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Scanning Electron Microscopy and Energy Dispersive Spectroscopy Analysis of Calciotraumatic Lines in Rat Labial Dentin After Acute Exposure to Strontium Chloride

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SCANNING ELECTRON MICROSCOPY AND ENERGY DISPERSIVE SPECTROSCOPY ANALYSIS OF CALCIOTRAUMATIC LINES IN RAT LABIAL DENTIN AFTER ACUTE EXPOSURE TO STRONTIUM CHLORIDE

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Abstract

Rats were given strontium chloride ($SrCl_2$) intraperitoneally at a dose of 500 mg/kg. The upper incisors were removed after injection of strontium. These incisors were studied by scanning electron microscopy - energy dispersive spectroscopy analysis (SEM-EDS) and light microscopy to examine the calciotraumatic lines of strontium in the rat incisor labial dentin.

At 24 hours after injection of strontium, the calciotraumatic response was observed in the predentin using hematoxylin and eosin (H-E) staining. At 5 days, three layers of calciotraumatic lines were present in the labial dentin using an H-E staining and backscattered electron imaging in the SEM. The external layer consisted of unmineralized dentin, the intermediate layer of relatively unmineralized dentin, and the internal layer of unmineralized dentin. By SEM-EDS analysis, strontium was detected in these layers. The elemental dot map showed that the external and internal unmineralized layers had a low calcium content. The magnesium concentration was higher in the internal unmineralized layer than the external unmineralized layer.

Key Words: Dentin, rat, strontium, calciotraumatic line, scanning electron microscopy, energy dispersive spectroscopy.

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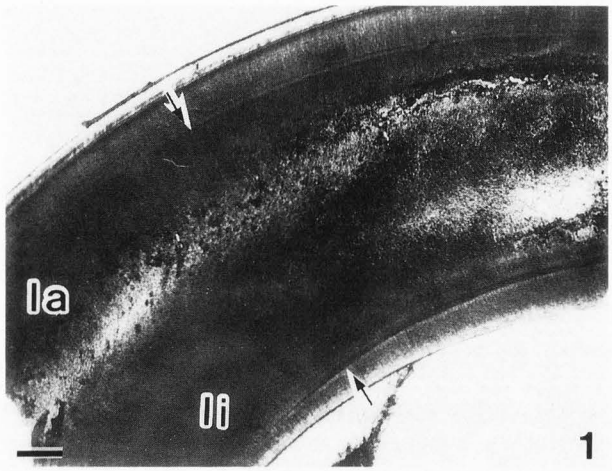
Introduction

Strontium is similar to Ca in its chemical and biological behaviour, and is therefore a suitable tracer to examine the transport of Ca [11]. Sr has a beneficial effect on caries resistance [4]. The acute exposure to the strontium ion induces a calciotraumatic response to the dentin of rat, and the response has been widely discussed [5, 6, 7, 9, 10, 22, 23, 24, 25, 26]. Ogawa *et al.* [18] reported that two hypomineralized layers were consistently identified in the labial dentin, but the lateral, medial and lingual walls revealed only a single hypomineralized layer. Appleton [3] demonstrated that the appearance of the unmineralized layer reflects the differences in structure between labial and lingual dentin. In the rat incisor dentin, differences were found between the lingual and the labial dentin in the inorganic composition and phosphoprotein composition [20, 21], the odontoblast and pulp capillaries morphology [19], and the mineralization front and calcospherite morphology [16]. Mishima *et al.* [17] have reported the presence of differences in the mineralization pattern between the lingual and the labial dentin. The globular calcification occurs in the labial dentin [15]. In this study, the effect of strontium chloride on labial dentin formation was examined by scanning electron microscopy - energy dispersive spectroscopy analysis (SEM-EDS).

Materials and Methods

Thirty male Wistar-strain rats weighing 150-250 g were given strontium chloride ($SrCl_2$) intraperitoneally at the dose of 500 mg/kg. After 6 hours, 12 hours, 24 hours, 2 days, 5 days, 7 days, and 15 days, the upper incisors were extracted and immediately fixed in 10% neutral formaldehyde solution for 24 hours. Decalcified and undecalcifed specimens were then prepared.

