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Original

Aspects Regarding Fluoride Treatment for Reinforcement and Remineralization of Apatite Crystals

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Abstract: The purpose of the present study was to investigate whether fluoride (F) ions are really capable of the repair (remineralization) of damaged crystals and useful for reinforcing the quality (i.e. modify the crystal structure) of tooth enamel using transmission electron microscopy and Raman microprobe analysis. Additionally, carbonic anhydrase activity was measured in immature enamel tissue to compare the harmfulness of F ions to that of cadmium (Cd) ions during the process of crystal nucleation by means of differential gas pressure analysis. Electron micrographs indicated no signs of remineralization of artificially damaged crystals after incubation in a remineralizing solution and further revealed that treatment with acidulated phosphate fluoride (APF) gel caused crystal dissolution rather than crystal improvement. Regarding crystal structure modification, Raman microprobe analysis revealed that no up-shift of PO_4^{3-} ν_1 band assigned to human sound enamel crystals occurred when APF gel was used. Furthermore, fluorapatite crystals were not generated by daily intake of F ions in developing rat tooth enamel. A differential gas pressure method demonstrated that the harmfulness of F exposure during the nucleation process of calcified hard tissues was much greater than that of Cd exposure. These results demonstrate that F treatments have no effect on improving crystal quality or remineralization and are inconsistent with the purpose of public health.

Keywords: Crystal modification, Electron microscopy, Fluoridation, Raman microprobe analysis, Remineralization.

Introduction

Recently, we demonstrated that fluoride (F) intake caused crystal structure defects in calcified hard tissues[1]. The World Health Organization, however, still supports F delivery programs such as water fluoridation, salt fluoridation, and milk fluoridation in an effort to protect against dental caries, although these methods have ambiguous beneficial effects[2, 3]. The repair of carious lesions by F treatment, in particular, is one such ambiguous effect, and many researchers have focused on the beneficial effect of F treatment on this event, suggesting that the demineralized enamel crystals may be restored. Despite a great deal of research, however, the experimental procedures employed by many research groups have failed to generate convincing evidence regarding whether damaged crystals are truly restored by immersion in a purported remineralizing solution [4-12].

F ions are generally thought to be capable of transforming hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, into acid-resistant fluorapatite, $Ca_{10}(PO_4)_6F_2$. Furthermore, the F agent acidulated phosphate fluoride (APF) gel has been thought to cause minimal crystal dissolution and to exchange of OH with F ions under low pH condition [13-19]. Based on these conceptions, F schemes have been believed to improve the quality of tooth enamel for the past several decades [13]. Additionally, F intake has been considered to affect bone crystal structure and change bone mineral content during mineralization, leading to more acid resistant fluorapatite crystals to bone resorption. Because of this belief, until recently NaF was used to treat osteoporosis [20-26]. To address these controversial issues, we designed the present study to directly verify whether the damaged crystals were actually repaired and whether the replacement of OH with F ions occurs. The present study also aimed to clarify whether daily intake of F ions produce fluorapatite in calcified hard tissues.

Exposure to Cd ions also caused crystal defects similar to those resulting from F exposure[1, 27]. In particular, Cd exposure is well known as a risk factor for developing itai-itai disease accompanied by osteomalacia and osteoporosis [28]. This similarity of crystal structure defects led us to speculate that daily exposure to F ions may be a latent risk factor for bone disease[1] because the

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mechanism of crystal formation between enamel, dentin and bone is basically the same [29, 30]. Despite some studies postulating a relationship between F in drinking water and an increased risk of bone fractures[31-33], many people remain unaware of the bone damage posed by F exposure. Considering this possibility, we also conducted experiments to investigate the harmfulness of F exposure during hard tissue formation compared to that of Cd. To assess the harmfulness of unwanted chemicals on calcified hard tissues, we examined carbonic anhydrase activity in developing rat tooth enamel because this enzyme, not alkaline phosphatase, plays a key role in initiating the nucleation process for central dark line formation[1, 27-30, 34-38].

Materials and methods

Materials

To compare crystal structures between sound, carious and fluorosed human tooth enamel, samples were embedded in Araldite 502 resin without fixation and subjected to electron microscopy.

To assess the effect of APF treatment on crystal structure, human sound tooth enamel was subjected to electron microscopy and Raman microprobe analysis. Shark (*Prinace glauca*) tooth enameloid was used as a reference for fluorapatite.

The use of human teeth was approved by the Ethics Committee of our institution.

Male Sprague-Dawley rats were used to clarify whether daily intake of F was able to produce fluorapatite during the development of tooth enamel. The use of animals was approved by the Animal Care and Use Committee of Meikai University.

A commercially available APF gel that contained approximately 2% F in the form of NaF (about 9000 ppm as F ions) was purchased (Bee Brand Medico Dental Co. LTD.).

Electron microscopy of remineralization and APF treatment

Procedures were carried out directly on thin sections of enamel crystals mounted on collodion-coated platinum grids to assess the effectiveness of F ions for remineralization of damaged enamel crystals. The pieces of sound tooth enamel were embedded in Araldite 502 resin without fixation. Thin sections were obtained using a Porter-Blum MT2-B ultramicrotome (Sorvall Inc. U.S.A.) equipped with a diamond knife. The sections were then mounted on collodion-coated platinum grids. Partial demineralization of crystals on grids was carried out by immersion in an acetate buffer solution (pH 4.0 to 4.6) for approximately 10 to 30 s depending on the color reflections of the sections. After rinsing with distilled water, sections were incubated in a remineralizing solution for 2 weeks at 37ºC as described [4, 5].

