Chapter 2

Tissue Reaction to Root Canal Filling Material Embedded Subcutaneously in Rats

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《**Abstract**》

The reaction of subcutaneous connective tissues to root canal filling material pastes, made chiefly of calcium hydroxide, has been studied by means of radiography, histopathology, and electron microscopy. Radiography has revealed that embedded paste displays a well-demarcated radiopaque mass just after embedding, then gradually disappears over time. The resulting images have shown granulation tissue appearing, as well as and the phagocytosis by macrophages and giant cells, as observed histopathologically. Furthermore, there have been heterotopic calcifications within the proliferating granulation tissues in some cases, with electron microscopic examinations revealining phagocytosis. In the present study, the ultrastructural aspects of initial calcification in rat subcutaneous tissues elicited by root canal filling material paste, made up of calcium hydroxide and iodoform with the addition of silicone oil, were examined. The embedded paste caused heterotpic calcifications, which can be divided into two types: dystrophic calcification; and matrix vesicle calcification, which resembles that of bone tissue. These data suggest that the paste may elicit the differentiation of osteoblasts and/or cementoblasts from mesenchymal cells in periodontal ligament.

Introduction

The basis for success following root canal therapy is the ability of the periapical connective tissue to undergo repair. The closure of the apical foramen by osteoid or cementoid hard tissue deposition is usually referred to as biologic filling. We have shown biologic filling after root canal

treatment with two different root canal fillings and have given evidence that the root canal filling material Vitapex (Neo Dental Chemical Products Co., Ltd., Tokyo, Japan), made up of calcium hydroxide and iodoform with the addition of silicone oil, caused cells in the periodontal ligament to differentiate into osteoblasts and/or cementoblasts [1].

The aim of this chapter, therefore, is to present the examination results of tissue reactions and the resorption of subcutaneously embedded root canal filling materials paste in rats and mice [2, 3].

Animal experiment and examination methods

A small amount of some root canal filling material pastes, chiefly made of calcium hydroxide Vitapex, were embedded into the dorsal or abdominal subcutaneous connective tissue of rats or mice. Vitapex consists of calcium hydroxide and iodoform with the addition of silicone oil, which means Vitapex is an oil-based calcium hydroxide paste. At intervals of 4 days to 4 months, the animals were killed after the operation. The paste and the surrounding connective tissues were removed together, immediately fixed and embedded in paraffin for histopathological examination. The sectioned specimens were stained with the following reagents: hematoxylin and eosin, van Gieson's stain, and von Kossa's stain.

For electron microscopic examination, the specimens fixed with Karnovsky's solution were post-fixed with 1% osmium tetraoxide and embedded in Epon 812 resin (TAAB, UK). Ultrathin sectioned were stained with uranyl acetate and lead citrate (U-Pb) and ruthenium red (RR) stains and examined ultrastructually and electron-microanalytically. Furthermore, alkaline phosphatase activity was examined by means of the lead citrate method, and calcium localization was observed with the use of the potassium pyroantimonate method.

Diffusion, absorption and phagocytosis of the paste

Soft X-ray photography showed the embedded paste as a clearly distinct bordered radiopaque region just after embedding (Figure 1). The radiopaque region was gradually diffused and the border became unclear from the surrounding radiopaque granules. The center of the radiopacity became smaller over time, and then the radiopacity became weak and finally almost disappeared with the passage of time, but there were small radiopaque granules appearing within the embedded area (Figure 1).

As observed histopathologically, granulation tissue appeared around the embedded area. There was no necrotic tissue.

A slight inflammatory reaction of plasma cells and lymphocytes was noted, without neutrophilic leucocyte infiltration. In some cases, macrophages infiltrated mainly within the granulation tissues. These macrophages had von Kossa's positive granules in their cytoplasms. This means the macrophages phagocytosed the components of calcium hydroxide in the paste embedded.

After a few weeks, the embedded paste was divided into small gathering masses by the proliferating granulation tissue, in which foam cells having clear cytoplasm were observed. The foam cells increased in number over time. Furthermore, in these granulation tissues, there were some giant cells, in whose cytoplasm there were numerous von Kossa's positive granules.

In the case of water-based calcium hydroxide paste, there were necrotic and degenerative histopathological changes, as well as foreign body reactions or the proliferation of granulation tissue, in specimens after the embedding into the subcutaneous connective tissues. In particular, a few days after the embedding, necrotic changes occurred around the embedded paste [4, 5]. Upon examination of the 1-week specimens, granulation tissue rich in macrophages