The left incisors were decalcified with 10% formic acid-formalin; they were dehydrated and cut at a thickness of approximately 4 μm after being embedded in paraffin wax. The decalcified sections were stained with hematoxylin and eosin (H-E) and silver. They were



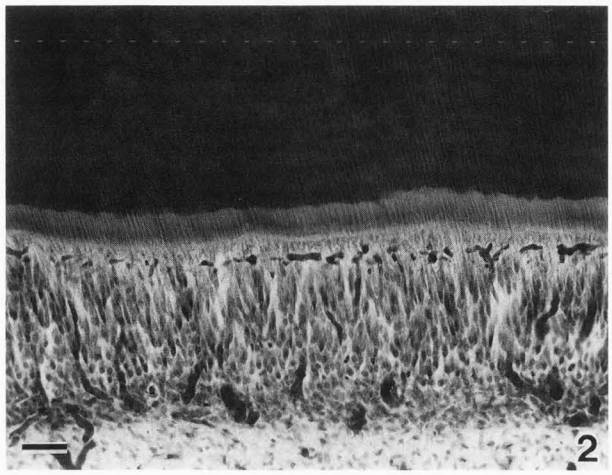


Figure 1. Longitudinal ground section of a rat incisor at 15 days after injection of strontium chloride; la: labial dentin; li: lingual dentin; arrows: calciotraumatic lines. Bar = 400 μm.

Figure 2. Longitudinal decalcified section of labial dentin at 24 hours after injection of strontium chloride. A slightly darker stained layer is observed in the predentin. Hematoxylin and eosin stain. Bar = 40 μm.

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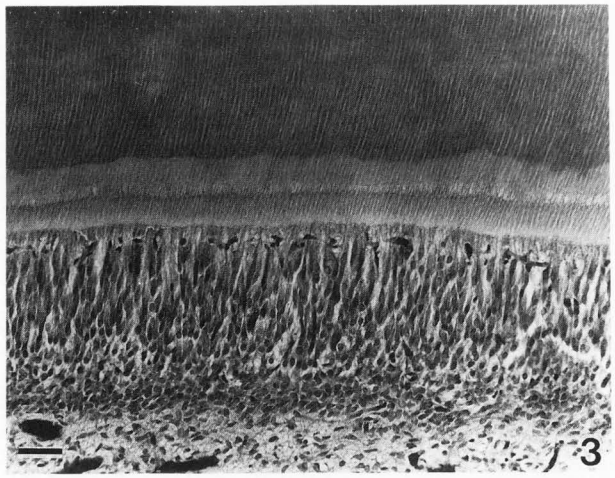
observed by light microscopy.

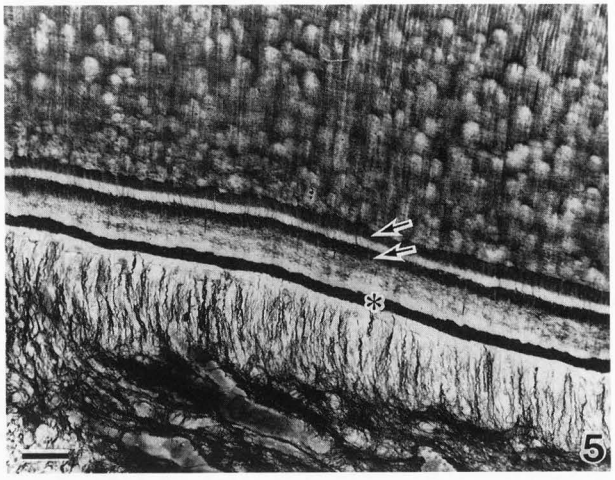
Longitudinal ground sections about 200 μm thick along a plane parallel to longitudinal axes were prepared from right incisors using an Isomet low-speed saw (Buehler Co, Evanston, IL, USA). The slices were ground and polished to prepare 40-80 μm ground sections for study by light microscopy. For scanning electron microscopy (SEM), the incisors were halved parallel to their longitudinal axes using an Isomet low-speed saw. The cut surface was ground with a whetstone initially and then with diamond paste (0.25 μm in particle diameter), after which the specimens were subjected to ultrasonic cleaning for 3 minutes, and washed well with distilled water. The specimens were dehydrated in a series of ethanol, substituted with isoamyl acetate, and subjected to critical-point drying with carbon dioxide. The polished surface was carbon-coated in the ion coater. The polished specimens were examined by scanning electron microscopy and SEM-EDS analysis. SEM observation was carried out by both secondary electron and backscattered electron imaging techniques using the JEOL T-200 and HITACHI S-2500Δ operated at an accelerating voltage of 15 kV and 25 kV. Energy dispersive X-ray microanalysis was carried out by spot mode analysis with a JED-2001 (JEOL, Tokyo) attached to the SEM. The analytical conditions were: accelerating voltage of 15 kV, beam-sample incidence angle of 90°, takeoff angle of 23.5°, and counting time of 100 seconds. X-ray spectra were analyzed using a JEOL sprint program and data were expressed as atomic percents for each element detected. The JEOL sprint program is a standard method. $SrCl_2$, fluorapatite ($Ca_{10}(PO_4)_6F_2$) and gypsum ($CaSO_4$) were used as standard specimens. Elemental dot map of image for phosphorous, calcium, and magnesium was displayed on the calciotraumatic lines.