Enamel tissue embedded on resin blocks was used to determine the effect of APF gel on tooth. Thin sections before and after APF treatment were obtained from the same block. Thin sections without APF treatment were obtained in advance. Then the cutting plane with exposed enamel tissue was placed in APF gel for 30 min or more. The block was then rinsed lightly with distilled water and dried at room temperature. In an additional method to assess the effectiveness of APF, thin sections of enamel crystals were treated with APF gel for 30 s on collodion-coated platinum grids. They were then rinsed and dried at room temperature. These specimens were examined under a JEM 100CX transmission electron microscope (Jeol Ltd. Tokyo, Japan) at an accelerating voltage of 80 kV.

Raman microprobe analysis

Human tooth enamel and shark enameloid were sliced using an Isomet precision saw (Buehler Ltd. USA). The sliced human tooth samples were placed in APF gel for 30 s to 1 week. They were then rinsed lightly with distilled water and dried at room temperature. In an animal experiment, 3-week-old rats were used to clarify whether daily intake of F was capable to produce fluorapatite during the development of tooth enamel. The experimental animals were given free access to drinking water containing 2 mg/L fluoride ions (NaF) for 6 months. After obtaining thick sections of incisor enamel tissue, the immature enamel was subjected to analysis. Each sample was analyzed using a laser Raman microprobe spectrometer (HoloLab5000R, Kaiser Optical Systems Inc). The laser beam was focused onto a sample with a spot size of 1 μm through a microscope objective. The scanning range analyzed in this study comprised the PO_4^{3-} ν_1 band ranging from 800 to 1200 cm^{-1} because crystal conversion is easily evaluated by the PO_4^{3-} ν_1 band up-shift.

Differential gas pressure method

To compare the harmfulness of F and Cd exposures during the crystal nucleation process, rats were fed water containing either F (2 ppm) or Cd (20, 40, 100 ppm) ions. Immature enamel tissues collected from 8-week-old rat incisors were prepared for analysis of enzymatic activity. After removing the adhering blood and surrounding soft tissues, the incisors were briefly rinsed with cold saline solution. The immature developing enamel tissues were scraped from the incisors with razor blades. Enzymatic activity was measured at a differential gas pressure [39]. Each sample of immature enamel tissue was lyophilized, pulverized, and then suspended in distilled water. A 0.1-mL aliquot of the suspension containing 1.0 mg enamel powder was tested for enzymatic activity using a Warburg flask. One milliliter of 0.2 M phosphate buffer (pH 6.8) was introduced into the main chamber, and 1.0 mL of 0.1 M sodium hydrogen carbonate substrate solution was introduced into a side arm. To the experimental main chamber, 0.1 mL of tissue suspension was added, while the same volume of distilled water was added to the blank chamber. Flasks were kept on crushed ice for 5 min to stabilize the internal pressure of both experimental and blank chambers. During this process, the valve

