bridges (P) had formed, but osteodentine tissue was not observed. A large defect of pulp tissue was found in the exposed area. (HE, X170).

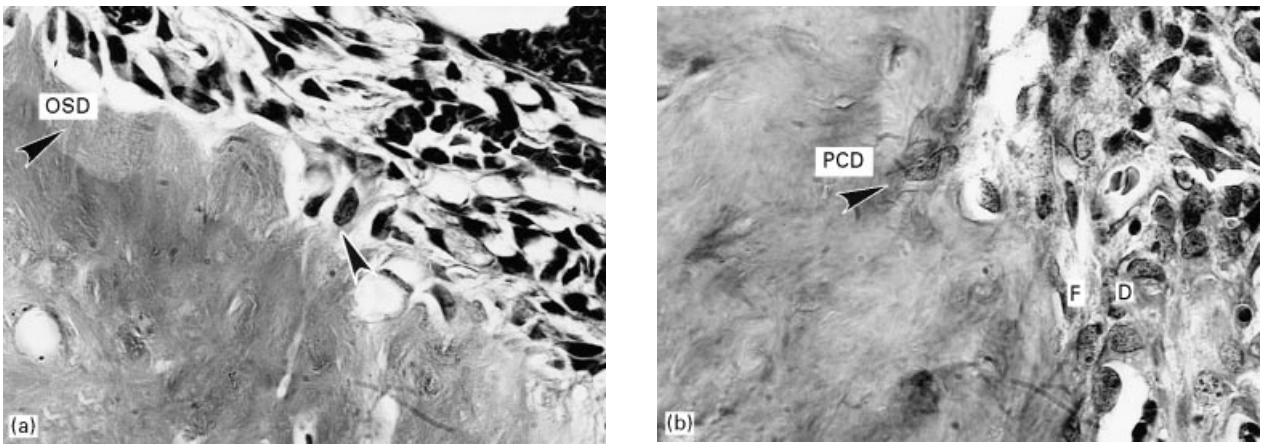

Figure 8(a) Light micrograph of pulp tissue 28 days after of HAp-sol injection. The osteodentine like structure (OSD) and a higher calcified tubular dentine bridge were observed. (HE, X800). (b) Light micrograph of pulp tissue 28 days after HAp-800 °C suspension injection. A relatively poorly calcified dentine bridge (PCD) without the dentine tubules was observed. (HE, X800).

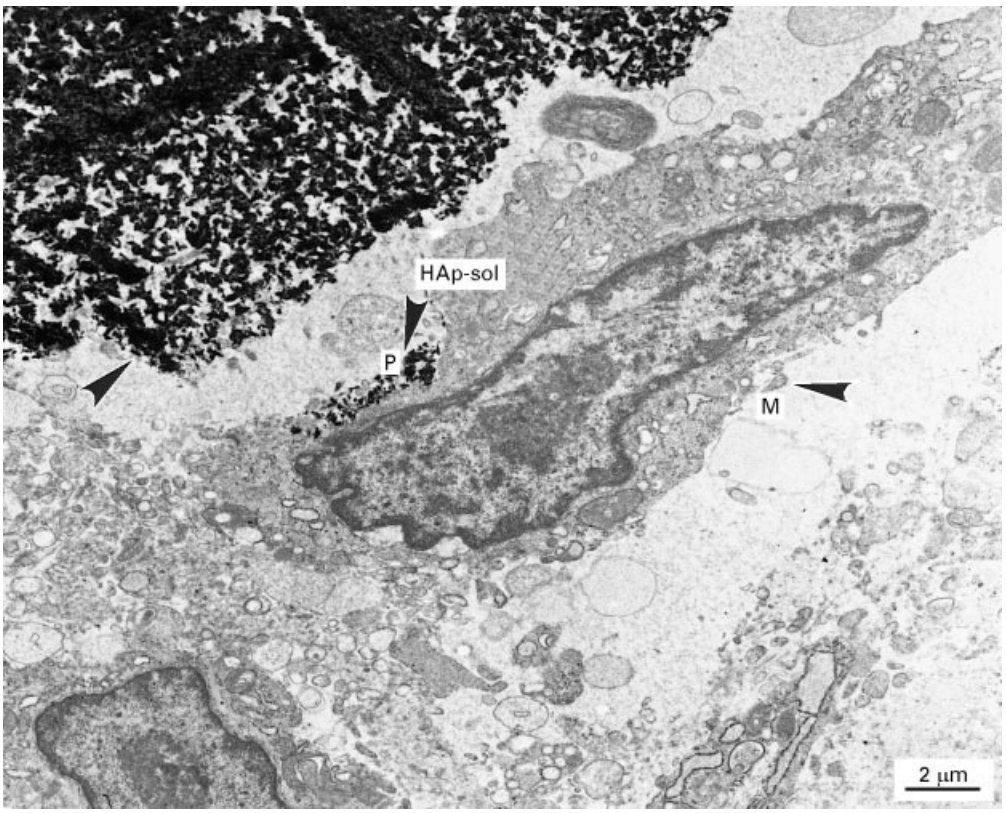

Figure 9 Transmission electron micrograph of pulp cells 7 days after HAp-sol injection. Macrophages (M) containing phagosomes (P) of hydroxyapatite particles were observed.

