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Effect of Experimental Fluorosis on the Surface Topography of Developing Enamel Crystals

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Abstract
Dental fluorosis is an increasing problem, yet the precise mechanism by which fluoride exerts its effects remains obscure. In the present study, we have used atomic force microscopy to image and quantitate surface features of enamel crystals isolated from specific developmental stages of fluorotic and control rat incisors. The results showed a significant decrease in crystal surface roughness with development in control tissue. Crystals from fluorotic tissue were significantly rougher than controls at all stages of development, did not decrease in roughness during the later stages of their development and had many morphological abnormalities. These data clearly demonstrate an effect for fluoride on enamel crystal surfaces which could reflect changes in the nature and distribution of growth sites and/or in mineral-matrix interactions. These would be expected to affect crystal growth during maturation, resulting in the characteristic porous appearance of fluorotic lesions in mature teeth.

The clinical effect of elevated dietary fluoride is the presence of white opacities on the tooth surface [Den Besten and Giambro, 1995]. According to its severity, increasingly greater areas of the tooth surface may be involved [Dean, 1934; Thylstrup and Fejerskov, 1978] and in its most severe form, post-eruptive effects, such as exogenous staining and mechanical damage, may lead to discoloration and loss of tissue (e.g. pitting) [Fejerskov et al., 1974]. These effects are due to the porous nature of the fluorotic tissue which, because of its similarity with the maturation stage of normal enamel development, has prompted the suggestion that fluorosis may represent an arrested tissue maturation [Fejerskov et al., 1977].

During its development, enamel passes through a number of discrete stages which have been defined both chemically and histologically [Robinson et al., 1981]. In the earliest stage of amelogenesis (the secretory stage), the extracellular matrix into which the young crystals are initially deposited is protein-rich, containing approximately 30% protein by weight [Deakins, 1942]. During this stage, the developing crystals appear as long, thin ribbons, extending from the amelodentinal junction towards the retreating ameloblasts at the enamel surface [Nylen et al., 1963; Cuisinier et al., 1992]. With increasing developmental age, the protein matrix is degraded and removed [Fincham et al., 1982] and the ameloblasts begin to lose their secretory characteristics (the transition stage) [Marsland, 1951, 1952; Pindborg and Weinmann, 1959]. Matrix removal leaves a

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porous, highly hydrated tissue in which the crystals grow, mainly in thickness, to achieve the final dimensions characteristic of the mature tissue [reviewed by Robinson et al., 1997]. This is the maturation stage which is white and opaque in appearance on drying, due to its porosity [Hiller et al., 1975]. The maturation stage is also accompanied by changes in the concentrations of a number of both major inorganic constituents such as calcium and phosphate ions and also minor components including fluoride, which accumulate early in maturation [Weatherell et al., 1975]. Any inhibition of the crystal growth process during this developmental stage would therefore be expected to result in the eruption of immature tissue with a white, chalky appearance. The latter is characteristic not only of fluorosis but also of a number of enamel dysplasias [Small and Murray, 1978; Witkop, 1989].

Numerous studies have attempted to determine the effects of fluoride on the processes described above, placing particular emphasis upon the events which occur during the maturation stage [reviewed by Robinson and Kirkham, 1990; Den Besten and Giambro, 1995]. However, a significant effect for fluoride at the relatively low serum concentrations associated with fluorosis has yet to be demonstrated.

In a previous report, we have described the use of the atomic force microscope (AFM) to image individual crystals isolated from the secretory and maturation stages of enamel development and reported the effects of fluoride on the appearance of crystals taken from the maturation stage [Kirkham et al., 1998]. The AFM provides a significant advance in high resolution imaging of biological material as samples can be imaged under fluid without the need to fix, stain, embed or coat and quantitative data can be obtained at the subnanometre level in all three dimensions. However, no such quantitative measurements were obtained in the preliminary study and crystals were not isolated from every developmental stage, precluding comparisons of any effect for fluoride throughout enamel development.

The aim of the present study was to use the AFM to generate quantitative as well as qualitative data describing the size and surface roughness of enamel crystals from all stages of development in control and fluorotic tissue.