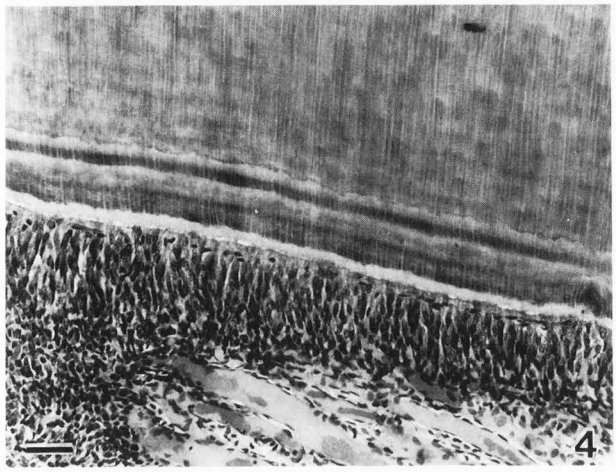
Results

Figure 1 shows the longitudinal ground section of an incisor 15 days after injection of strontium chloride. There were calciotraumatic lines (arrows) in both the labial (la) and lingual dentin (li). In the labial dentin, two dark layers were observed. Towards the root apex, the lingual calciotraumatic lines gradually disappeared. The calciotraumatic lines in the labial dentin were much clearer than those in the lingual dentin. In H-E stain, a slightly darker stained layer was observed in the labial predentin at 24 hours after injection of strontium chloride (Figure 2). Two days after injection of strontium chloride, the pale stained layer and slightly darker layer were observed in the labial dentin (Figure 3). Figure 4 shows the H-E stained section 5 days after the injection of strontium chloride. Three layers of calciotraumatic lines could be observed in the labial dentin. The external first-formed layer was about 10 μm wide and pale stained, the second-formed layer was dark stained and of about the same width and the internal third-formed layer was about 10 μm wide and pale stained. In silver stain, two dark stained layers (arrows) were observed in the labial dentin (Figure 5). These dark stained layers of the silver stain corresponded to the pale stain layer of the H-E stain.

Figure 6 shows a secondary electron image of the labial dentin 5 days after injection of strontium chloride.