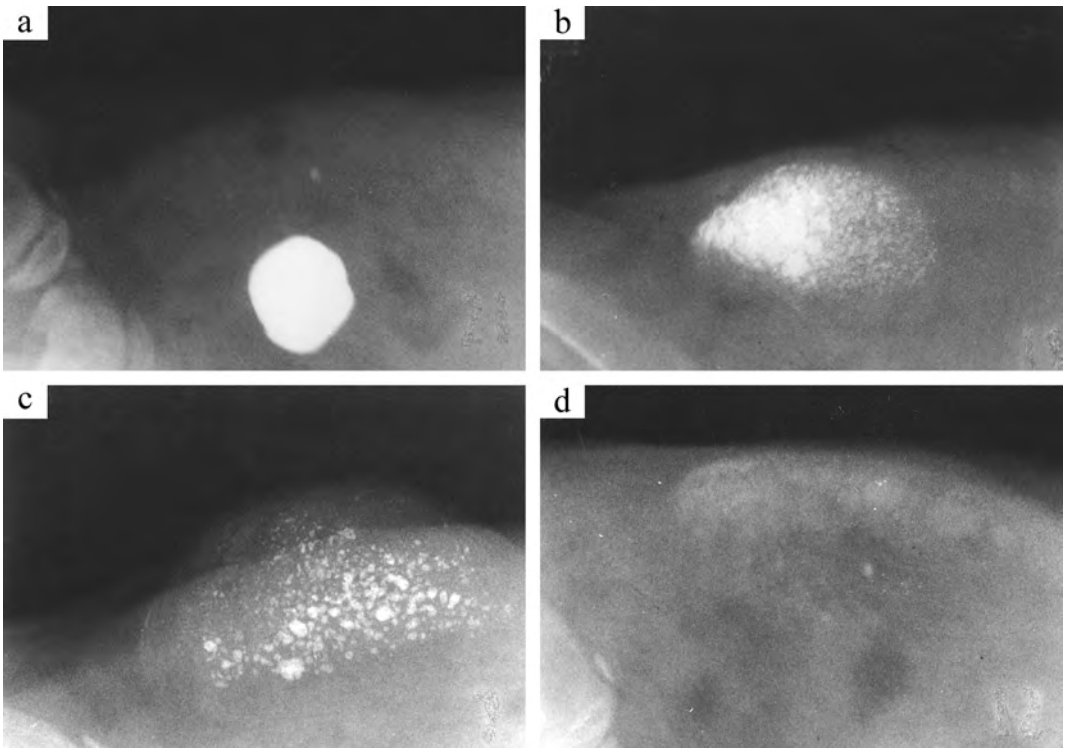

Figure 1 X-ray photographs of the embedded material in the abdominal subcutaneous connective tissue in rats: just after the embedding (a), 2-week specimen (b), 3-week specimen (c) and 5-week specimen (d).

and giant cells was observed, and inflammatory cells, lymphocytes and plasma cells were reduced in number. At 3 weeks, no necrotic or degenerative changes due to the paste were seen; however, a foreign body reaction or granulation tissue was observed. Finally, only some inflammatory cell infiltration was seen in the 4-month specimens.

On the basis of the histopathological findings, we think that immediately after subcutaneous embedding of these pastes, calcium hydroxide, a major component of the paste, causes necrotic and/or degenerative changes in the tissue surrounding the embedded site because of the high alkalinity of water-based paste. However, there were no necrotic reactions caused by the oil-base paste Vitapex.

Electron microscopically, there were a number of electron-dense structures within the macrophages, with many cytoplasmic foldings, proliferating around the embedded paste. The electron-dense structures existed among the folding and in the cytoplasm (Figure 3, a, b). These electron-dense materials were thought to be calcium salt originating from the embedded calcium hydroxide component of the paste. These structures increased in number with the passage of time. During the same period in other sites there were lipid-like droplets within the macrophages (Figure 3, c, d). These droplets showed uniformity and moderate electron-density, and some of them had a comparatively light periphery. These cells were full in their cytoplasm and showed foam cells. The lipid-like droplets were considered to have originated from the silicone oil and other components of the embedded paste.

Furthermore, there were some foreign body giant cells within the proliferating granulation tissues (Figure 4). The nuclei wered irregular in shape. Mitochondria and rough-surfaced