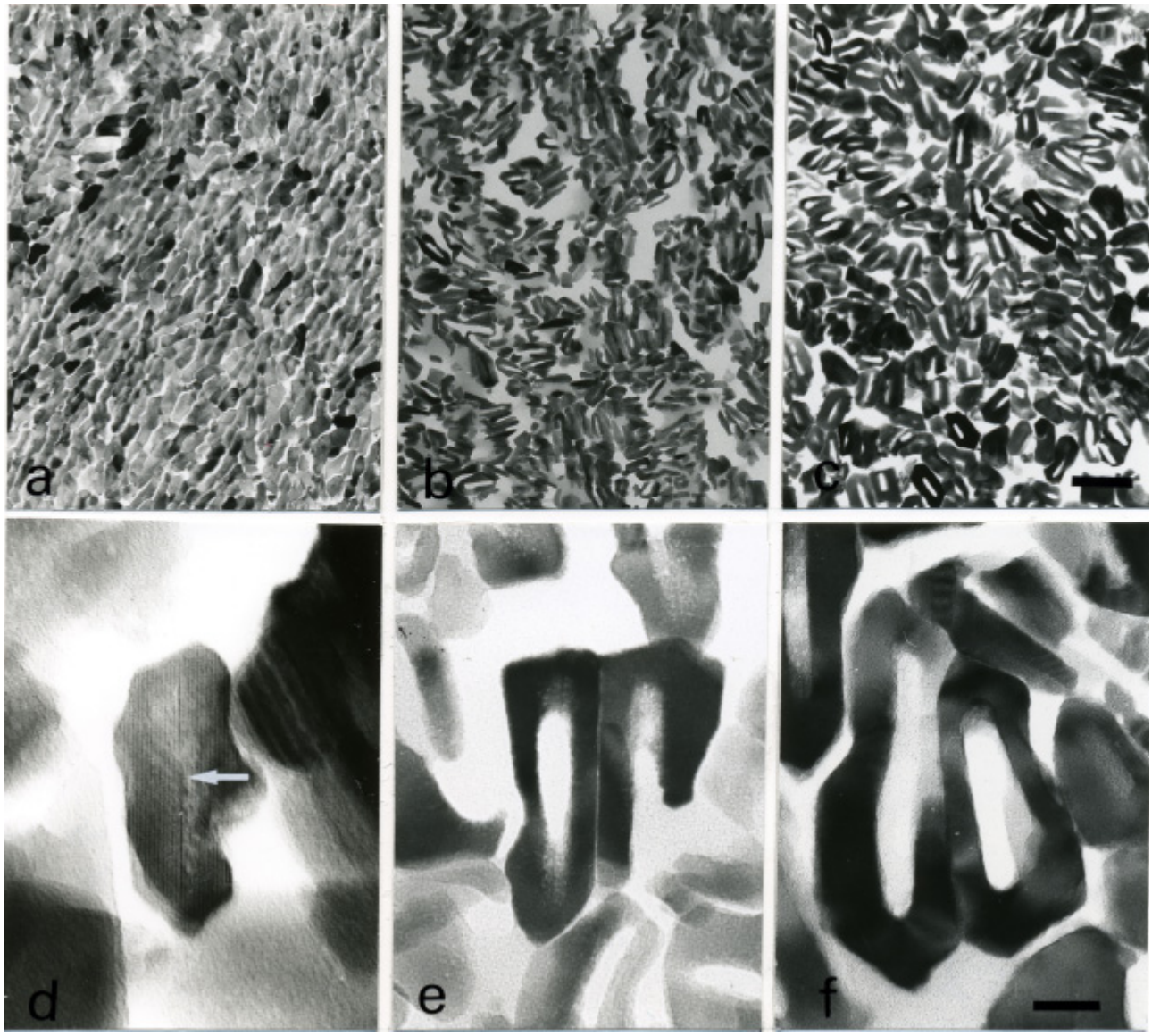

Figure 1: Electron micrographs of cross sections of sound (a and d), carious (b and e) and fluorosed human dental enamels (c and f), and higher magnification of the respective crystals (d–f). The centers of crystals of both fluorosed enamel and carious lesions show less acid resistance than the peripheral area. Arrow indicates the central dark line. a–c, scale bar = 160 nm; d–f, scale bar = 20 nm.

was kept open to equalize the internal and external pressure. Subsequently, the solutions were mixed and shaken. The valve was then immediately closed, and the enzymatic activity was recorded and processed by a computer. The gas pressures (Pa) generated by carbonic anhydrase activity over time were recorded every 30 s, for 3 min, from a starting point of 0 pascal (Pa). Values are expressed as the mean ± standard deviation (S.D.) of 4–6 experiments. Acetazolamide (2×10^{-5} M concentration in 0.2 M phosphate buffer solution) was used for the inhibitory test.

Results

Electron micrographs of sound, carious, and fluorosed human tooth enamel

Regarding the detailed crystal structures observed in this study, electron micrographs demonstrated central dark lines (CDLs) in the structures of sound human enamel samples. By contrast, crystals in fluorosed human dental enamel demonstrated the absence of CDLs, leaving void spaces in the centers of their structures and similar to those of carious lesions (Fig. 1).

Electron microscopy of remineralization and APF effect

The centers of thin sections of sound enamel crystals (Fig. 2a) on the platinum grids demonstrated dissolution and resembled those of carious lesions after immersion in an acetate buffer solution for about 10 to 30 s (Fig. 2b). Incubation in a