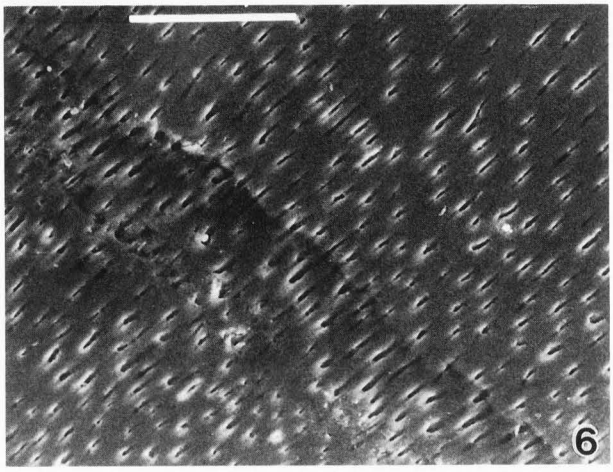


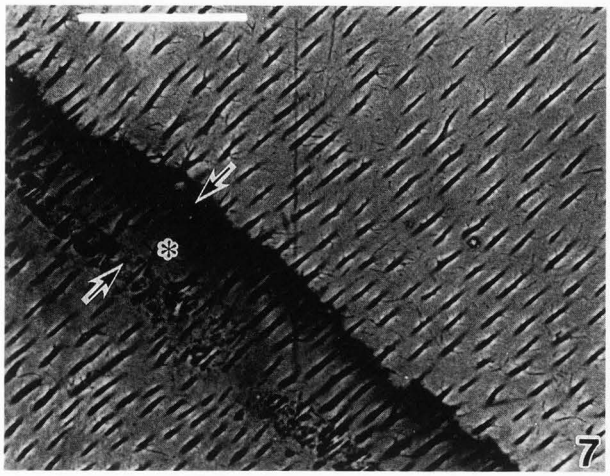
Figure 3. Longitudinal decalcified section of labial dentin at 2 days after injection of strontium chloride. Two layers of calciotraumatic lines are observed in calcified dentin. Hematoxylin and eosin stain. Bar = 40 μm.

Figure 4. Longitudinal decalcified section of labial dentin at 5 days after injection of strontium chloride. Three layers of calciotraumatic lines are observed in calcified dentin. Hematoxylin and eosin stain. Bar = 40 μm.

Figure 5. Longitudinal decalcified section of labial dentin at 5 days after injection of strontium chloride. Two dark stained layers (arrows) of calciotraumatic lines are observed in calcified dentin. Asterisk: predentin. Silver stain. Bar = 40 μm.

Figure 6. Secondary electron image of a ground section of calciotraumatic lines in labial dentin. Bar = 50 μm.

The calciotraumatic lines were observed in the labial dentin. The pre-injection dentin is shown on the right side, and post-injection dentin on the left side. The backscattered electron image (Figure 7) showed two unmineralized layers (arrows) more clearly than the secondary electron image. In the boundary of the external unmineralized layer and pre-injection dentin, small calcospherites were observed. These unmineralized layers correspond to the pale stain layer of the H-E stain. The layer of relatively unmineralized dentin (asterisk) was observed between these unmineralized layers. This layer corresponds to the dark stain of H-E stain. Five different spots in the labial dentin were analyzed by SEM-EDS (Figure 8); one each in the pre-injection dentin (1), external unmineralized layer (2), relatively unmineralized layer (3), internal unmineralized layer (4) and post-injection dentin (5). In the pre-injection dentin, Ca and P were mainly detected by EDS analysis, and Mg was also detected (Figure 9). In the external unmineralized layer, Ca and P were the major elements detected by EDS analysis (Figure 10). However, the Ca and P X-rays may be generated by electrons scattered from the calciotraumatic line into the surrounding mineral and not



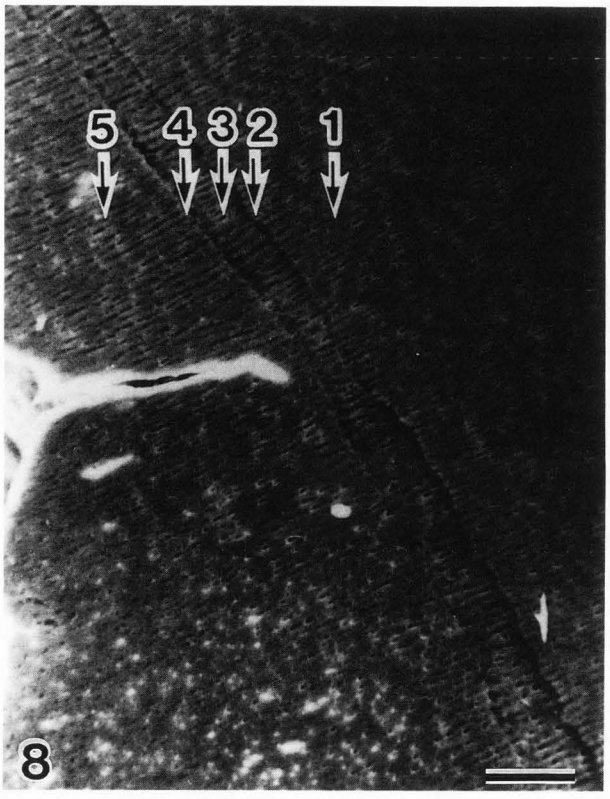
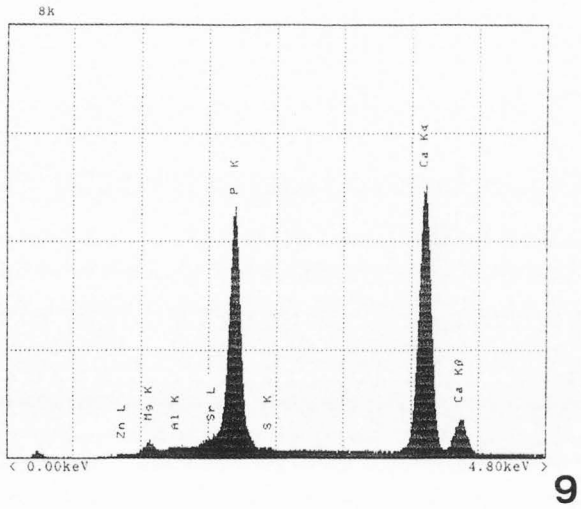