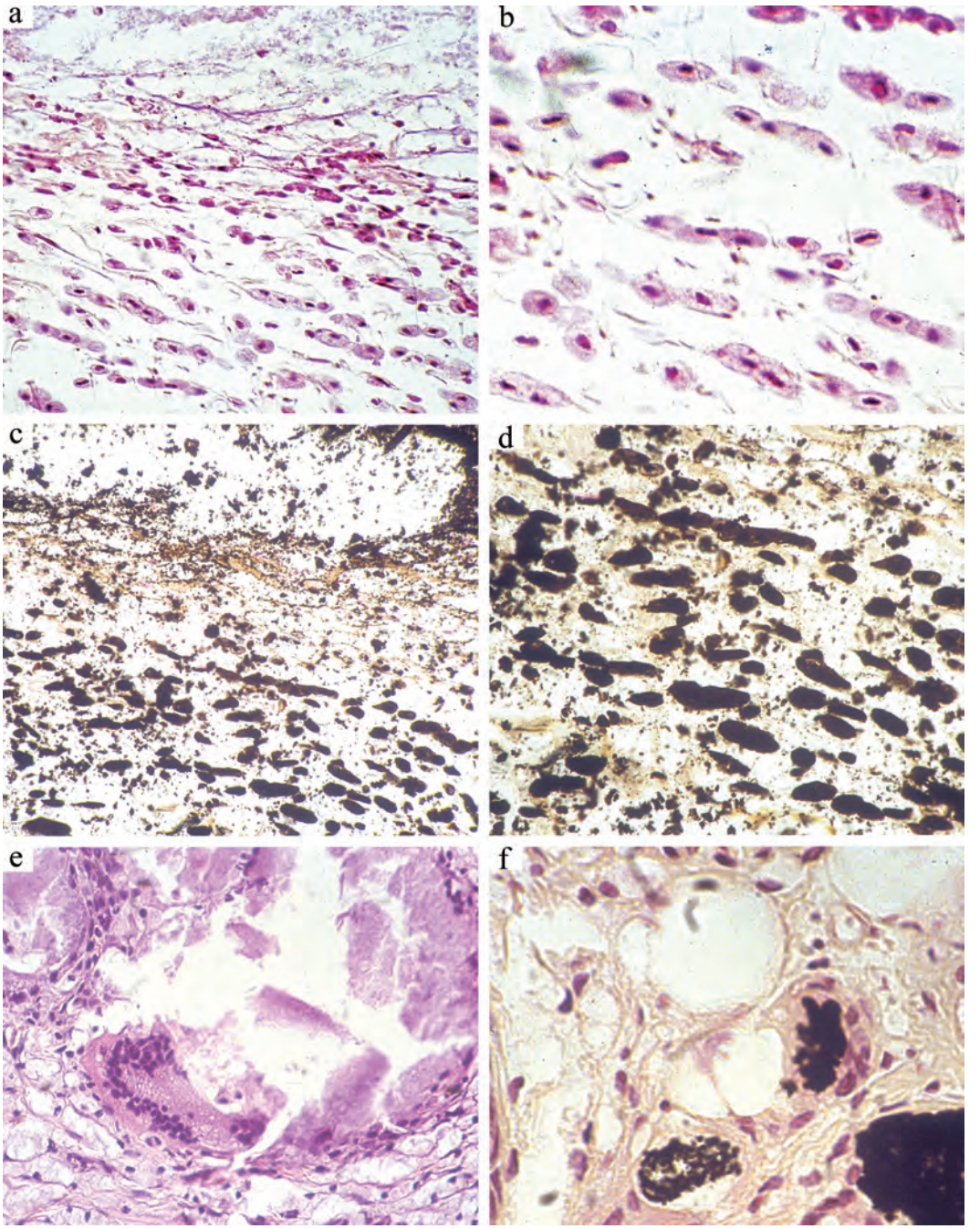

Figure 2 1-week specimen shows proliferating granulation tissue around the embedded paste. Macrophage rich area (a), and enlarged photograph (b). These macrophages contained Von Kossa's positive (calcium) granules (c); enlargement (d). Giant cells appearing in 7-week specimen (e) and which react to von Kossa's positive (f).

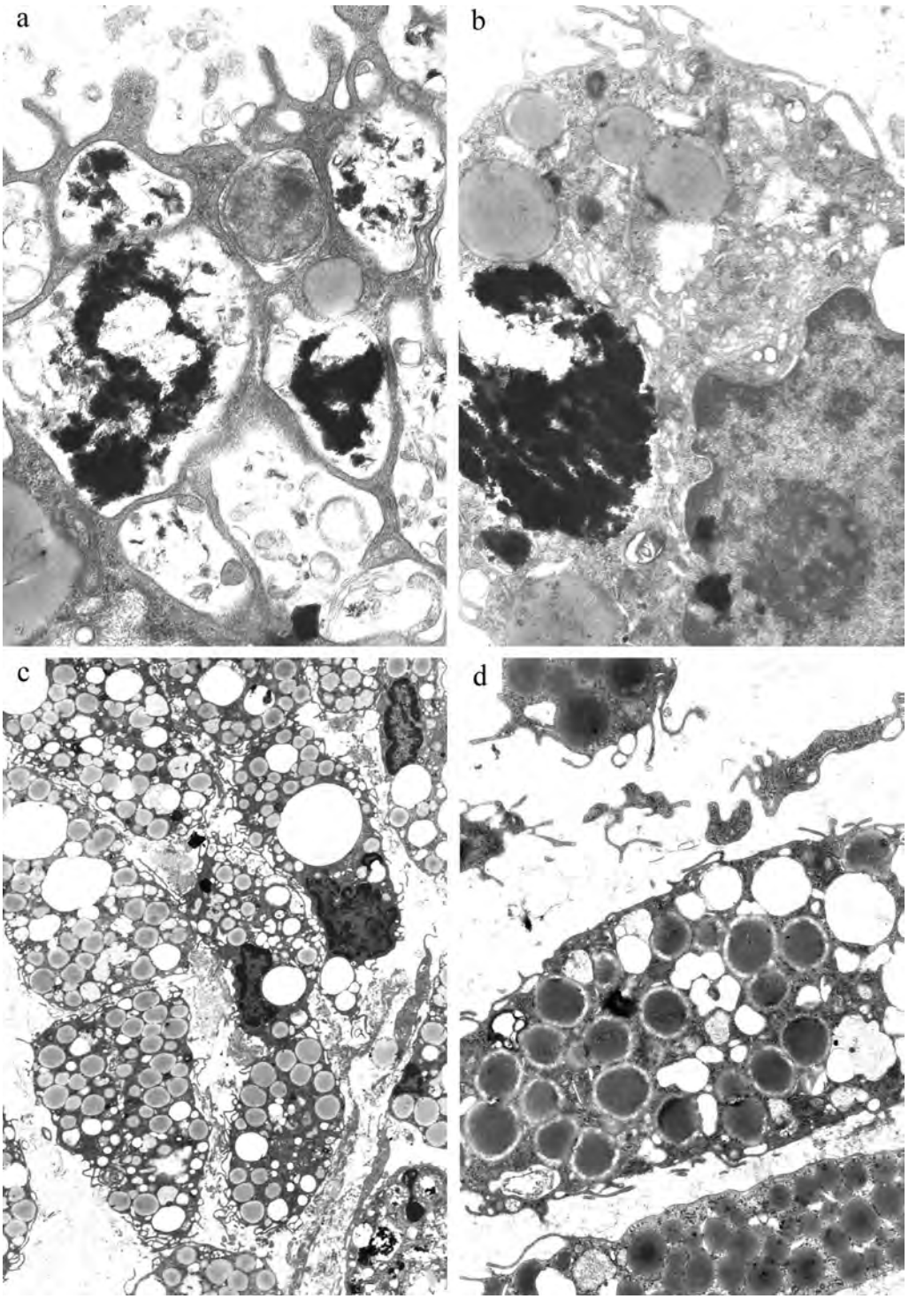

Figure 3 Histopathology of 2-week specimen shows phagocytosed macrophages with numerous cytoplasmic foldings (a) and the electron dense phagozomes in the cytoplasm of the macrophages (b). There are many lipid-like droplets within the cytoplasm of macrophage in 2-week specimen (c) and the droplets show uniform densities (d).