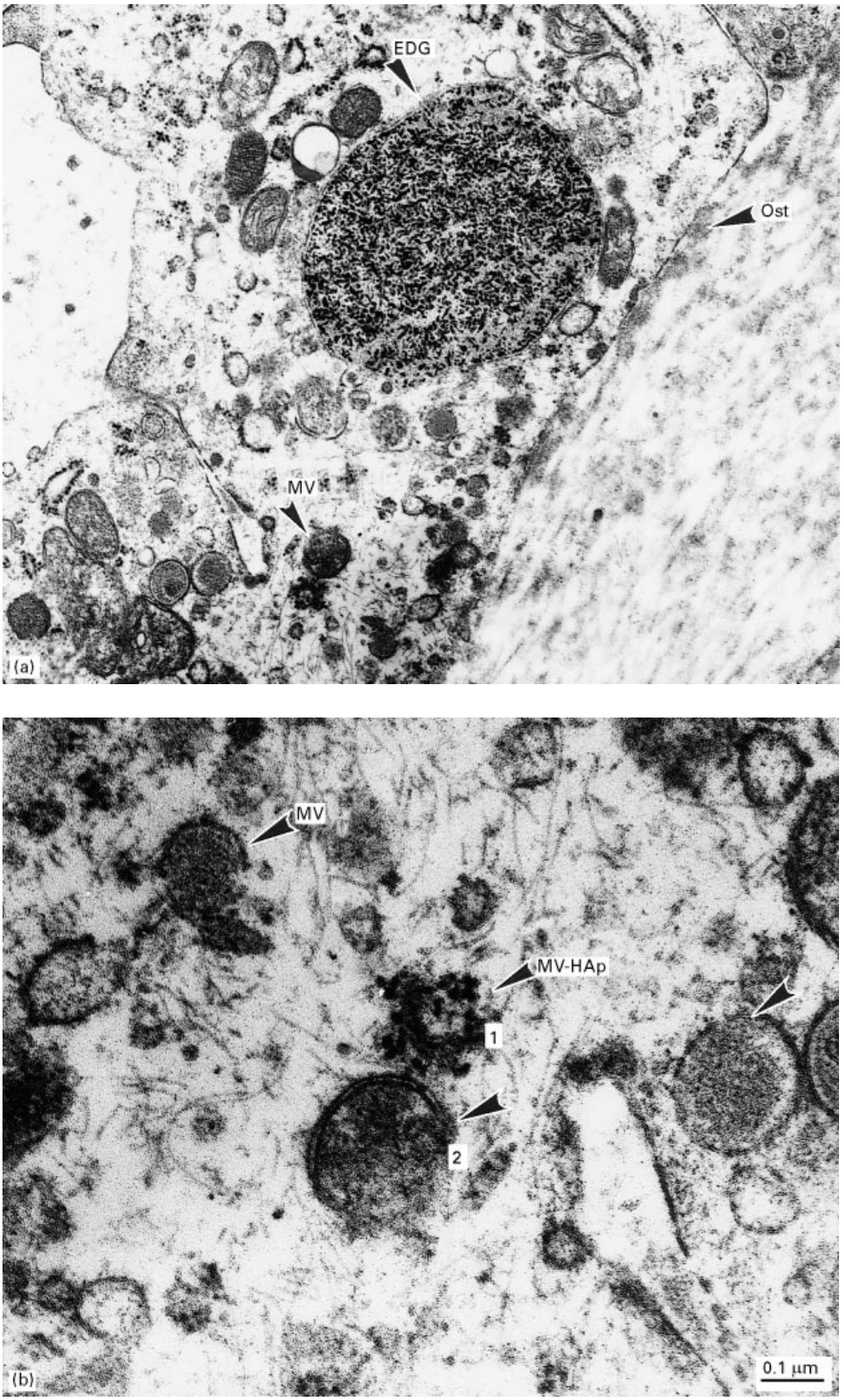

Figure 10(a) Transmission electron micrograph of pulp cells 7 days after HAp-sol injection. Electron-dense granular structures (EDg) and mantle dentine around collagen fibrils were observed. Matrix vesicles (MV) were seen in the extracellular matrix between these large cells and collagen fibrils and had diameters of 50–300 nm. (Ost) Oosteodentine. (b) A higher magnified view of (a). Matrix vesicles (MV) 1 and 2 contain hydroxyapatite crystals surrounding electron-dense granule structures (EDg). (Ost) Osteodentine.

extracellular matrix and the HAp particles (Fig. 12). However, in group B, electron-dense granular structures and mantle dentine were never observed within the dentine tissue (Fig. 13).