Figure 7. Backscattered electron image of a ground section of calciotraumatic lines in labial dentin. Backscattered electron image shows two unmineralized layers (arrows) more clearly than the secondary electron image. Asterisk: relatively unmineralized layer. Bar = 50 μm.

Figure 8. Scanning electron micrograph of calciotraumatic lines. Five different spots in the labial dentin were analyzed by SEM-EDS. **1**: pre-injection dentin; **2**: external unmineralized layer; **3**: relatively unmineralized layer; **4**: internal unmineralized layer; and **5**: post-injection dentin. Bar = 50 μm.



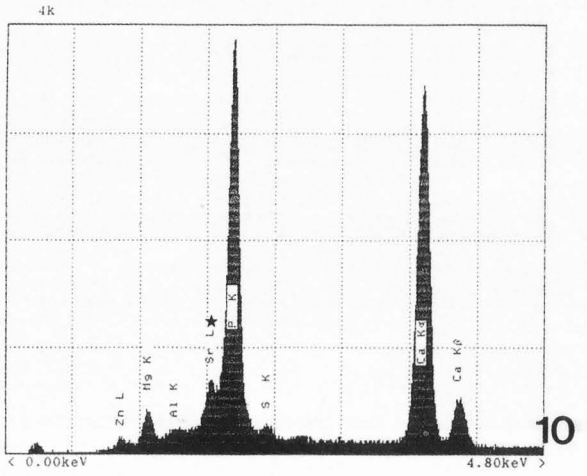


Figure 9. EDS spectrum of the pre-injection dentin.

Figure 10. EDS spectrum of the external hypomineralized layer. Asterisk: Sr.

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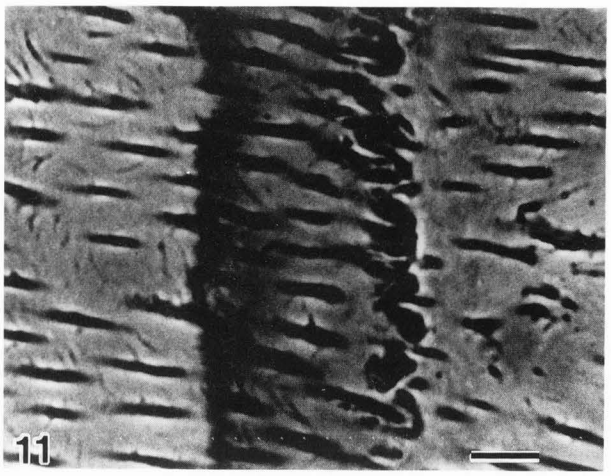
in the calciotraumatic line itself; therefore, we use the term "unmineralized," even though we cannot exclude some mineral present in the calciotraumatic lines. Mg, Al, S, Zn and Sr were also detected in small amounts. The atomic percent of Sr was 2.4. In the relatively unmineralized layer, Ca and P were the major elements detected by EDS analysis, and Mg, S and Sr were also detected (2.3 atomic percent). In the internal unmineralized layer, Ca and P were the major elements detected by EDS analysis, and Mg, Al, S, Zn and Sr were also detected. As compared with the external unmineralized layer, the amount of Sr was small (1.9 atomic percent). Figure 11 shows a backscattered electron image of the calciotraumatic lines, with the pre-injection dentin on the