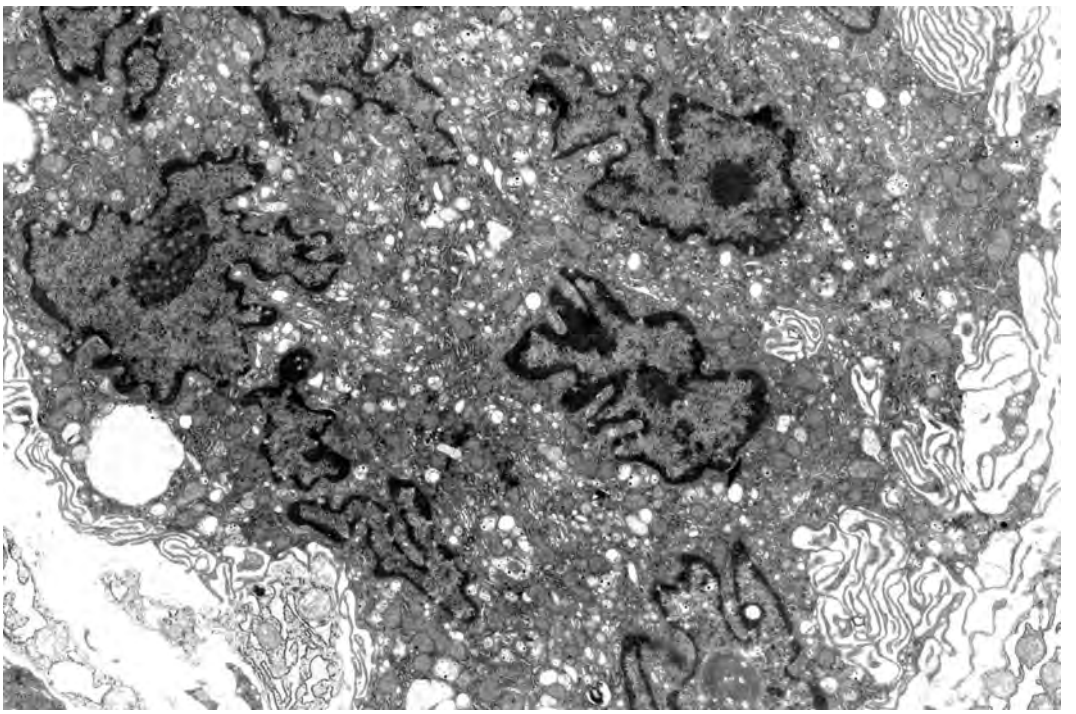

Figure 4 Giant cell appearing in the granulation tissue has many irregular nuclei.

endoplasmic reticulum were observed as full in the cytoplasm.

Within a short period of time, however, the calcium hydroxide reacted with the tissue fluids to form non-soluble calcium salts, such as calcium phosphate. The calcium salt did not cause any damage even though it was not phagocytosed by macrophages and giant cells. Granulation tissue rich in cells, including foreign body giant cells, proliferated around the embedded site. Since the 4-month specimens showed many foci of calcification in the fibrous tissues, we propose that the calcium foci persisted for a long time.

Calcification and hard tissue formation

Soft X-ray photography of one-month specimens showed the embedded paste almost to have totally disappeared with the passage of time. In one-month specimens, there were small radiopaque granules that appeared within the embedded area (Figure 1, d).

The embedded paste was divided into small masses; it was gradually resorped by macrophages and it disappeared with the passage of time. Histopathologically, granulation tissue associated with embedded the paste strongly reacted to von Kossa' s stain in one-week specimens. Calcified and/or hard tissue formation occurred within the embedded sites as many hematoxylin deeply-stained structures associated with the calcium hydroxide component of the embedded paste. At this stage, alkaline phosphatase activity appeared strongly in the granulation tissues (Figure 5, a). In some cases, hematoxylin-stained irregular calcified matrices were formed. There were no cyto-inclusions in the matrices. In the cases of embedded meterial attached to bone, bone tissue formation occurred (Figure 5, b). Occasionally, a specific case showed that bone-like matrix had formed (Figure 5, c). In this case, osteocyte-like cyto-inclusion was noticed in the Schmorl's staining (Figure 5, e). The matrix was stained red by van

Gieson's staining. Therefore, the inclusion cell was determined to be an osteocyte, suggesting that the tissue is bone. These tissue reactions correspond to the following clinical effect: if the calcium hydroxide past is applied as root canal filling material, there is some periodontal tissue remodeling.