Materials and Methods

Preparation of Crystals

Twenty-four male Wistar rats (age = 3 weeks at start of experiment) were divided into two groups. In the fluorosis group, animals were provided with 75 ppm F^- (as NaF) in drinking water ad libitum for 3 weeks, after which the erupted incisor enamel appeared typically fluorotic [Angmar-Månsson and Whitford, 1984]. Control animals were given tap water (0.3 ppm F^-) for the same period of time. All animals were housed together and maintained on the same standard laboratory diet. Individual crystals from specific stages of enamel development were then obtained from mandibular incisor teeth of both fluorotic and control animals following dissection from the mandibles. Particles of enamel were microdissected from specific developmental stages using the beginning of the white opaque enamel as a marker for the onset of the maturation stage as described previously by Hiller et al. [1975]. All developing enamel was removed from each tooth and the tissue pooled according to developmental stage, giving three samples: secretory enamel, transition stage enamel and maturation stage enamel. All detectable traces of matrix protein were removed from the enamel samples using a sequential extraction procedure as described by Robinson et al. [1997] and the isolated crystals spread on to mica as described by Kirkham et al. [1998].

Atomic Force Microscopy

AFM was carried out as described previously [Kirkham et al., 1998] using a Nanoscope III AFM (Digital Instruments) equipped with a 16×16-μm scanner and 25-μm silicon nitride cantilevers. Images were obtained in oscillating mode at 0.2 Hz below resonance with drive amplitudes in the range 300–950 mV. Measurements of crystal width and height were made using the software provided. Surface roughness measurements (R_a) were obtained over a length of 50–100 nm in an approximately central line parallel to the crystal c axis. At least 50 crystals from each developmental stage were measured in order to achieve a final mean roughness value. R_a roughness was calculated using the Nanoscope 4.23 software and is defined here as:

$$R_a = \frac{1}{L_x L_y} \int_0^{L_y} \int_0^{L_x} |f(x,y)| \, dx \, dy,$$

where f(x,y) is the surface relative to the centre plane and L_x and Ly are the dimensions of the surface. Therefore the calculated surface roughness in this case is the mean value of the surface relative to the centre plane and takes into account any changes in the slope of the crystal surface.

The results of the roughness measurements for each developmental stage and for control and fluorotic tissue were analysed statistically using an unpaired t test.

Results

Control Enamel Crystals

The typical appearances of developing enamel crystals from each stage of enamel development are shown in figures 1 and 2. A low power view of the AFM field, showing the spread of crystals (maturation stage) is shown in figure 3. Secretory stage crystals were characteristically narrow and thin (table 1) in comparison with later stages and were irregular in outline, with numerous surface features, including apparently globular domains measuring approximately 25 nm in diameter (fig. 1a). During transition, the crystals were generally wider, slightly thicker and smoother in their