After 28 days, in group A, newly formed osteodentine had developed adjacent to the HAp particles and odontoblast-like cell. Within the odontoblast-like cell, well-developed endoplasmic reticules (ER)

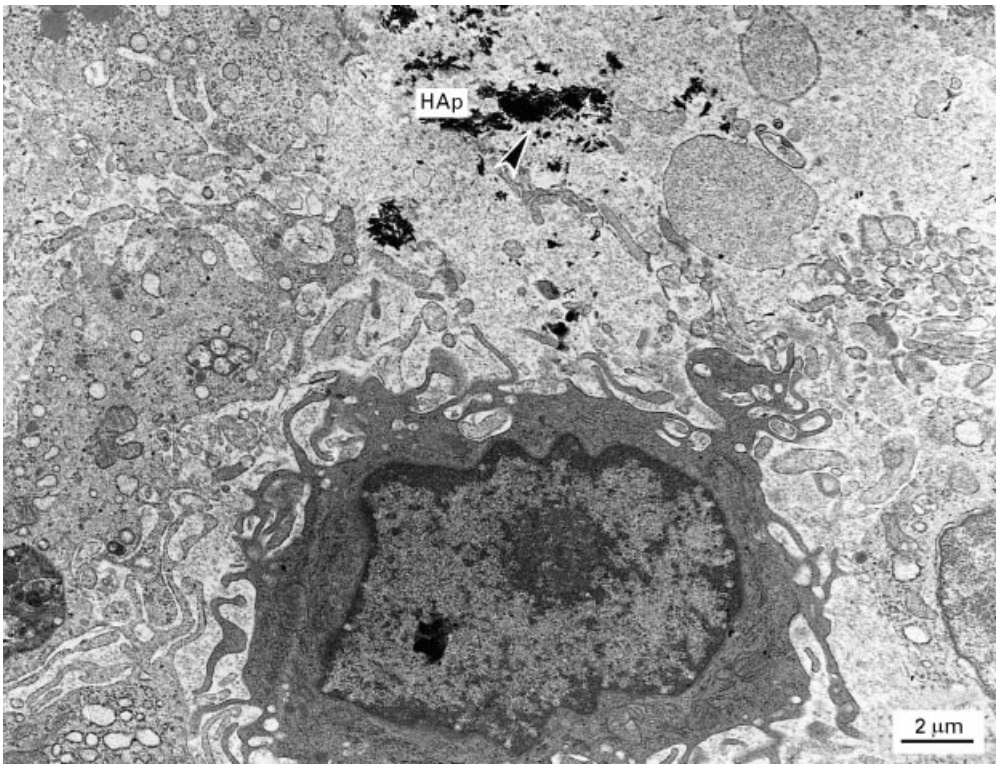

Figure 11 Transmission electron micrograph of pulp cells 7 days after HAp-800°C injection. Macrophages containing phagosomes of hydroxyapatite particles were not observed. (HAP) HAp-800 °C.

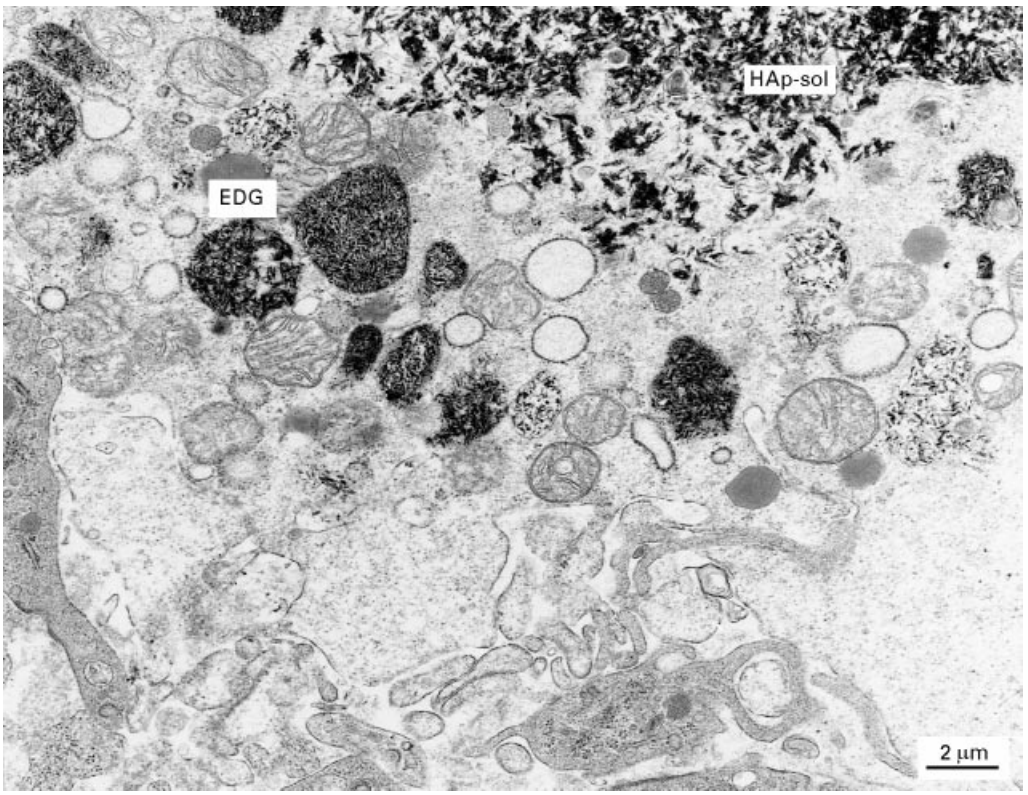

Figure 12 Transmission electron micrograph of pulp cells 14 days after HAp-sol injection. Electron-dense granule structures (EDg) were more developed between extracellular matrix and HAp particles.