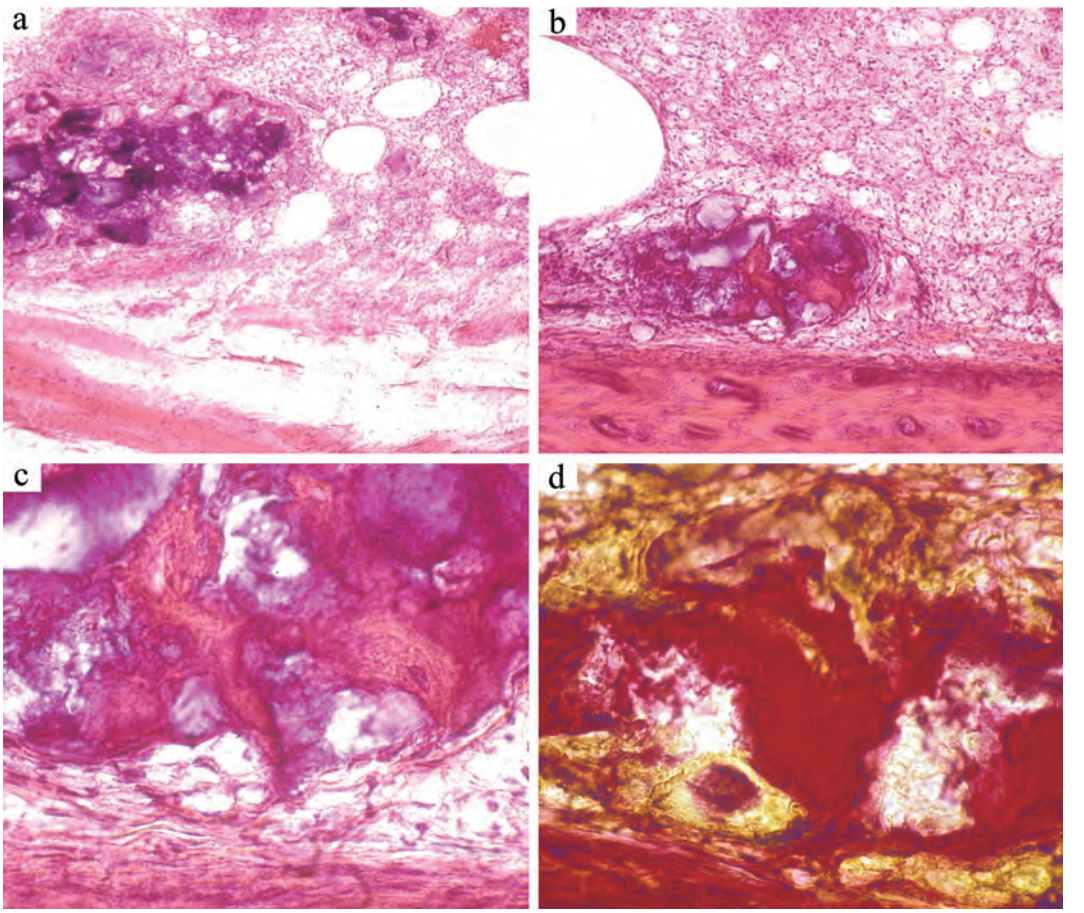

Figure 5 In one-week specimen, In hematoxylin-stained irregular calcified matrices (a, b) was associated with bone tissue (c); the enlargement stained van Gieson's stain (d) showing osteocytes.

As observed with electron microscopy, in the early period (about 1 to 2 weeks later), collagen fibers had formed around the paste, and needlelike electron-dense crystals had appeared on these collagen fibers. However, no evidence of matrix vesicle formation was observed in these areas. Two or more weeks after embedding, the vesicle appeared in the ground matrices of the granulation tissues (Figure 6) and caused calcification (Figure 7, a).

Many small granules of glycosaminoglycans were distributed around the collagen fibers (Figure 7, b). Large granules that reacted positively to RR stain for glycosaminoglycans, especially those attached to the collagen fibers, were in these granulation tissues, but they had disappeared from the vesicles of the calcified zone (Figure 7, c). In areas of sparsely distributed collagen fibers, vesicles that contained electron-dense materials (Figure 7, c, e) and some organelles and cell debris (Figure 7, c) were found. In later stages, the growth of electron-dense crystals beyond the calcified vesicles was observed (Figure 7, d).

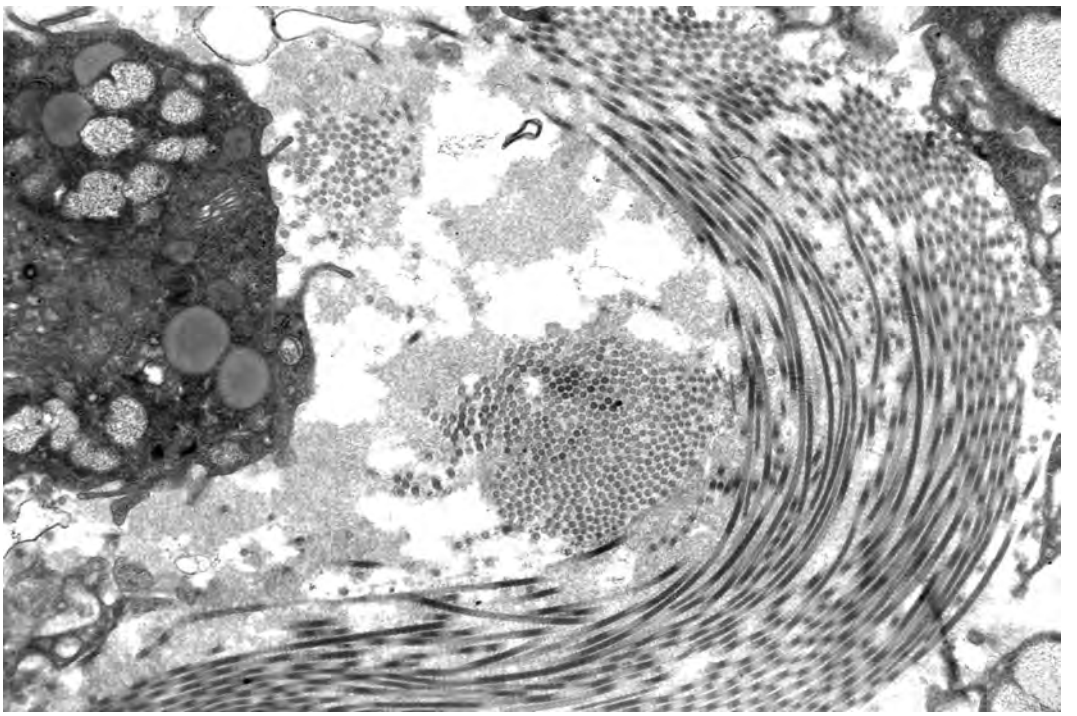

Figure 6 Fine granular and fibrilar matrices were distributed in the granulation tissue.

These calcifies areas were covered with a unique structure made up of electron-dense substances that were RR-positive (Figure 8, a, b). In some cases, these unique structures had a membranous appearance (Figure 10), and one of them covered large rod-shaped crystals (Figure 8, c, d).

Cytochemical techniques demonstrated alkaline phosphatase activity and heavy calcium deposition in the vesicles of the calcified zone (Figure 9, a, b, c). Furthermore, calcium deposition was detected (Figure 9, d).

By means of electron probe microanalysis (EPMA), the electron-dense materials associated with calcification were shown to be composed mostly of calcium and phosphorus (Figure 10, a-e).