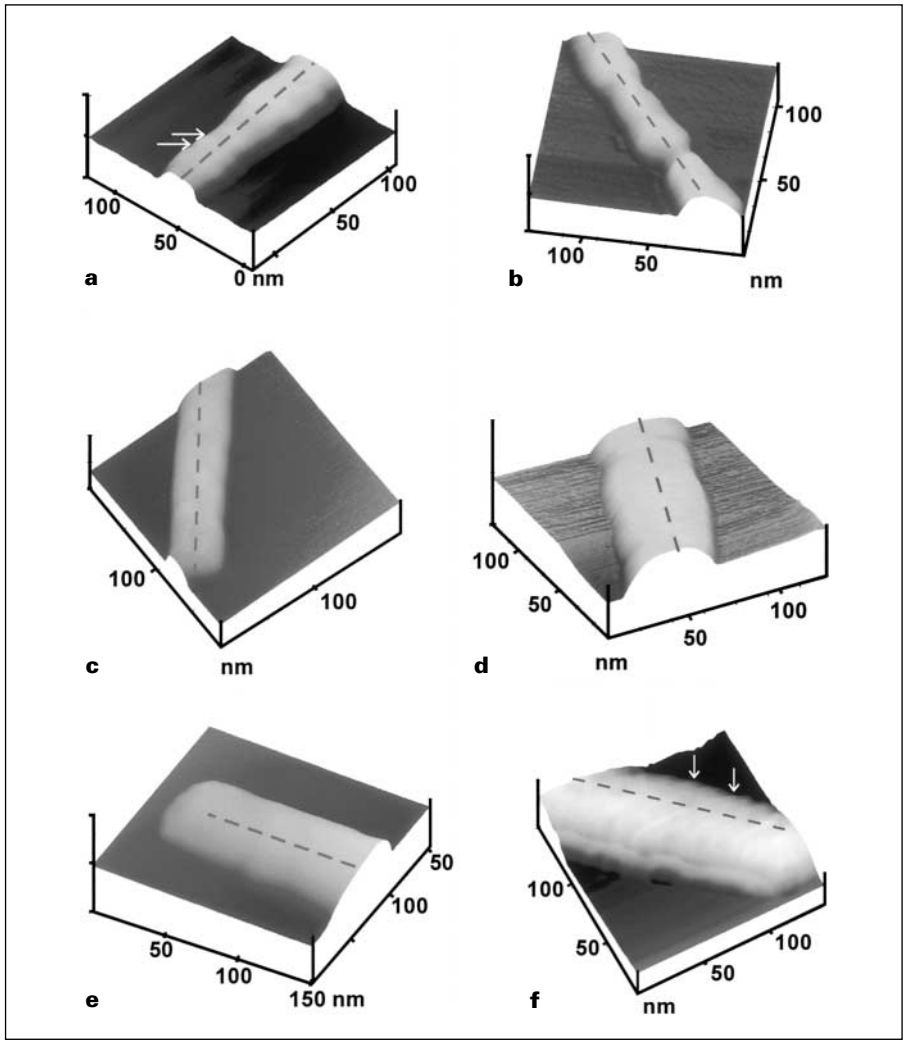

Fig. 1. Three-dimensionally rendered typical AFM images of hydroxyapatite crystals from control and fluorotic rat incisor enamel at different stages of development. Dotted lines indicate position of line scans obtained from original height image and shown in figures 2 a–f. **a** Control crystal from secretory stage. Globular subunits of structure are indicated (arrows). **b** Fluorotic crystal from secretory stage showing irregular outline. **c** Control crystal from transition stage. **d** Fluorotic crystal from transition stage. **e** Control crystal from maturation stage. **f** Fluorotic crystal from maturation stage showing ridges running perpendicular to c axis (arrowed) [a and c are modified from Fig. 1, Kirkham et al., 1998 © OPA (Overseas Publishers Association) N.V. with permission from Gordon and Breach Publishers].

surface topology (table 1, fig. 1b). The globular subunits of structure which had been a feature of the secretory stage crystals were no longer apparent. However, surface irregularities were still observed, including steps with dimensions up to 3 nm and the outlines of the crystals, though more uniform than those of the secretory stage, remained irregular in areas and retained some evidence of tapering. Maturation stage crystals were larger, particularly in their thickness dimension and markedly smoother (table 1, fig. 1c). Many crystals showed little surface irregularity other than occasional 0.8 nm steps [Kirkham et al., 1998], which approximated to the dimension of one unit cell for hydroxyapatite. These crystals had regular outlines and were not tapered. There was a significant decrease in surface roughness with developmental stage (table 1).

Fluorotic Enamel Crystals

The typical AFM appearances of developing enamel crystals from fluorotic tissue are shown in figures 1 and 2 together with control crystals from the same stage of development. Fluorotic crystals increased in width and height with developmental stage in a similar way to that seen for controls (table 1). However, the effect of fluorosis was clearly apparent at all developmental stages. Secretory stage fluorotic crystals appeared to be a more exaggerated version of the controls, exhibiting greater irregularities in their outlines and increased surface roughness. There was also a tendency for the crystals to be narrower and thinner in comparison with control crystals from the same stage, though this was not significant when analysed statistically (table 1). This same effect was observed in the transition stage, where greater numbers of unusual surface features were observed