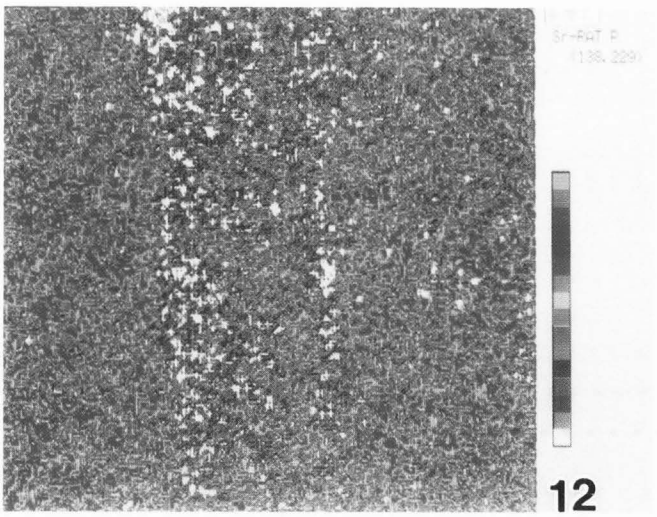
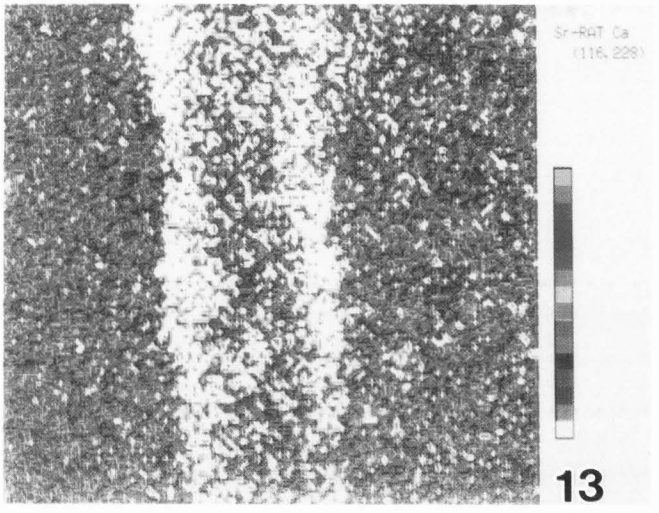
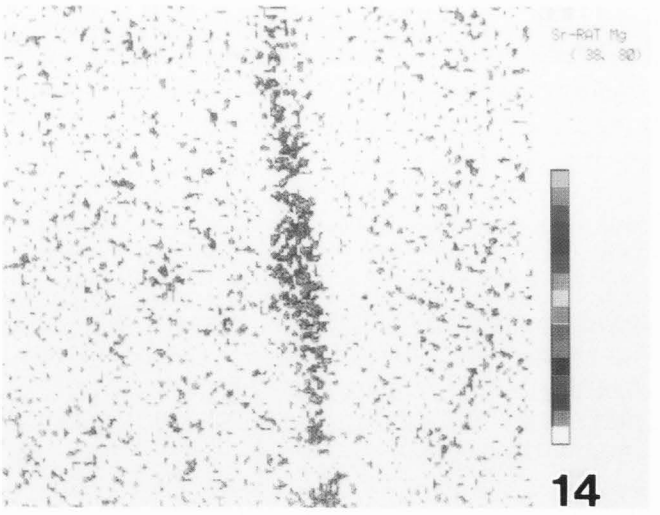


Figure 11 (above). Backscattered electron micrograph of calciotraumatic lines. Pre-injection dentine left. Bar = 10 μm.

Figurea 12-14 (at right) Elemental dot maps showing the distribution of phosphorus (**Fig. 12**), calcium (**Fig. 13**), and magnesium (**Fig. 14**). Linear scale: white = lowest content; and yellow = highest content.





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left side, and post-injection dentin on the right side. In this area, we performed the elemental dot map analysis. Figure 12 shows the elemental dot map showing the distribution of phosphorus. The external and internal unmineralized layers had a low content of phosphorus. The next elemental dot map shows the distribution of calcium in calciotraumatic lines (Figure 13). This elemental dot map of calcium shows a similar pattern as that of phosphorus. However, the calcium content in the relatively unmineralized layer was slightly lower. The external and internal unmineralized layers clearly had low calcium content. The last elemental dot map shows the distribution of magnesium (Figure 14). The Mg and P contents were higher in the internal unmineralized layer than in the external unmineralized layer (Figs. 12 and 14).