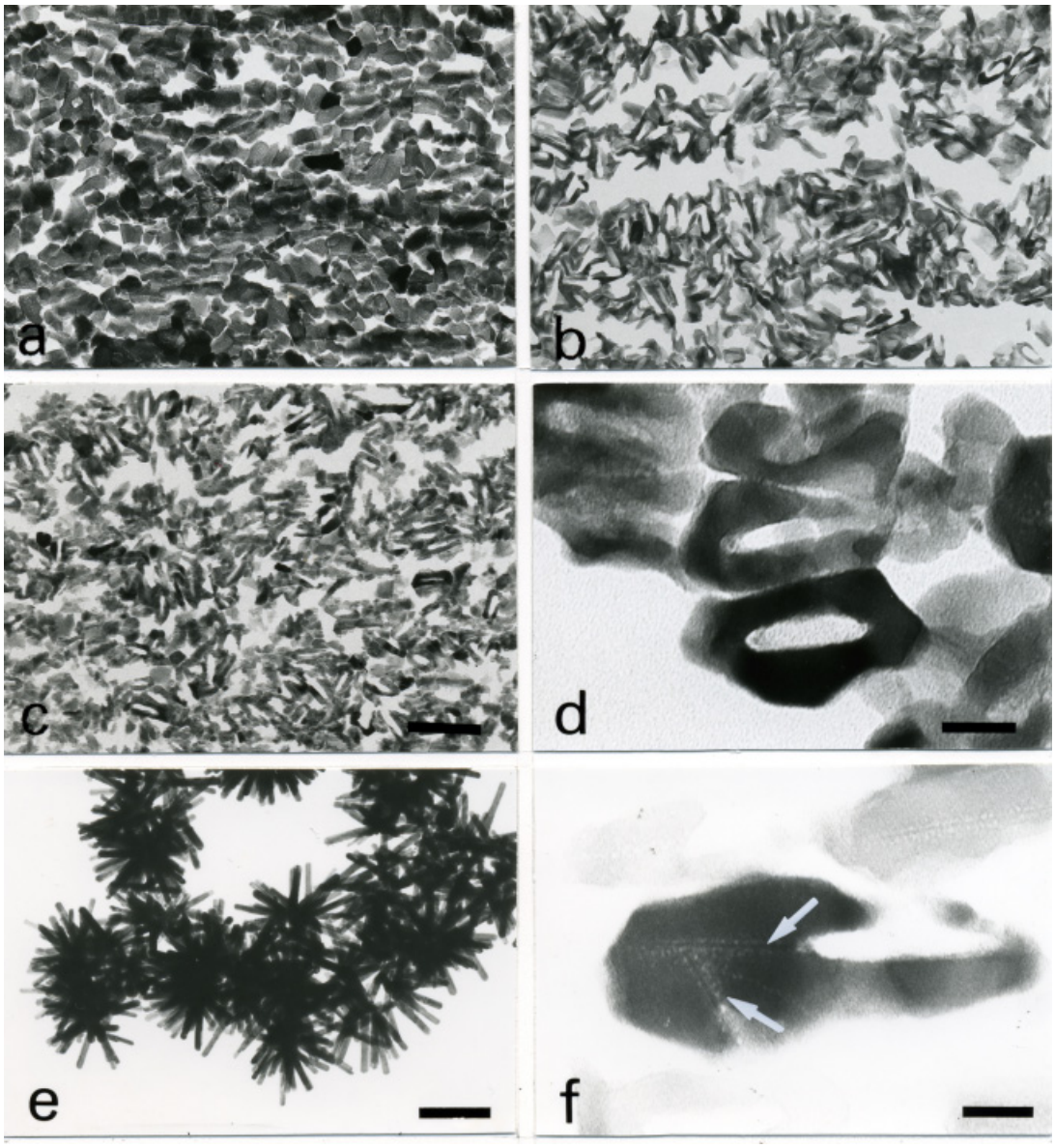

Figure 2: Electron micrographs of crystals before and after incubation with a remineralization solution. There is no evidence that remineralization took place. Sound enamel crystals (a), artificially demineralized enamel crystals (b). Low (c) and higher (d) magnifications of demineralized crystals after immersion in a remineralizing solution. Newly formed deposits after immersion in a remineralization solution (e). A unique crystal showing 2 central dark lines in its structure without immersing in a remineralization solution (f). a–c, scale bar = 200 nm; e, scale bar = 1 μm; d and f, scale bar = 20 nm.

remineralizing solution for 2 weeks had no observable effect on repairing the damaged crystals on electron micrographs (Figs. 2c and d). Instead, irregular long and hexagonal-shaped deposits were observed on the collodion membrane (Fig. 2e). These newly formed deposits also appeared on the damaged crystals (data not shown). Thus, these newly formed deposits were clearly irrelevant to crystal repair. As a supplemental finding, we observed unique crystal with 2 CDLs intersecting at a 60-degree angle among demineralized crystals without incubation in remineralizing solution (Fig. 2f).

Regarding the effectiveness of APF gel on the thin enamel sections embedded in resin blocks, electron micrographs clearly demonstrated that APF gel treatment caused only crystal damage and no restoration events were observed regardless of treatment duration (Fig. 3). In addition, less than 30-s exposure was sufficient to dissolve the crystals, which were about 100 Å in thickness (data not shown).

Raman microprobe analysis

The results of comparing the Raman spectrum of the PO_4^{3-} ν_1 band across samples are compiled in Table I. Raman microprobe analysis revealed that the PO_4^{3-} ν_1 band assigned to human sound enamel crystals was positioned at 959.5 ± 0.1 cm^{-1}. After F treatment, however, the value of enamel crystals PO_4^{3-} ν_1 bands

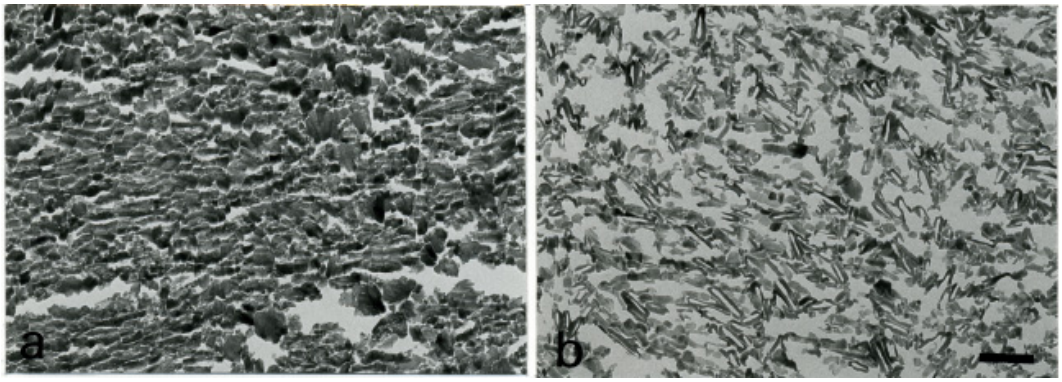

Figure 3: Electron micrographs of crystals before (a) and after (b) APF gel treatment. APF treatment caused only crystal damage. a and b, scale bar = 300 nm.