and mitochondria (MT) in the distal part of the cytoplasm were seen. Higher magnification views, as seen at bottom left and top right in Fig. 14 show newly formed ostoedentine adjacent to the HAp particles and an electron-diffraction pattern identified as HAp particles. These electron-diffraction patterns had *d*-spacing of 002 and 211 in hydroxyapatite structure. In group B, newly formed osteodentine

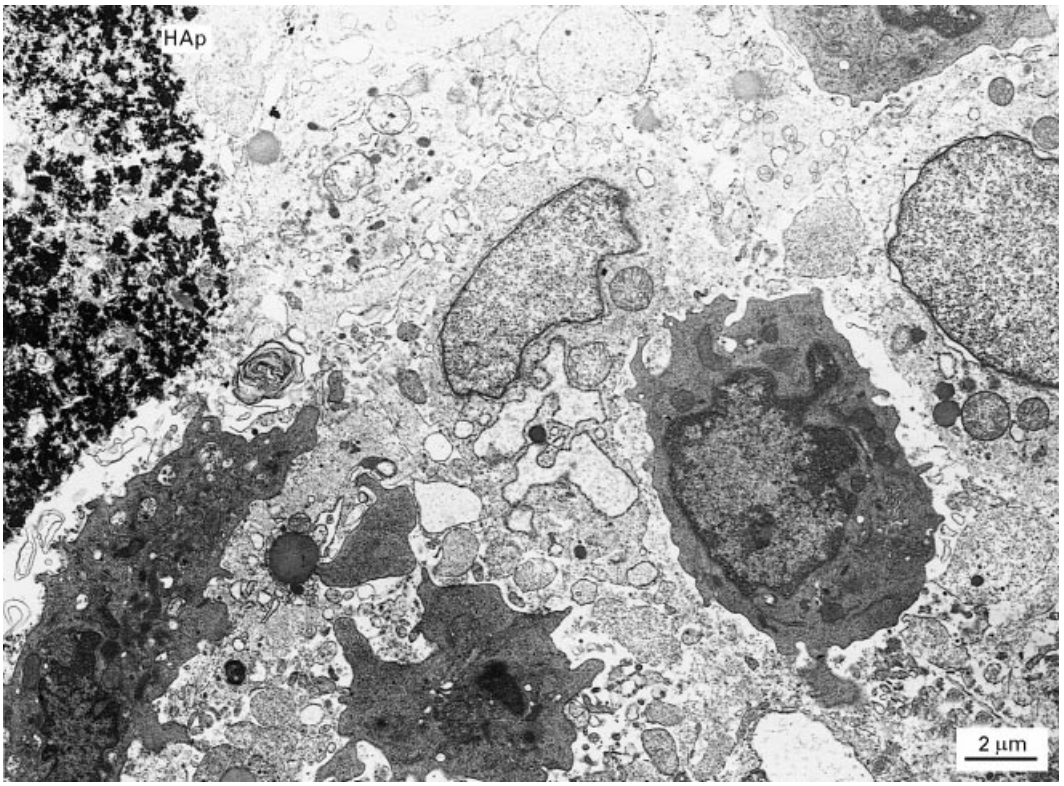

Figure 13 Transmission electron micrograph of pulp cells 14 days after of HAp-800°C injection. Needle-like crystals and granular structures were never observed. (HAP) HAp-800°C.