Histopathological investigations of tissue reactions to root canal fillings have already been conducted [2-8]. However, very few reports on the ultrastructural appearance of these calcification sites caused by the materials have been published [2, 3]. As mentioned above, two types of calcification were observed in the granulation tissues formed around the embedded paste. In one type, needle-like electron-dense crystals appeared on the collagen fibers, and this was considered to be dystrophic calcification caused by the paste. The other type involved calcification associated with various vesicles. Through cytochemical analysis, this type of calcification was verified in alkaline phosphatase activity and in calcium deposition in these vesicles in the calcified zone. On the basis of these findings, these vesicles were considered to be matrix vesicles and to cause calcification. The fact that cell debris and organelles were distributed in the calcified zone lead us to believe that those matrix vesicles may have been derived from degenerative cellular structures in the granulation tissues. In these calcification sites, unique structures that consist of electron-dense materials sometimes appeared to be

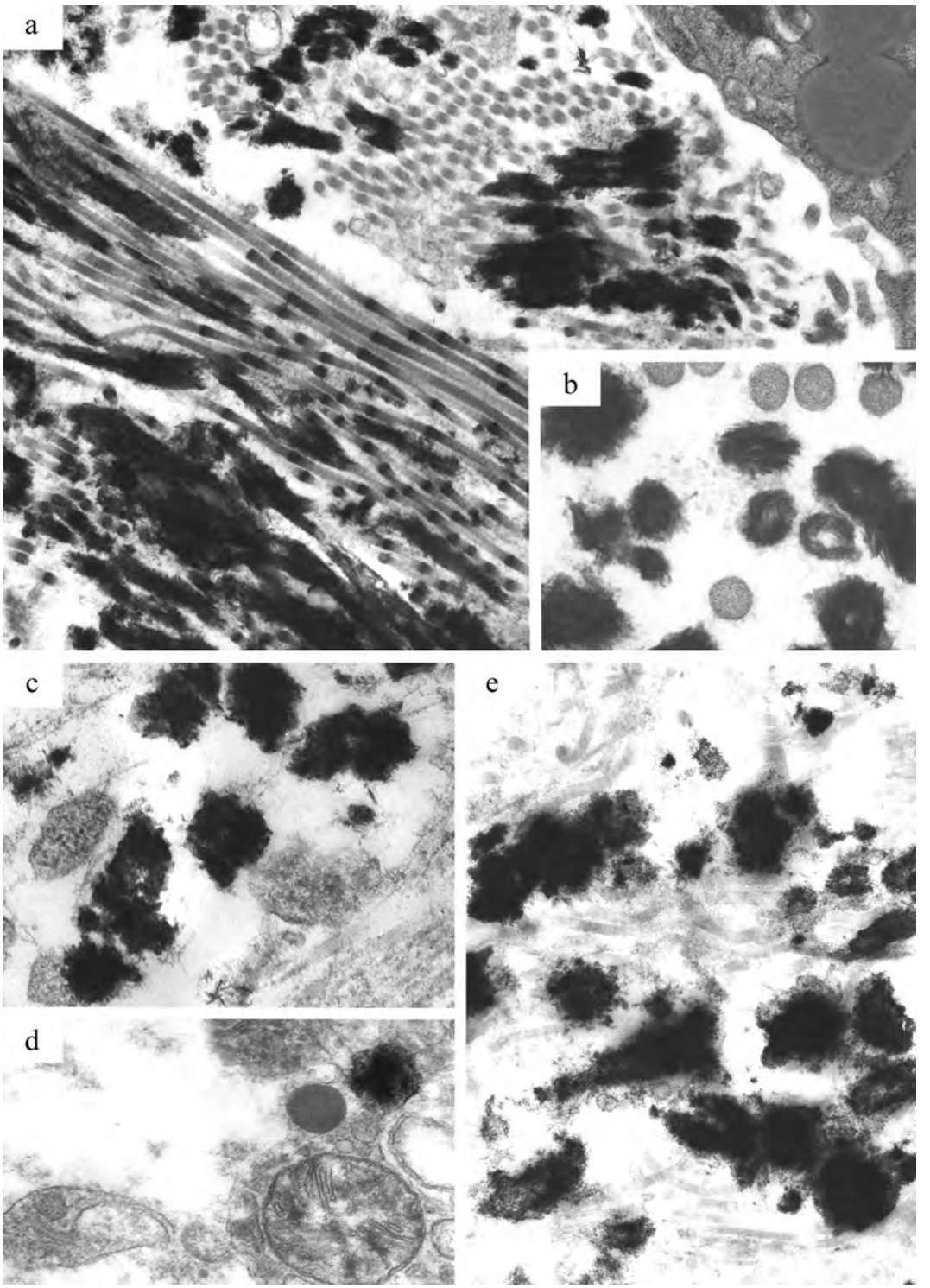

Figure 7 Electron dense crystals appearing on the surface of the collagen fibers (a), a cross sectional view (b), varied vesicles among in collagen fibers (c), mitochondria appearing among the varied vesicles (d), and calcification appearing association with the matrix vesicles (e).