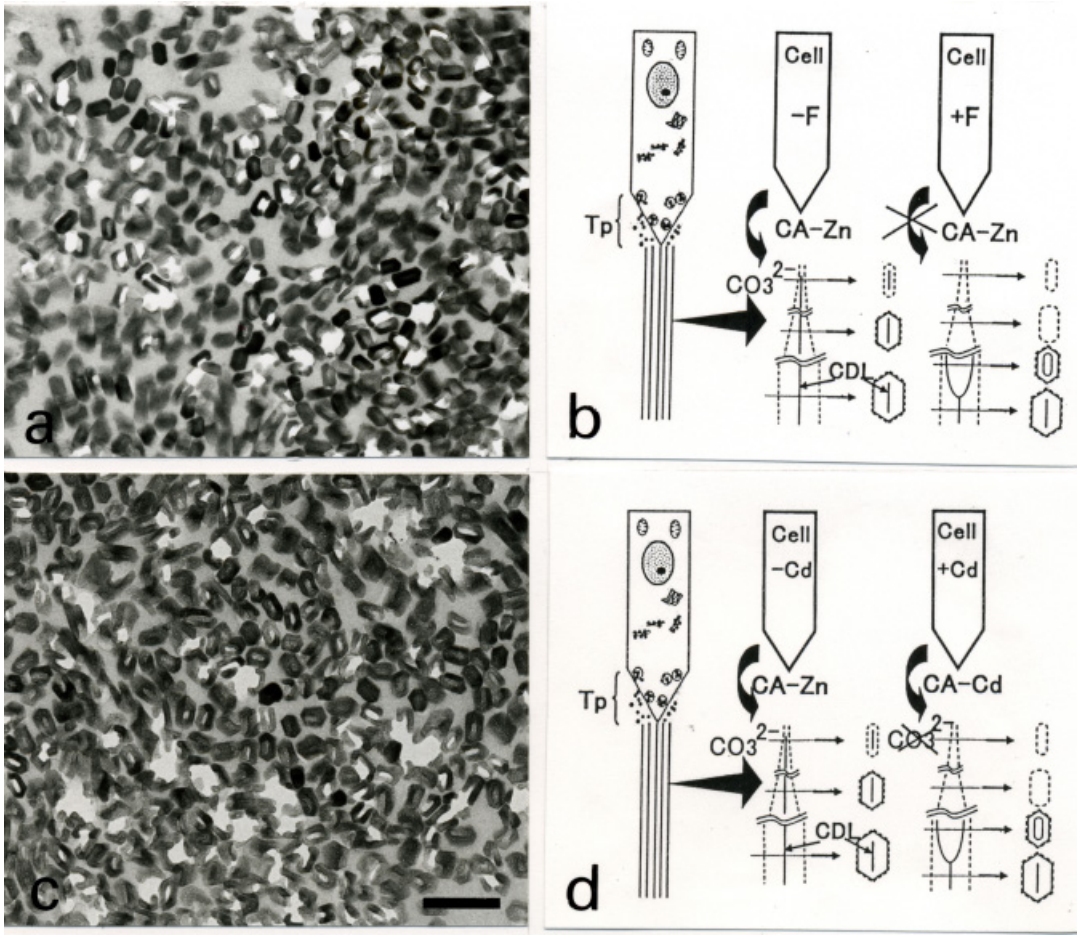

Figure 4: Crystal structural defects caused by F or Cd exposures and schematic diagrams of their mechanisms. Each ribbon-shaped crystal (lines) is created beneath an ameloblast (cell) Tomes' process (Tp). Both F and Cd ions interrupt the supply of carbonate ions needed to initiate central dark line formation. Left side: Electron micrographs of enamel crystals exposed to F (a) or Cd (c) ions. Right side: Schemes for the mechanisms of crystal structural defects caused by F (b) or Cd (d) exposure. CA: carbonic anhydrase. a and b, scale bar = 100 nm.

did not up-shift to that of fluorapatite crystals regardless of treatment duration. An animal experiment was conducted to determine whether F ions arising from daily F uptake are involved in the formation of fluorapatite. In control animals, the value of the PO_4^{3-} ν_1 band assigned to apatite crystals was 960.7 ± 0.4 cm^{-1}, and no up-shift of PO_4^{3-} ν_1 band was detected in the experimental animals given water containing fluoride for a period of at least 6 months.

Comparison of enzymatic activity between F- and Cd-exposed enamel samples

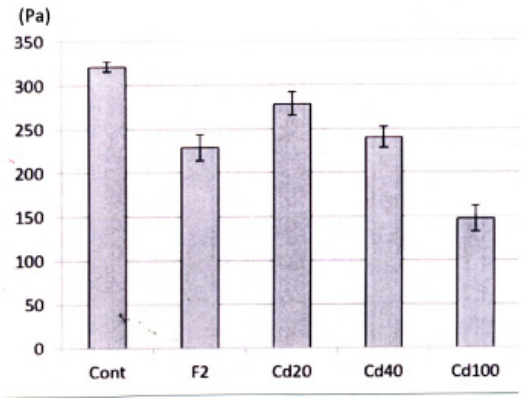

Figure 5: Comparative study of enzymatic activity in immature enamel tissue affected by F or Cd exposures. The harmfulness of 2 ppm F is commensurate with that of 40 ppm Cd ions. The unit of vertical axis is expressed as pascal (Pa). Cont: control, F2: 2 ppm F, Cd20: 20 ppm Cd, Cd40: 40 ppm Cd, and Cd100: 100 ppm Cd.

As shown in Fig. 4, both F and Cd exposure caused similar crystal structure defects in developing rat tooth enamel. Therefore, we conducted a comparative study of carbonic anhydrase catalytic activity between F- and Cd-exposed developing rat tooth enamel. Differential gas pressure analysis revealed the harmful effects of 2 ppm F on the crystal nucleation process to be approximately equivalent to 40 ppm Cd, showing a 20-fold greater effect on enzyme activity than that of Cd (Fig. 5). The enzymatic activity was also inhibited by the presence of acetazolamide at a concentration of 2×10^{-5} M in 0.2 M phosphate buffer (data not shown)

Discussion

Many dental researchers and organizations still advocate F schemes as an effective strategy to prevent tooth decay. Fluorosed enamel crystals are said to be more acid resistant than sound crystals. As shown in Fig. 1e, the central area of crystals clearly demonstrated less acid resistance than the peripheral area viewed from a carious lesion. Similarly, the fluorosed enamel crystals demonstrated voids in the centers of their crystals (Fig. 1f). Therefore, increased acid resistance with the dental fluorosis caused by F exposure may be merely a superficial phenomenon, despite the widely held belief that F use has beneficial effects on caries prevention if the amount of F ions is kept below a certain level. Even as many researchers endeavor to determine the optimal F intake level, our previous and present findings raise doubt about F schemes for caries prevention from a scientific viewpoint [1)], indicating that there is no safe level of fluoride.

The mechanism of crystal formation is crucial to understanding how crystal structure defects occur. Our previous findings indicate that each crystal develops within the organic envelope structure [1, 27, 29, 30, 35, 38)], which consists of an inner mineral zone, comprised of calcium, phosphate and magnesium ions, and a surrounding

Table 1. The value of PO_4^{3-} v1 bands analyzed by laser Raman microscope from tooth enamel before and after fluoridation, shark tooth enamel, and developing rat incisor enamel with and without F intake.