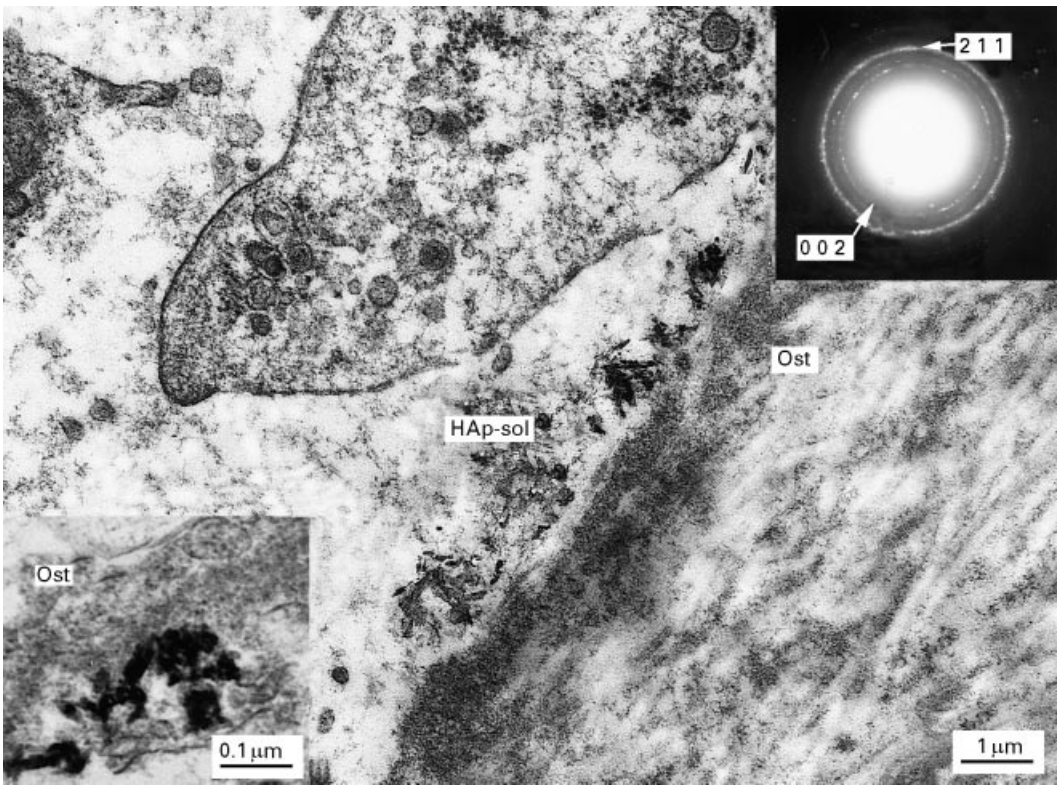

Figure 14 Transmission electron micrograph of pulp cells 28 days after HAp-sol injection. Newly formed osteodentine (OSD) had developed adjacent to the HAp particles and odontoblast-like cells. The higher magnified views at bottom left and top right, show newly formed ostoedentine (OSD) adjacent to the HAp particles and an electron-diffraction pattern identified as HAp particles. These electron diffraction patterns had *d*-spacings of 0 0 2 and 2 1 1 of hydroxyapatite.

was observed in the distal part of the cytoplasm of the odontoblast and HAp particles was seen between the odontoblast and newly fomed osteodentine (Fig. 15).

3.4. Enzyme cytochemistry

3.4.1. ACPase activity

After 7 days post-operatively, in group A, ACPase activity was detected along all the biosynthesizing

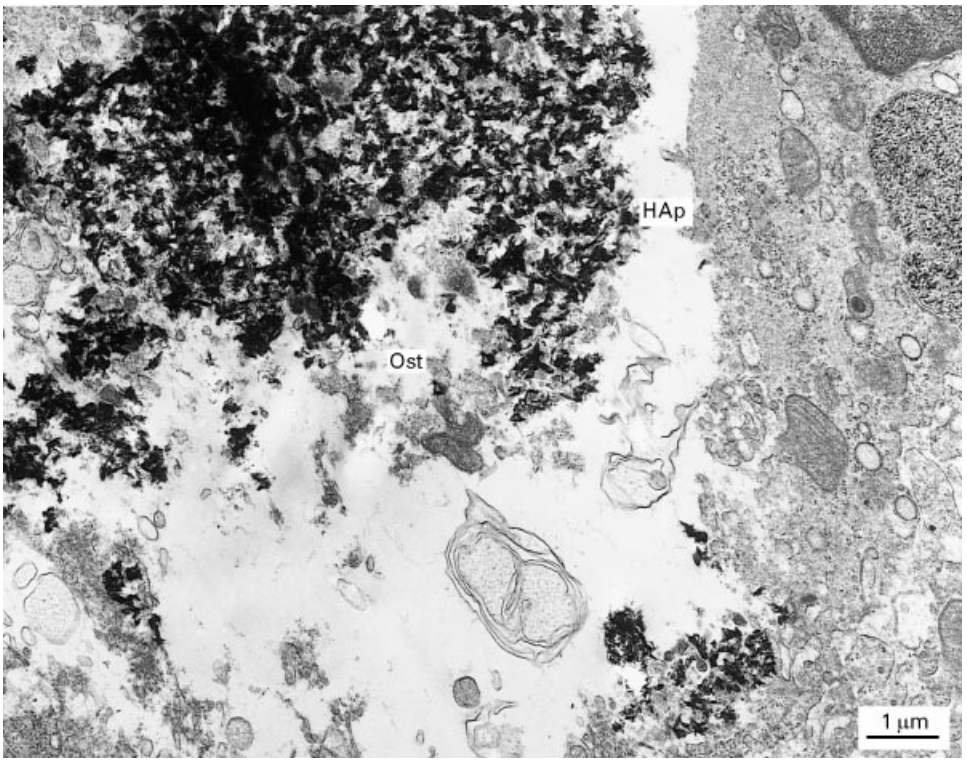

Figure 15 Transmission electron micrograph of pulp cells 28 days after HAp-800°C injection. Newly formed osteodentine (OSD) between the distal part of the cytoplasm of the odontoblast and HAp particles was seen between the odontoblast and newly formed osteodentine.

