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Original Article

Why does fluorosed dentine show a higher susceptibility for caries: An ultra- morphological explanation

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Background-Chronic endemic dental fluorosis is a malformation of teeth caused by excessive ingestion of fluoride during period of tooth development. Apatite crystallites and collagen fibrils determine the caries susceptibility related properties of dentine. Information relating the effect of dental fluorosis with dentine crystallite and collagen structure is currently unavailable. This study examined structural aberrations of apatite crystallites and collagen fibrils in human fluorosed dentine.

Method- Superficial dentine of thirty six normal (Thylstrup-Fejerskov index - 0) and thirty six severely fluorosed (Thylstrup-Fejerskov index - 7 to 9) extracted human molars from individuals living in an area which is endemic for dental fluorosis were morphologically analyzed using transmission electron microscopy with selected area electron diffraction and dynamic force microscopy.

Results- A statistically significant enlargement in the size of crystallites was found in fluorosed dentine ($p<0.05$). These crystallites were not homogenously arranged. Selected area electron diffraction revealed a lower density of crystallites in fluorosed dentine. Randomly distributed collagen fibrils showed a less complex lay out in contrast to normal dentine tissue.

Conclusion- Ultrastructure aberrations of the dentine explain the caries susceptibility of the tissue and suggest the necessity of special approach in dental caries treatment for patients with chronic severe endemic dental fluorosis.

Key words: Dentine, Fluorosis, Collagen, Apatite

INTRODUCTION

Fluoride has a high affinity for calcified tissues and is concentrated in dental and bone structures[1]. At elevated concentrations, fluoride disturbs the mineralization process[2]. Chronic endemic dental fluorosis is a malformation of teeth caused by excessive ingestion of fluoride, possibly in the water supply (usually 2 to 8 ppm) during the period of tooth development. Fluorosed enamel is characterized by an outer hypermineralized layer consisting of many large and extremely small crystallites which are occasionally attached to each other. The subsurface hypomineralized enamel is composed of sparsely arranged large crystallites and a few small crystallites[3]. An increase in the roughness of enamel crystallites in fluorotic enamel has also been reported[4]. Several epidemiological studies have been carried out to assess the relationship between dental fluorosis and dental caries experience[5,6]. However the relationship varies on additional social factors. With in vitro studies the tendency for caries formation[7] and restorative

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treatment options for fluorosed enamel are reported to be deviated from the normal tissue[8].

Distinct changes in mineralization pattern are not confirmed in the fluorotic enamel. Conversely, the underlying fluorotic dentine exhibits accentuated incremental growth patterns. After cessation of enamel secretion, a substantial variation of mineral content can be observed in dentine with occasional bands of interglobular dentine[9,10]. In our previous studies we found that mild and moderate fluorosed dentine are significantly susceptible for caries formation than the normal dentine[7] and the present dentine bonding systems offer lower bond strengths to this fluorosed dentine tissue[11].

To improve the understanding of the pathogenic mechanism and diversity of caries formation in fluorosed dentine, detailed studies of dentine structure changes occurring in human dental fluorosis are needed. However, there is little information relating dental fluorosis with dentine crystallite and collagen fibrillar structure. Thus, this study was designed to examine the existence of structural aberrations of apatite crystallites and collagen fibrils in severely fluorosed human dentine by means of dynamic force microscopy (DFM) and transmission electron microscopy (TEM) with selected area electron diffraction (SAED). The hypothesis tested was that there are differences in the ultrastructure of mineral crystallites and collagen fibrils between normal dentine and severely fluorosed dentine which may have made the structure prone to caries formation.

MATERIALS AND METHODS

Seventy two extracted human molar teeth from subjects between the age of 30 and 40 living in endemic areas for fluorosis in Sri Lanka, with 3ppm fluoride in drinking water were used for this study[4]. The teeth were caries-free and had been extracted due to periodontal reasons. Informed consent was obtained from the subjects for the use of their extracted teeth in this study, based on a protocol that had been reviewed and approved by the Tokyo Medical and Dental University, Japan. The teeth were wrapped in water-soaked cotton, stored at 4℃ in a refrigerator and used within 2 months following extraction. Prior to laboratory processing, the extracted teeth were analyzed for dental fluorosis severity according to the Thylstrup-Fejerskov index-TFI[13]. Thirty six normal teeth with TFI 0 and thirty six severely fluorosed teeth with severity between TFI 7 and TFI 9 were selected based on their clinical appearance[8]. The occlusal enamel was removed and 1x1x3 mm superficial dentin specimens were prepared from a plane 2mm away from buccal and distal surfaces using a slow speed diamond saw (Isomet, Buehler, Lake Bluff, IL) under water cooling.