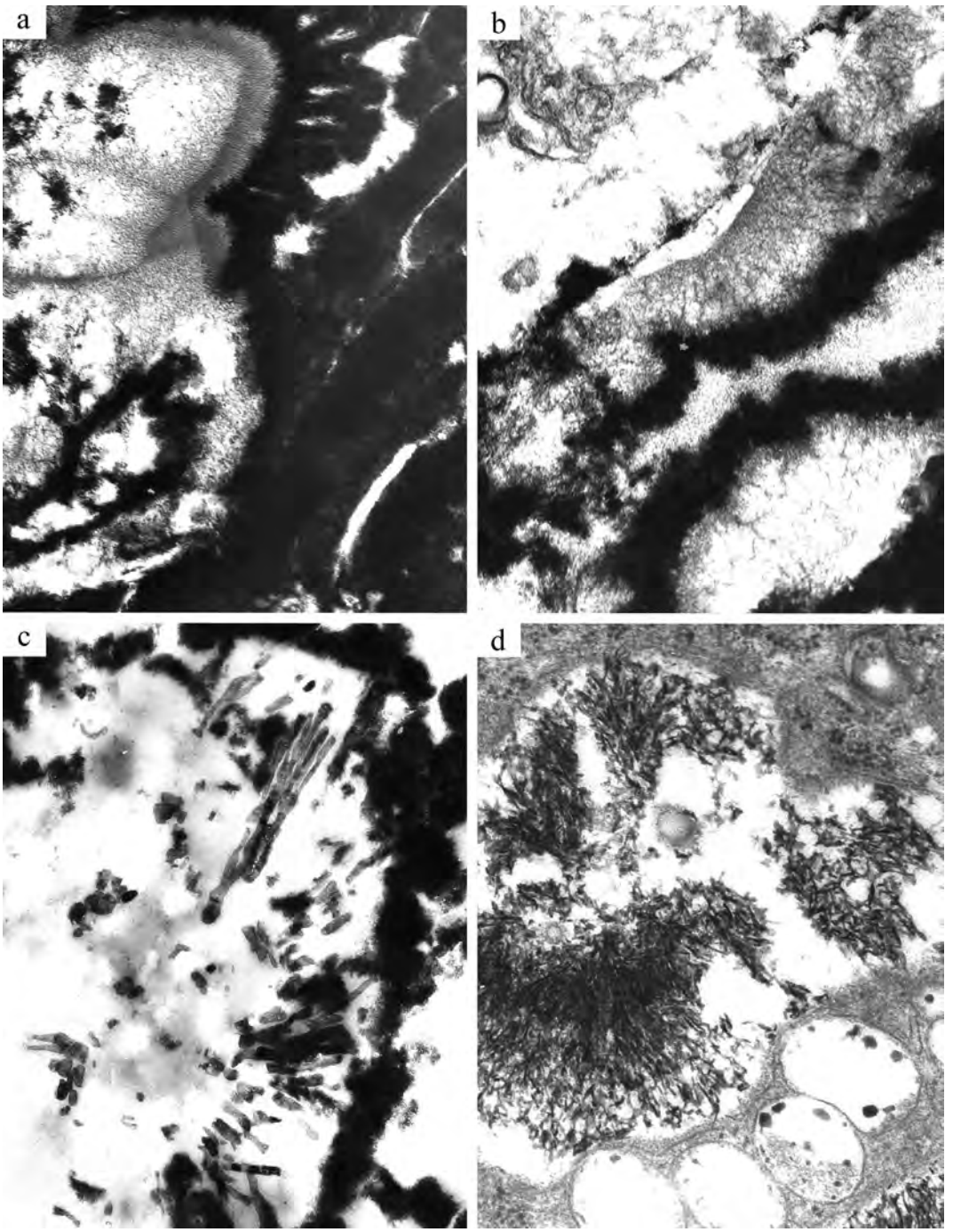

Figure 8 Band-like structure in the calcified zone (a), matrix borders (b), crystals within the band-like structure (c), and crystals forming the granule-body (d).