Samples	PO_4^{3-} ν_1 value
Human tooth enamel before fluoridation	959.5 ± 0.1 cm^{-1}
Human tooth enamel after 1 week fluoridation	959.5 ± 0.1 cm^{-1}
Shark tooth enamel	963.2 ± 0.2 cm^{-1}
Developing rat incisor enamel	
Control	960.7 ± 0.4 cm^{-1}
F intake	960.7 ± 0.2 cm^{-1}

Values are mean ± standard deviation (S.D.) of 5 experiments.

thin outer organic layer [1, 27, 29, 30, 37-38)]. Magnesium, ions are thought to inhibit the mineralization process [40)]. At the initial stage of crystal development, carbonate ions supplied by carbonic anhydrase initiate crystal nucleation by binding to magnesium (Mg) ions [1, 27, 37, 38)], resulting in the formation of the Mg-CO_3 compound huntite, $CaMg_3(CO_3)_4$ [41)]. This event may promote development of the first lattice line by reactivated calcium and phosphate ions. Then the first lattice line along with the Mg-CO_3 compound may create the CDL, which is the nucleation site of apatite crystals, followed by crystal growth. Eventually, each crystal coated by the thin organic layer possesses a CDL in its structure. If the supply of carbonate ions is insufficient due to exposure to unwanted chemicals [1, 27)], Mg ions may retain their inhibitory effect at the central area of the crystal. However, the peripheral area escapes the influence of Mg ions and can grow continuously from the already formed crystal surface. Eventually, this process may help to create the voids in the centers of enamel crystals [1, 27)].

Calcium and phosphate ions are thought to move in and out of the crystal structure in the oral environment, and if such ionic reversible behaviors really occur, their chemical reaction could contribute to the beneficial effects of fluorapatite formation and remineralization. In this regard, therefore, if the replacement of OH with F ions occurs during F treatment, PO_4^{3-} ν_1 band up-shift should take place. Raman microprobe analysis, however, could not provide any evidence of ionic replacement regardless of the duration of F treatment. Although similar Raman analyses have been conducted by other research groups, those studies did not address the modification of crystal structure associated with ionic replacement in tooth enamel and dentin crystals [42, 43)]. Instead, they described the possibility of CaF_2 formation. Even though CaF_2 was formed on the crystal surface [18, 42, 43)], this did not improve crystal quality and may remain as a contributing factor to the crystal structure defects in calcified hard tissues. Another research group reported that Raman analysis of F-treated bone revealed a peak up-shift of PO_4^{3-} ν_1 value from 961 to 964 cm^{-1} [7)] and regarded this up-shift as evidence of crystal modification. However, their experimental fluoridation procedure was conducted with the mixed

solution consisting of phosphate, calcium and F ions. Under these experimental conditions, we speculate that this procedure may contribute to the formation of artificial deposits similar to those observed in our present remineralization experiment (Fig. 3e).

F intake is generally believed to produce fluorapatite in calcified hard tissues in place of hydroxyapatite. Our Raman analysis, however, revealed that the replacement of OH with F ions did not occur in rat developing enamel (Table I). This finding may be due to 2 different calcification mechanisms whereby the formation of crystal either with CDL or without CDL is strictly regulated by each hard tissue with the except of pathological calcification [44]. One pathway, mediated by the precursor mineral octacalcium phosphate (OCP), may have appeared around the Cambrian period [45]. This OCP pathway is facilitated by the presence of F ions [46] that contribute to the creation of fluorapatite observed in the tooth apparatus of conodonts, fish enameloid, and other structures according to previous reports [45, 47-50]. The other pathway involves CDL formation, which may have evolved up to the Silurian period [45] and is observed in ordinary hard tissues such as tooth enamel, dentin, and bone [29, 30, 51, 52]. Judging from Raman analysis, the CDL-bearing pathway may not employ F ions during crystal formation, suggesting that F ions may merely remain as a minor mineral in the calcified tissues with not involvement in the crystal lattice. Consequently, F intake is unlikely to increase the crystal quality of calcified hard tissues including enamel due to these differing mechanisms.

We define remineralization as the repair of damaged crystals. In this study, we conducted an experimental procedure on thin sections to directly evaluate remineralization. However, electron microscopy did not provide convincing evidence of remineralization (Figs. 2c and d). Instead of restoration of the damaged crystals, newly formed deposits appeared after incubation in a remineralizing solution for 2 weeks (Fig. 2e). These newly formed deposits were irrelevant to the repair of the damaged crystals. Therefore, we hypothesize that the appearance of these newly formed deposits may provide misleading evidence of the effect that the damaged crystals were repaired [8]. Although a previous study regarded the presence of 2 CDLs intersecting at 60° in one crystal as evidence of crystal fusion during the remineralizing process [8], we also observed similar crystal prior to immersion in a remineralizing solution (Fig. 2f). This finding indicates that the presence of this unique crystal does not necessarily indicate the restoration of damaged crystals. F ions in APF gel are thought to combine with calcium and phosphate ions released from the enamel surface under acid conditions, followed by reprecipitation to form acid-resistant crystals. However, we could not confirm any event of reprecipitation; instead, APF gel only caused the dissolution of the crystals (Fig.3b).