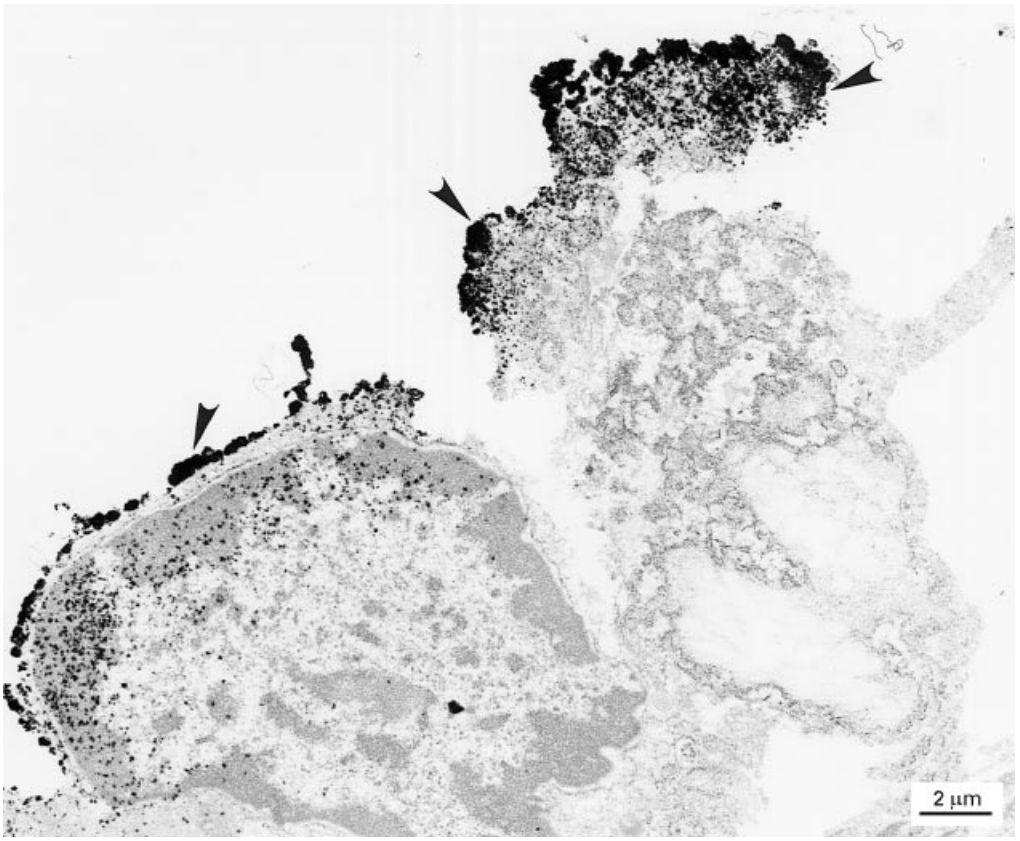

Figure 16 Transmission electron micrograph of ACPase activity in pulp cells 7 days after HAp-sol injection. ACPase activity was detected along all the biosynthesizing pathways, including the perinuclear space, Golgi complex, vesicles and vacuoles of odontoblast-like cells attached to the HAp particles.

pathways, including the perinuclear space, Golgi complex, vesicles and vacuoles of odontoblast-like cells attached to the HAp particles. Extracellular ACPase activity was also demonstrated around the ruffled border area (Fig. 16). In group B, ACPase activity was detected only along membranes of odontoblast-like cells and membranes of macrophages (Fig. 17).

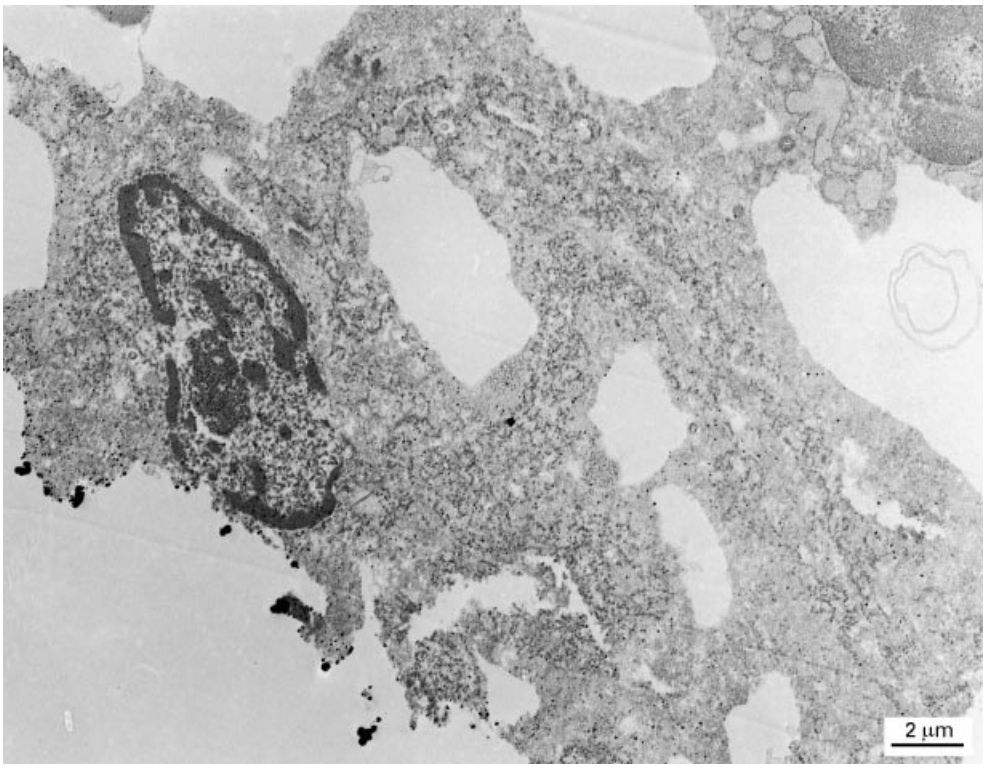

Figure 17 Transmission electron micrograph of ACPase activity in pulp cells 7 days after HAp-800 °C suspension injection. ACPase activity was detected only along membranes of odontoblast-like cells and membranes of macrophages.