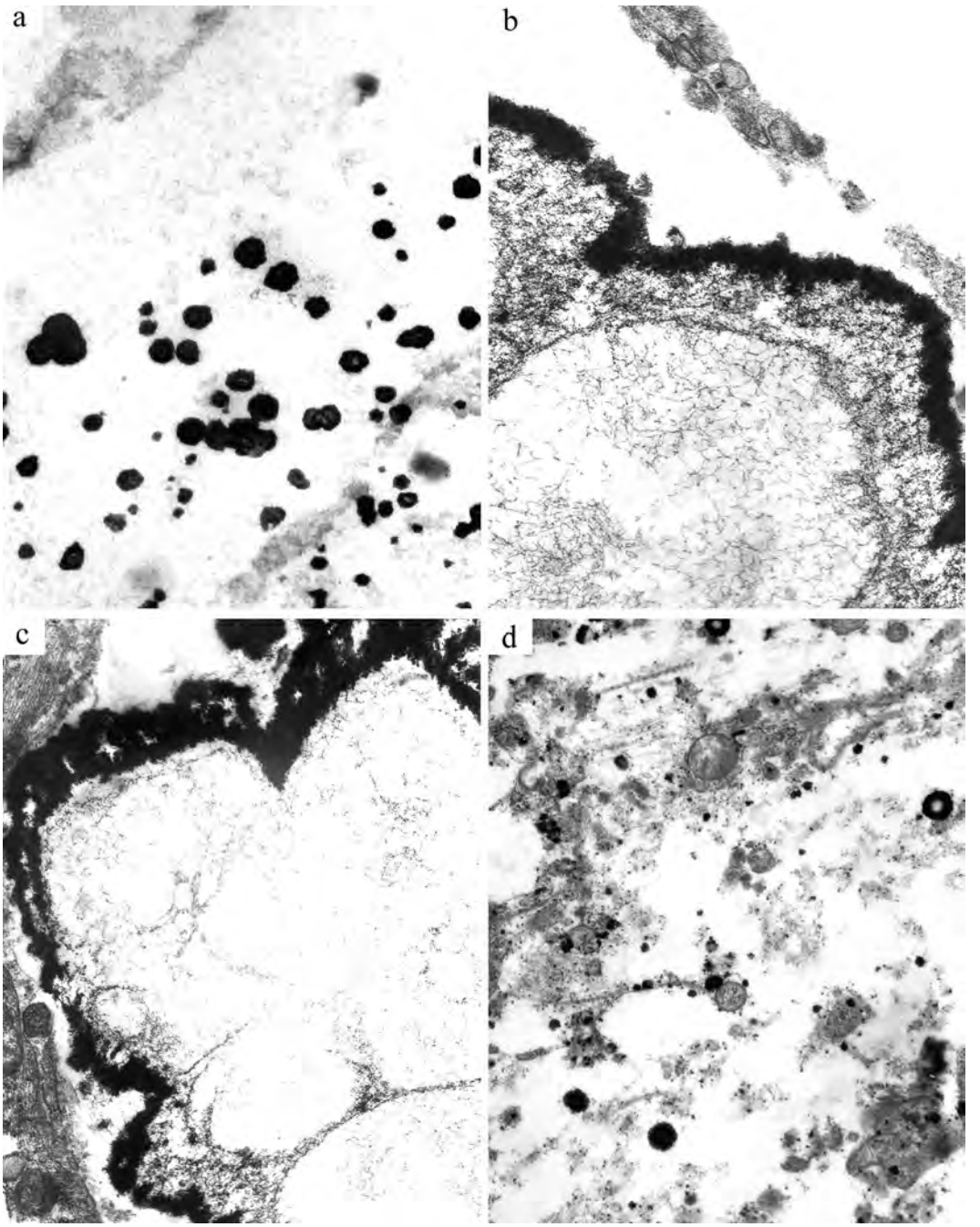

Figure 9 ALP activity appearing in the matrix vesicles (a), ALP spearing in the band-like structure (b, c), and calcium detected in the matrix vesicles (d).

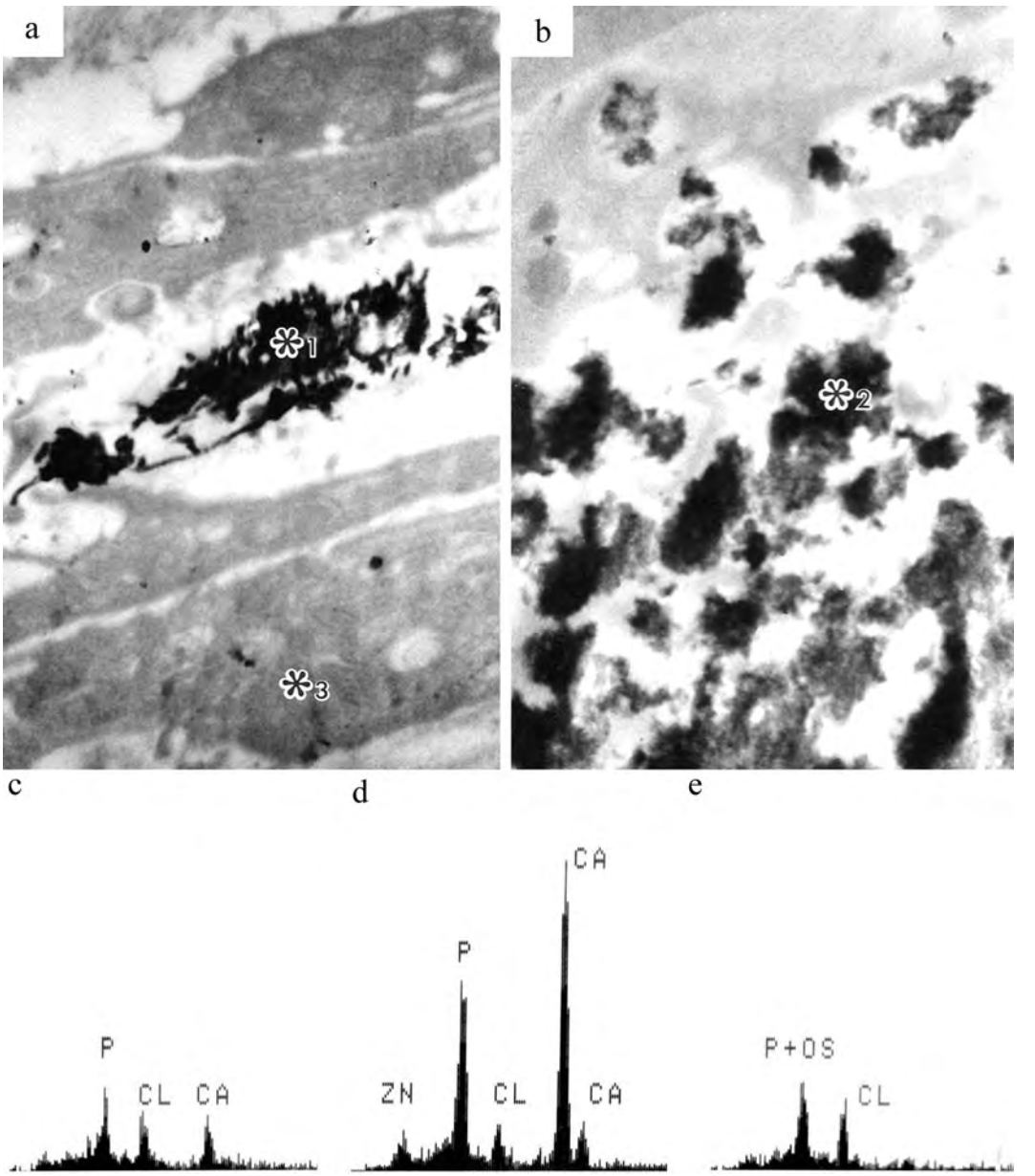

Figure 10 STEM image of dystrophic calcification of attached collagen bundles (a), matrix vesicle calcification area (b), and analysis results of *1 in a (c), *2 in b (d), and *3 in a (e).

surrounded by a membranous structure. These structures were thought to be the lamina limitans [9], which demarcates the zone in which the matrix has been changed by substances generated during the destruction of matrix vesicles. It may, therefore, be considered a buffer zone. Moreover, the appearance of glycosaminoglycans in the matrices of the calcified areas of the granulation tissue was the same as in the early stages of osteogenesis.

Therefore, the root canal filling material Vitapex was recognized to cause matrix vesicle calcification resembling that of bone tissue. The data suggest that this could be an excellent

biologic material with an induction effect leading to differentiation of cells in the periodontal ligament into osteoblasts and/or cementoblasts.

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