Discussion

At the 24th hour after injection of strontium, the calciotraumatic response was observed in the predentin using hematoxylin and eosin (H-E) staining. A few days after injection of strontium, three layers of calciotraumatic lines were present in the labial dentin using H-E staining and backscattered electron imaging in the SEM. The external layer consisted of unmineralized dentin, the intermediate layer of relatively unmineralized dentin and

the internal layer of unmineralized dentin. This relatively unmineralized layer has lower calcification than normal dentin. The layer matched the dark stain of H-E stain. This may explain why the density of hematoxylin is not parallel with the degree of calcification. We did not observe the hypermineralized dentin reported by Irving and Weinmann [10]. We confirmed the presence of an indistinct band of relatively unmineralized dentin within the unmineralized layer [3].

Strontium was detected in these unmineralized layers using SEM-EDS analysis. Aoba [1] reported that Sr^{2+} occupies the Ca^{2+} position in the hydroxyapatite. Sr is easily incorporated into the apatite [13]. Marie *et al.* [14] and Grynpas and Marie [8] showed that Sr does replace Ca in the bone mineral apatite. Sr ions have been suggested as substitutes for Ca ions in the dentin apatite crystal. LeGeros [13] demonstrated that Sr substitution in the apatite caused no significant difference in crystallinity but some loss of resolution in the absorption bands of the PO_4 groups in the infra-red (IR) spectra. Sr-substituted apatite was shown to be more soluble than Sr-free apatite [13]. Hirayama [9] reported that the size of the dentin apatite crystals in the hypomineralized dentin was smaller than that of the normal dentin apatite using X-ray diffraction analysis. The elemental dot map shows that the external and internal unmineralized layers had a low content of calcium. This suggests that Sr may be reflected in the mineral deposition of dentin. The magnesium content was higher in the internal unmineralized layer as compared to the external unmineralized layer. Mg may play an important role in the formation of the internal unmineralized layers [12].

Krefting *et al.* [11] showed that the uptake of Sr into the blood occurred rapidly after injection of Sr. The Sr/Ca ratio reached its maximum after 10 minutes in the serum and after 20 minutes in the extracellular space of the growth plate cartilage in the rat tibia [11]. We estimated that Sr reached the odontoblast within a relatively short time after Sr injection and then rapidly damaged the odontoblast. Grady and Yaeger [7] demonstrated differences in collagen fiber orientation in the hypomineralized areas. Ogawa *et al.* [18] reported that the internal hypomineralized layer showed a different texture from the external layer; sparse collagen fibrils seemed to contain substantial minerals; granules stained with ruthenium red among collagen fibrils seemed to represent glycosaminoglycans or proteoglycans. Appleton [2, 3] suggested that Sr affects the organic matrix so that the nucleating centre is modified, thereby inhibiting crystallite deposition. Grynpas and Marie [8] suggested that Sr has an effect on the proteoglycans. The presence of proteoglycans included in the dentin matrix may be one of the factors controlling the growth of the dentin apatite. The glomerulus structure in the kidney was injured by Sr injection (Mishima, unpublished data). This may cause a decrease in the serum calcium concentration. We suggest that the external unmineralized layer arises from the odontoblast directly damaged by Sr, and the internal unmineralized layer arises from the obstruction of calcium metabolism in the body.

Acknowledgments

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Discussion with Reviewers

E.-R. Krefting: In calcium-apatite, only up to 10% of calcium content can be exchanged for strontium (Bang and Baud, 1972). May this be an additional reason for the hypomineralization in your experiment as you use relatively high Sr does?

Authors: Yes, we think that this may be an additional reason for the hypomineralization. Sr may be reflected in the mineral and organic matrix deposition of dentin.

E.-R. Krefting: In Figure 13, in the hypomineralized calciotraumatic lines, the calcium content is often very low (white), but there are some yellow spots indicating the highest Ca-content possible. May these spots reflect the sparse collagen fibrils containing substantial mineral you mention in the discussion or is there another reason for this finding?

Authors: Yes, these spots may reflect the sparse collagen fibrils containing substantial mineral, or may correspond to the small calcospherites in the unmineralized dentin.

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