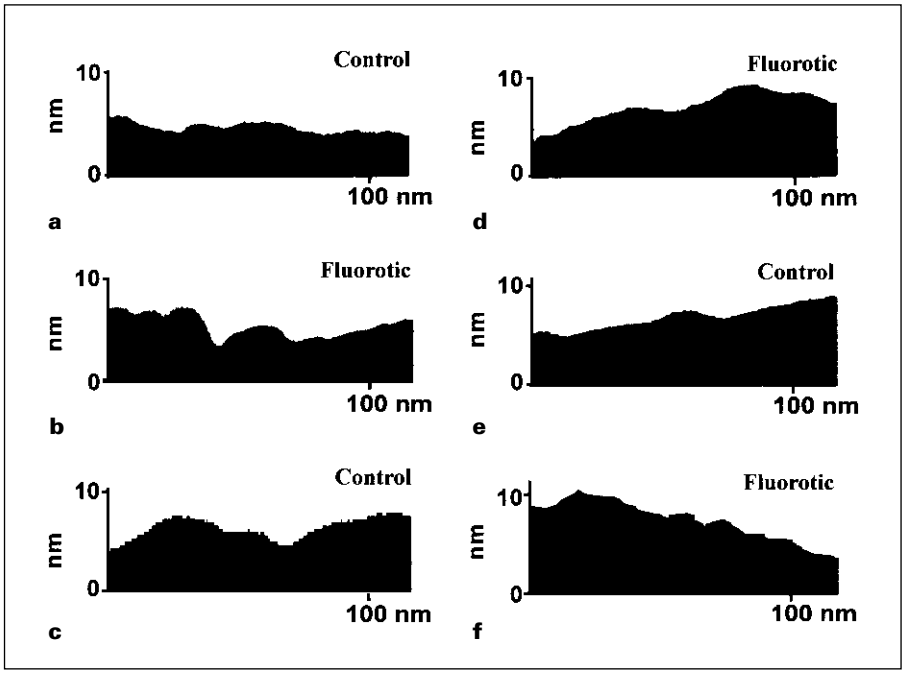

Fig. 2. Line scans obtained from AFM quantitative height images showing topographical detail of hydroxyapatite crystals from control and fluorotic rat incisor enamel at different stages of development. **a** Control crystal from secretory stage. **b** Fluorotic crystal from secretory stage. **c** Control crystal from transition stage. **d** Fluorotic crystal from transition stage. **e** Control crystal from maturation stage. **f** Fluorotic crystal from maturation stage showing ridges running perpendicular to c axis (arrowed).

and the outlines of the crystals were much more irregular, with frequent tapering to extremely narrow 'waists'. Maturation stage crystals were also obviously different in fluorotic tissue compared with controls of similar developmental age. They had increased surface roughness, largely attributable to the appearance of regular and frequent 'ridges' across the crystal surface. Fluorotic crystals were significantly rougher than controls at each developmental stage ($p < 0.01$, table 1). In addition, although there was a significant decrease in surface roughness from secretion to transition in the fluorotic tissue, there was no significant difference in surface roughness between transition and maturation stage fluorotic crystals (table 1).

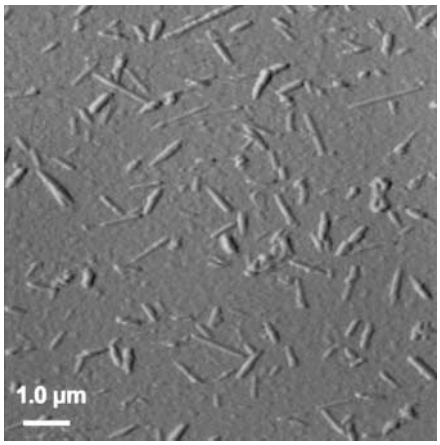

Fig. 3. Typical low power view of maturation stage enamel crystals in the AFM.

Discussion

These results clearly demonstrate an effect of fluorosis on the surface topography of enamel crystals during development. In addition, they corroborate and provide quantitative data to support our preliminary findings which described some of the surface features of secretory and maturation stage control crystals and the abnormal appearance of fluorotic crystals from the maturation stage [Kirkham et al., 1998].

Previous investigators have suggested that fluoride affects ameloblast proliferation and differentiation [Kerley and Kollar, 1977; Holland and Hongslo, 1979], ameloblast morphology [Smith et al., 1993], matrix composition and degradation [DenBesten and Crenshaw, 1984; DenBesten and Hefferman, 1990] and matrix-mineral binding [Aoba et al., 1989]. However, many of these results have been equivocal or only present at very high levels of fluoride exposure (notably in the culture fluid for in vitro work) which are not typical of those generally associated with dental fluorosis. Fewer workers have examined the effects on the enamel

Table 1. Comparison of crystal widths, heights and surface roughness (R_a) at each stage in enamel development for control and fluorotic enamel (mean ± SD). At least 50 crystals were measured for each parameter. Data compared using unpaired t test.

Developmental stage	Widt, nm		Height, nm		R_a, nm	
	control	fluorotic	control	fluorotic	control	fluorotic
Secretion	28.0±1.5	27.0±1.0	8.8±0.8	6.5±1.0	0.6±0.06	1.1±0.06[c]
Transition	41.0±1.5[a]	41.0±3.0[a]	11.3±0.7	9.2±0.8[a]	0.4±0.03[a,c]	0.6±0.03[a]
Maturation	55.0±3.0[b]	50.0±2.0[b]	19.0 ± 2.0[b]	21.0±1.0[b]	0.3 ± 0.02[b]	0.6±0.04[c]

[a] Significantly different to secretory stage crystals.
[b] Significantly different to transition stage crystals.
[c] Significantly different to control crystals. All other comparisons were not significantly different.

mineral itself, though it has been reported that the quality of the mineral is affected by exposure to high fluoride concentrations, with higher concentrations of magnesium [Robinson et al., 1983] and, in the case of fluorosed crystals from bone, increased manganese and decreased carbonate, citrate and zinc [Kanwar and Singh, 1981]. Bronckers et al. [1984a, b] and Lyaruu et al. [1986], using a tooth organ culture system, demonstrated that fluoride affected matrix mineralisation in a complex way, disrupting the growth of existing crystals and inhibiting deposition of crystals in newly secreted matrix, suggesting that fluoride may be able to interfere with matrix nucleating sites and presumably affect matrix-mineral interactions.