To raise awareness of the harmfulness of F schemes, we compared carbonic anhydrase activity of F exposed animals with that of Cd exposed ones. Exposure to environmental cadmium is well known to cause itai-itai disease, which is accompanied by osteomalacia and osteoporosis. Our previous study revealed that crystal structure defects caused by Cd exposure are a contributing factor for the development of itai-itai disease[27]. The present study has also demonstrated that both F and Cd ions interrupt the supply of carbonate ions needed for CDL formation, causing similar crystal structure defects (Figs. 4a and c). As shown schematically in Fig. 4b and d, F ions inhibit the synthesis of carbonic anhydrase, whereas Cd ions may reduce catalytic activity by replacing zinc with Cd ions, eventually hampering hard tissue formation [27]. Concerning the harmfulness of F on the crystal nucleation process, differential gas pressure analysis clearly indicated that the inhibitory effect of F on enzymatic activity was much greater than that of Cd (Fig. 5). Therefore, the plausible relationship between F exposure through unnecessary F schemes such as water fluoridation and the risk of development of bone disease should be considered.

Acknowledgments

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References

1. Kakei M, Sakae T, Yoshikawa M, and Tamura N. Effect of fluoride ions on apatite crystal formation in rat hard tissues. Ann Anat 189: 175-185, 2007
2. WHO. Guidelines for drinking-water quality, 3rd edition incorporating 1st and 2nd addenda. Vol. 1. Recommendations. Geneva, 2008, pp375-377b
3. WHO. Milk fluoridation for the prevention of dental caries, ed by Banoczy J,Petersen PE and Rugg-Gunn AJ,World Hralth Organization, Geneva, 2009, pp67-161
4. Takuma S. Demineralization and remineralization of tooth surface - an ultrastructural basis for caries prevention. J Dent Res 59: 2146-2156, 1980
5. Silverstone L M, Wefel J S, Zimmerman B F, Clarkson B H and Featherstone M J. Remineralization of natural and artificial lesions in human dental enamel in vitro. Caries Res 15: 138-157, 1981

6. Robinson C, Shore R C, Brookes S L, Strafford S, Wood S R and Kirkham J. The chemistry of enamel caries. Crit Rev Oral Biol Med 11: 481-495, 2000
7. Freeman J J, Wopenka B, Silva M J and Pasteris J D. Raman spectroscopic detection of changes in bioapatite in mouse femora as a function of age and in vitro fluoride treatment. Calcif Tissue Int 68: 156-162, 2001
8. Miake Y, Saeki Y, Takahashi M and Yanagisawa T. Remineralization effects of xylitol on demineralized enamel. J Electron Microsc 52: 471-476, 2003
9. Aoba T. Solubility properties of human tooth mineral and pathogenesis of dental caries. Oral Dis 10: 249-257, 2004
10. Yamagishi K, Onuma K, Suzuki T, Okada F, Tagami J, Otsuki M and Senawangse P. A synthetic enamel for rapid tooth repair. Nature 433: 819, 2005
11. Ten Cate J M, Buijs MS, Miller CC and Exterkate R A M. Elevated fluoride products enhance remineralization of advanced enamel lesions. J Dent Res 87: 943-947, 2008
12. Mäkinen K K. Sugar alcohols, caries incidence and remineralization of caries lesions: A literature review. Int J Dentistry Article 2010: 1-23, 2010
13. Brudevold F, Savory A, Gardner D E, Spinelli M and Speirs R. A study of acidulated fluoride solution-1 in vitro effects on enamel. Arch Oral Biol 8: 167-177, 1963
14. Key M I, Young R A and Posner A S. Crystal structure of hydroxyl-apatite. Nature 204: 1050-1052, 1964
15. Brudevold F and Deepaola P F. Studies on topically applied acidulated phosphate fluoride solution at Forsyth Dental Center. Dent Clin North Am 1966 Jul. 299–308, 1966
16. Mellberg J R. Fluoride uptake by intact human tooth enamel from acidulated fluoride-phosphate preparations. J Dent Res 45: 303-306, 1966
17. Nelson K G and Higuchi W I. Mechanism of fluoride uptake by hydroxyapatite from acidic fluoride solutions: Theoretical considerations. J Dent Res 49: 1541-1548, 1970
18. Chander S, Chiao C C and Fuerstenau W. Transformation of calcium fluoride for caries prevention. J Dent Res 61: 403-407, 1982
19. Fan Y, Sun Z and Moradian-Oldak J. Effect of fluoride on the morphology of calcium phosphate crystals grown on acid-etched human enamel. Caries Res 43: 132-136, 2008
20. Farley S M G, Wergedal J E, Smith. L C, Lundy M W, Farley J R and Baylink D J. Fluoride therapy for osteoporosis: characterization of the skeletal response by serial measurements of serum alkaline phosphatase activity. Metabolism 36: 211-218, 1987
21. Balena R, Kleerekoper M, Foldes J A, Shih M-S, Rao DS, Schober H C and Parfitt A M. Effects of different regimens of sodium fluoride treatment for osteoporosis on the structure, remodeling and mineralization of bone. Osteoporosis Int 8: 428-435, 1988
22. Lehmann R, Wapniarz M, Hofmann B, Pieper B, Haubitz I and Allolio B. Drinking water fluoridation: Bone mineral density and fracture incidence. Bone 22: 273-278, 1998
23. Grynpas M D. Fluoride effects on bone crystals. J Bone Miner Res 5: Suppl 1, S169-175, 1990
24. Grynpas M D and Rey C. The effect of fluoride treatment on bone mineral crystals in the rat. Bone 13: 423-429, 1992
25. Farrerons J, Rodríguez de la Serna A, Guañabens N, Armadas L, López-Navidad A, Yoidi B, Renau A and Vaqué J. Sodium fluoride treatment is a major protector against vertebral and nonvertebral fractures when compared with other common treatment of osteoporosis: a longitudinal, observational study. Calcif Tissue Int 60: 250-254, 1997
26. Rubin C D, Pak C Y C, Adams-Huet B, Genant H K, Li J and Rao S. Sustained-release sodium fluoride in the treatment of the elderly with established osteoporosis. Arch Intern Med 161: 2325-2333, 2001
27. Kakei M, Sakae T and Yoshikawa M. Mechanism of cadmium induced crystal defects in developing rat tooth enamel. Proc Jpn Acad Ser B 85: 500-507, 2009
28. Friberg L, Elinder C-G, Kjellström T and Nordberg G F. Cadmium and Health: A Toxicological and Epidemiological Apprasial, vol. Ⅱ (Effects and Response). CRC Press Inc, Boca Raton, FL, USA, 1986, pp111-158.
29. Nakahara H and Kakei M. The central dark line in developing enamel crystallite: An electron microscopic study. Josai Shika Daigaku Kiyo 12: 1-7, 1983
30. Nakahara H and Kakei M. TEM observations on the crystallites of dentin and bone. Josai Shika Daigaku Kiyo 13: 259-63, 1984
31. Sowers M R, Clark M K, Jannausch M L and Wallace R B. A prospective study of bone mineral content and fracture in communities with different fluoride exposure. Am J Epidemiol 133: 649-660, 1991
32. Danielson C, Lyon J L, Egger M and Goodenough G K. Hip fractures and fluoridation in Utah's elderly population. JAMA 268: 746-748, 1992
33. Jacobsen S J, Goldberg J, Miles T P, Brody J A, Siters W and Rimm A A. Regional variation in the incidence of hip fracture: U.S. white women aged 65 years and older. JAMA 264: 500-502, 1999
34. Kakei M and Nakaraha H. Electroimmunoblotting study of carbonic anhydrase in developing enamel and dentin of the rat incisor. Jpn J Oral Biol 27: 357-361, 1985
35. Nakahara H and Kakei M. Central dark line and carbonic anhydrase: Problems relating to crystal nucleation in enamel. In: Tooth Enamel Ⅳ, ed by Fearnhead R W and Suga S, Elsevier, Amsterdam, 1989, pp 42-46.
36. Nakahara H and Kakei M. Ultrastructural and protein aspects