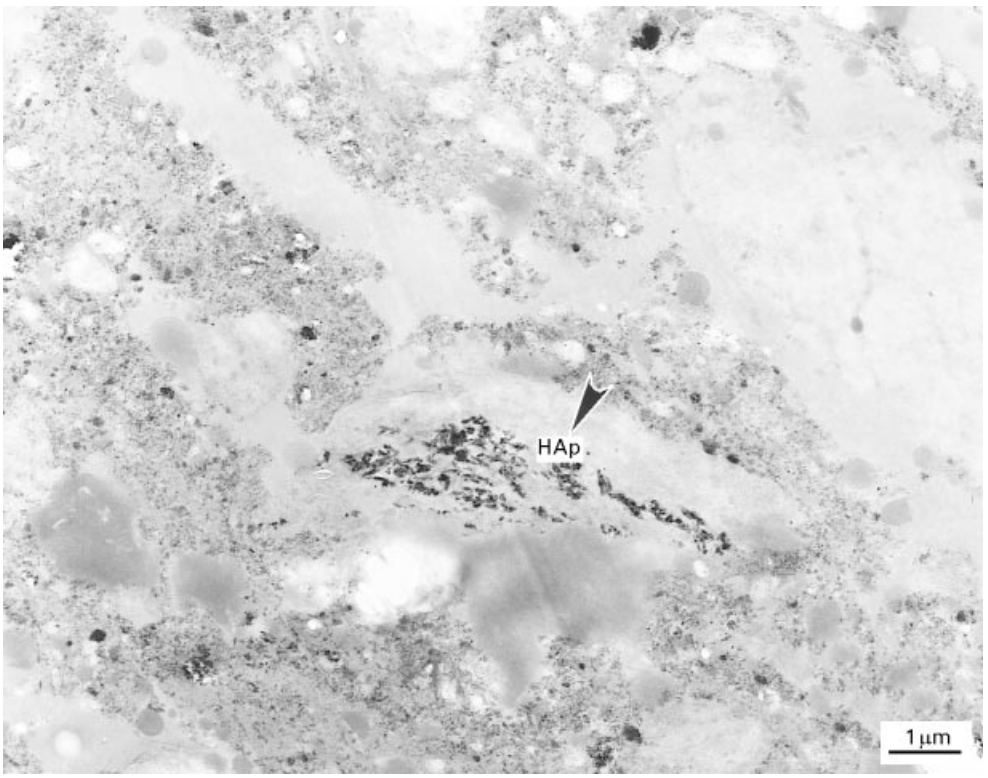

Figure 18 Transmission electron micrograph of LDH activity in pulp cells 7 days after HAp-sol injection. Hydroxyapatite particles HAp were observed.

3.4.2. LDH activity

After 7 days post-operatively, in group A, LDH activity was localized along the inner membranes of the crest of the mitochondria, as small dots on the plasma membrane and in some cases along the inner lamellae of the Golgi apparatus. HAp particles were observed in odontoblast (Fig. 18). In group B, LDH activity was not observed (Fig. 19).

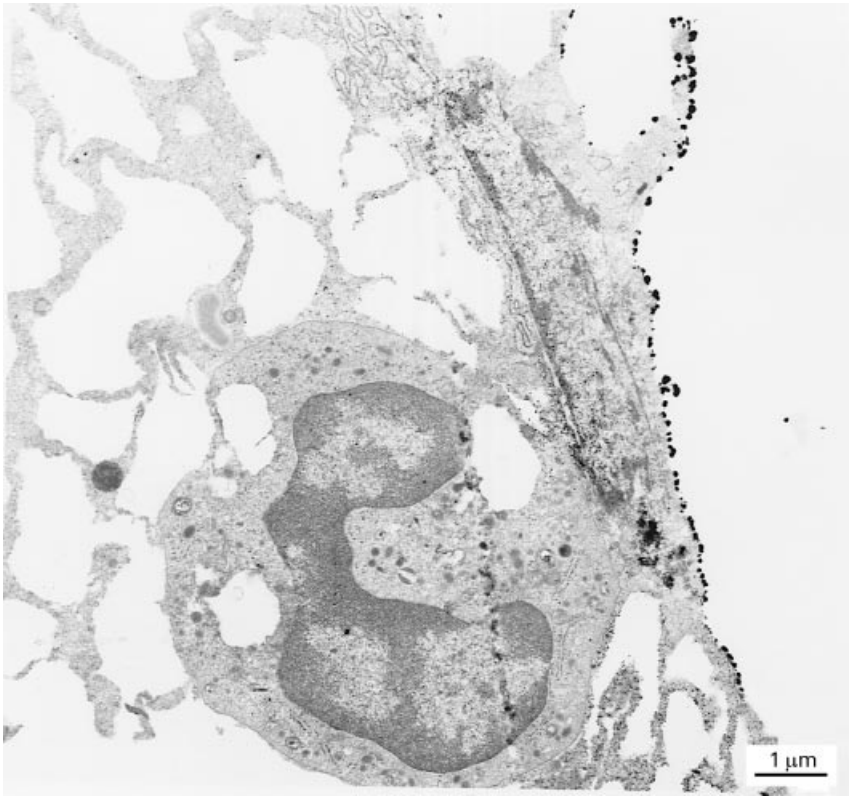

Figure 19 Transmission electron micrograph of LDH activity in pulp cells 7 days after HAp-800°C injection. LDH activity was not observed because there was no phagocytosis.