The results presented here demonstrate that fluorosis has a marked effect on enamel crystal surface topography and ultrastructural morphology at all stages of development, strongly suggesting an influence on crystal growth. It is not known whether this is a direct effect – due to alterations of crystal chemistry affecting crystal growth sites – or an indirect effect via an influence on matrix-mineral interactions. The sizes of the crystals reported here for control enamel (table 1) are in the same range as those reported previously for different stages of developing rat enamel [Selvig and Halse, 1974; Daculsi and Kérébel, 1978], though absolute measurements for width are not possible using AFM due to tip convolution (height data is not affected). The decrease in surface roughness with increasing developmental age seen in control crystals suggests that incremental crystal growth during normal enamel development is accompanied by a decrease in surface features such as steps and grooves. These features are in general less than 5 nm in height, which represents only few unit cells for hydroxyapatite [Kay et al., 1964] and may correspond to the lattice defects detected using high resolution electron microscopy (HR-TEM) [Cuisinier et al., 1992; Shibahara et al., 1994] and high resolution secondary electron I scanning microscopy (HR-SEM) [Apkarian et al., 1990]. It is possible that such irregularities reflect discontinuities and areas of strain in the crystal which may represent growth sites. The differences between the younger crystals of secretion and those of the later maturation stage indicate a decrease in such sites with crystal growth. In this context, it is of interest to note that the maturation stage crystals from fluorotic tissue show no such decrease in surface roughness, echoing an earlier stage of development. Using HR-TEM, Kérébel et al. [1976] demonstrated a greater number of lattice defects in crystals derived from human mature fluorotic enamel compared with enamel crystals from normal tissue, which would be consistent with the findings reported here.

The significant increase in surface roughness at all stages of development in fluorotic tissue has important implications in respect of matrix-mineral interactions. Given that the fluorotic crystals were not significantly different in width or height compared with control, increased roughness would result in an increased surface area. Rougher crystals have been shown to bind increased amounts of protein in other tissues [Gathercole et al., 1996]. Aoba et al. [1989] demonstrated selective binding of specific amelogenin components (normally unbound) to fluorotic mineral in vitro. This does not rule out changes in surface chemistry, which may enhance the effect. Previous studies using synthetic minerals and proteins including albumin have demonstrated that fluorapatite had increased adsorption bond strength and additional numbers of adsorption sites compared with hydroxyapatite [Hay and Moreno, 1979]. In addition, Tanabe et al. [1988] showed greater adsorption of amelogenin breakdown products with increasing fluoride substitution into the hydroxyapatite lattice, suggesting that fluoride-induced increased lattice stability had decreased crystal surface energy.

No significant effect for fluorosis was observed on the size of enamel crystals from any developmental stage (table

1). This contrasts with previous reports, where fluorotic crystals from mature, human enamel were shown to be larger than those from normal tissue [Kérébel and Daculsi, 1976; Kérébel et al., 1976]. However, Fejerskov et al. [1974] reported no differences in crystal size in a further study of fluorosed (mature) human enamel. Our results are taken from the early maturation stage of the developing tissue and it is not known whether the observed effects continue into the mature tissue.

The increase in protein binding following fluoride incorporation into the crystals may be particularly important when considering the activity of mineral bound enzymes during maturation. The serine protease (which is the most active protease at this time) has been shown to bind to the mineral in vivo [Brookes et al., 1998]. Increased mineral binding would be expected to affect its activity, perhaps leading to failure to remove potential inhibitors of crystal growth which are known to be present during early maturation [Robinson et al., 1992]. Alternatively, failure to remove protein from the tissue per se may also affect tissue architecture on a larger scale, perhaps resulting in larger interprismatic spaces [Fejerskov et al., 1974; Kérébel and Daculsi, 1976]. These effects would then be expected to result in the eruption of immature porous tissue such as is seen in enamel fluorosis.

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