of apatite formation in vertebrate hard tissues. In: Origin, Evolution and Modern Aspects of Biomineralization in Plants and Animals, ed by Crick R E, Plenum Press, New York, 1989, pp 225-235.

37. Kakei M and Nakaraha H. Aspects of carbonic anhydrase and content during mineralization of the rat enamel. Biochim Biophys Acta 1289: 226-230, 1996
38. Kakei M, Nakahara H, Tamura N, Itoh H and Kumegawa M. Behavior of carbonate and magnesium ions in the initial crystallites at the early developmental stages of the rat calvaria. Ann Anat 179: 311-316, 1997
39. Kodama E. Development of a new device equipped with the deferential gas pressure sensor for the measurement of carbonic anhydrase activity and its application to experiments of high school biology. Master's thesis, Tokyo Gakugei University, 2007, pp 1-84.
40. LeGeros R Z. Apatites in biological systems. Prog Crystal Growth Charact 4: 1-45, 1981
41. Casciani F S, Etz E S, Newbury D E and Doty S B. Raman microprobe studies of two mineralizing tissues: Enamel of the rat incisor and the embryonic chick tibia. Scan Electron Microsc 2: 383-391, 1979
42. Tsuda H and Arends J. Detection and quantification of calcium fluoride using micro-Raman spectroscopy. Caries Res 27: 249-257, 1993
43. Tsuda H, Ruben J and Arends J. Raman spectra of human dentin mineral. Eur J Oral Sci 104: 123-131, 1996
44. Kakei M, Sakae T, Mishima H and Yoshikawa M. Ultrastructure of apatite crystals formed during vascular calcification in humans. J Hard Tissue Biol 18: 135-140, 2009
45. Kakei M, Sakae T and Mishima H. In: Biomineralization: from Paleontology to Materials Science, ed by Arias J L and Fernandez M S, Editorial Universitaria, Santiago, Chile, 2007, pp. 107-115.
46. Newesely H. Darstellung von "Oktacaleciummphosphat" (tetracalcium-hydrogentriphosphat) duch homogene Krisyallisation. Mh Chem 89: 1020-1023, 1960
47. Miake Y, Aoba T, Moreno E C, Shimoda S, Prostak K and Suga S. Ultrastructural studies on crystal growth of enameloid menirals in elasmobranch and teleost fish. Calcif Tissue Int 48: 204-217, 1991
48 Kakei M, Nakahara H, Kumegawa M, Mishima H and Kozawa Y. Ultrastructural study on the lattice images of calcium phosphate minerals in fossil tooth. In: Biomineralization (BIOM2001): formation, diversity, evolution and application, ed. by Kabayashi I and Ozawa H, Tokai University Press, Kanagawa, 2003, pp 364-368.
49. Mishima H, Kakei M, Yasui T, Miyamoto S, Miake Y and Yanagisawa T. Apatite crystal in hard tissue of Conodont fossils. Front Mater Sci China 2: 171-179, 2008
50. Barry J C and Kemp A. High resolution transmission electron microscopy of developing enamel in the Australian lungfish, Neoceratodus forsteri (Osteichthyes: Diponi). Tissue Cell 39: 387-398, 2007
51. Marshall A F and Lawless K R. TEM study of the central dark line in enamel crystallites. J Dent Res 60: 1773-1782, 1981
52. Nakahara H. Electron microscopic studies of the lattice image and central dark line of crystallites in sound and carious human dentin. Josai Shika Daigaku Kiyo 11: 209-215, 1982