4. Discussion

Ultrastructural and cytochemical characteristics of dentine-bridge formation after hydroxyapatite was injected into pulp tissue, have long been controversial [27,28]. The common hydroxyapatites, including sintered and calcined hydroxyapatite heated at high temperatures, have been used in previous investigations; however, unheated HAp-sol has not been injected into pulp tissue. The X-ray diffraction pattern of HAp-sol showed line-broadening caused by very small crystals of less than 0.1 μm. On the other hand, the pattern of the hydroxyapatite heated at 800 °C showed many sharp peaks which were caused by larger sized crystals 0.5 μm. There are differences in physical and chemical properties between sintered or calcined hydroxyapatite and HAp-sol.

The initial solubility in distilled water of the HAp-sol was twice that of HAp-800 °C, as the common hydroxyapatite. The solubility and the rate of dissolution depend on differences in crystal size or crystallinity of HAp [29]. After the dissolution, the pH of the HAp-sol was almost neutral. Therefore, the HAp-sol will be quickly phagocytosed by cells and dissolved in living bodies. This indicates that the HAp-sol could be a calcium and phosphate ion supplier which could cause dentine bridge formation. Fukuchi *et al.* [15, 16] revealed that macrophage cells are activated by phagocytosing the HAp-sol using a cell-culture technique. The cell activation may depend on the crystal sizes of hydroxyapatite microcrystals in the HAp-sol.

The microcrystal size in the HAp-sol was approximately 0.1 μm and the size and crystallinity are almost the same as those of biological apatites in bones and dentine of animals and humans. Thus the sizes will not disturb the environment in cells and living bodies.

It was considered that the dentine bridge formation by the HAp-sol injection into dental pulps is accelerated by odontoblast cell activation and calcium and phosphate ion supply.

Schroder [30] reported that the cause of necrosis is thought to be due to the alkalinity of calcium hydroxide, and the slighter the inflammation, the greater is the frequency of bridging. Sekine *et al.* [31] reported that necrotic layer has a direct relationship with the formation of the dentine bridge. Imai and Hayashi [32] reported Ca-BGP is slightly alkaline (pH about 8) and the necrotic tissue formed immediately after application of Ca-BGP was more superficial in the region of the exposure site than that after application of the $Ca(OH)_2$ (pH about 12.5). One of the goals in the present study was to clarify that the inflammation of pulp cavity was slighter in the HAp-sol than in HAp-800 °C. These findings suggest that the necrotic layer is related to the pH and solubility rate of HAp, and mechanical exposure. The pH 7.4 of the HAp-sol is neutral and is favourable to pulp tissue. A dentine bridge was formed 7 days after the application of HAp-sol, while it had still not formed at 14 days after the application of HAp-800 °C. Immediately after capping by HAp-sol, calcium and phosphate ions were quickly released in the pulp tissue. Then, osteodentine was formed below the HAp-sol. Generally,

the formation of tubular dentine began below the osteodentine after the vital pulp tissue had been recovered. Thus, the HAp-sol may induce the early formation of tubular dentine in the recovery process.

Yoshiki and Kurahashi [33] suggested that ACP enzyme seems to be localized in association with not only fibrillar elements but also the intercellular amorphous ground substance. Matsuzawa and Clarke Anderson [34] concluded that acid hydrolysis may be involved in the removal of proteoglycans from cartilage matrix prior to calcification and that the presence of proteoglycans may be inhibitory to calcification. Morphological and cytochemical evidence showing that osteodentine in rat pulp elicited by the HAp-sol can be characterized as odontoblastes, was obtained in the present study, because they are characterized by a ruffled border, a clear zone, multiple nuclei, mitochondrion-enriched cytoplasm, various vacuoles concentrated in the cytoplasm adjacent to the ruffled border, and positive reactions for tartar-resistant ACPase activities. One of the interesting results of this study is that the same ACPase activation can simultaneously respond to both the extracellular matrix and the membrane of odontoblast-like cells. ACPase activity was not detected in HAp-800 °C. The reasons for the differences in ACPase activity and the formation of osteodentine in pulp tissue after injection of HAp-sol and HAp-800 °C, need further cytochemical investigation.

5. Conclusions

X-ray powder diffraction patterns of HAp-sol showed line-broadening peaks of hydroxyapatite on the water halo, while those of HAp-800 °C showed sharp peaks. The initial solubility velocity of HAp-sol in distilled water was twice that of HAp-800 °C. Inflammation after injection of HAp-sol into dental pulp of rats was milder than after injection of HAp-800 °C. Osteodentine was quickly formed in the pulp within 7 days after HAp-sol injection. Odontoblast ACPase and LDH activities in the pulp after injection of HAp-sol were notably higher than after injection of HAp-800 °C. HAp-sol had excellent biocompatibility and is a safe biomaterial for use as dental pulp and is a source of calcium and phosphate ions, causing calcification through hydrolysis by acid phosphates (ACP). The HAp-sol can be used as a new injectable direct pulp-sealing agent.

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