Sample preparation for transmission electron microscopy (TEM)

Collagen fibrillar structure

Twenty four superficial dentine samples (twelve normal and twelve severely fluorosed) were randomly selected and fixed with 2.5% glutaraldehyde then demineralized in a 4% ethylenediamine tetra-acetic acid buffer for 10 days. After thoroughly rinsing in phosphate buffered saline (PBS) for three times, the demineralized dentin specimens were post-fixed with 0.1% osmium tetroxide. Further TEM sample preparation was performed in accordance with common procedures used for ultrastructural TEM examination of biological tissue. The fixed specimens were dehydrated in an ascending ethanol series (50-100%), immersed in propylene oxide as a transitional medium and embedded in an epoxy resin (TAAB 812, TAAB Laboratories, Aldermaston, United Kingdom). An ultramicrotome (Reichert/Leica Ultracut S, Vienna, Austria) and a diamond knife were used to prepare 100 nm thick sections. These demineralized sections were stained with 2% uranyl acetate for 10 min and Reynold's lead citrate for 5 min prior to examination with a transmission electron microscope at an accelerating voltage of 75 kV (H-600, Hitachi, Tokyo, Japan). The axial periodicities obtained from fibrils in each group were recorded.

Crystallite structure

Twelve normal and twelve severely fluorosed superficial dentine specimens were prepared in the manner as previously described, but without demineralization of the specimens. After epoxy resin embedding, 100 nm thick mineralized sections were prepared and observed without further staining using the H-600 TEM at 75 kV. In order to determine the ultrastructural changes in intertubular dentin crystals due to fluorosis, the plate width (short axis) and the length (long axis) of dentin crystallites were measured with a digital image analysis program at a magnification of x200000 to a precision of 0.1nm. The arrangement of crystallites was observed at a magnification of x100000.

To identify the type of crystallites, electron diffraction method was performed on a selected area (selected

area electron diffraction; SAED). The d-spacings of diffraction patterns were calibrated using the d-spacings of gold determined under identical conditions. Calculated the ratios between the major rings and compared these data with known data for apatite structure.

Sample preparation for dynamic force microscopy (DFM)

Collagen fibrillar arrangement

A dynamic force microscope (SP 13800N Probe Station S11, Seiko Instruments, Tokyo, Japan) was used to observe the demineralized intact collagen fibrils on intertubular dentine surfaces. The last twelve superficial dentin specimens from each of the normal dentine and severely fluorosed dentine groups were embedded in an epoxy resin and polished under water cooling with a series of silicon carbide papers of decreasing roughness up to 1500-grit. Each specimen surface was subsequently polished with diamond pastes (Struers, Ballerup, Denmark) from 6 μm to 0.25 μm grit size to produce a high gloss surface and ultrasonically cleaned in deionized water for 30 min. The polished specimens were then partially demineralized with 10 vol% citric acid for 10 s, rinsed and gently air dried. They were further treated with an aqueous solution of 3 vol% sodium hypochlorite for 120 s to remove noncollagenous proteins from the organic matrix[14]. After extensive rinsing with water, the specimens were immediately observed with tapping-mode DFM in air. Examination was conducted using a high-aspect-ratio non-contact cantilever and a silicon nitride tip radius of approximately 15 nm at a resonance frequency of 29.6 kHz. The scan rate was 0.5 Hz with a scan size 1.2 x 1.2 μm.

Statistical analyses

The length, width of the mineral crystallites and the axial periodicities of the collagen fibrils in the normal dentine group and the severely fluorosed dentine group were separately analysed for mean and standard deviations using the statistical software program SPSS for windows version 11. Statistical differences were examined using a two-tail unpaired t-test with the significance level preset at $p = 0.05$.

RESULTS

Crystallite structure

TEM micrograph revealed the ultrastructure of the fluorosed dentine and the control normal dentine (Fig 1). In normal dentine the mineral phase consisted of needle like crystallites which were packed together forming structures with less mineral dense regions between them (Fig 1 A arrows a) and a preferred orientation with their c-axes along the collagen fibril in a homogenous manner (Fig 1A arrows b). The length and width of the sound dentine intertubular crystallites was 114.8 ± 31.5 and 9.9 ± 3 nm respectively (Fig 1B). Ultrastructure of intertubular fluorosed dentine formed a distinct haphazard not homogenous arrangement of crystallites (Fig 1C arrows). The crystallites appeared more "obese" (Fig 1D) with length of 129.4 ± 21.1 and width of 21.4 ± 3.9 nm. The plate width of crystallites in normal and fluorosed dentine was statistically different ($p<0.05$).

The selected area electron diffraction pattern of sound intertubular dentine (Fig 2A) consisted of ring patterns. Measuring radii and indexing of the principal ring patterns, i.e. (002) $\approx$ 3.44 Å and (300/112 and 211) rings were (112) $\approx$ 2.78 Å, confirmed that the intertubular crystallites were apatite. Indexing of electron diffraction patterns obtained from fluorosed intertubular dentine (Fig 2B), the diffraction spots which formed the ring diffraction patterns which were not totally continuous confirmed the lower density of apatite crystallites in this area.

Collagen fibrillar structure and arrangement

TEM imaging (Figs. 3A and 3B) along the axes of the fibrils confirmed a characteristic periodicity of type 1 collagen in both fluorosed and normal tissue (Table 1). Branching could be observed in some fibers. Random distribution was revealed.

Fig 4 shows an overview of collagen fibril organization. Interlocking of gap and overlap regions of neighboring fibrils are observed in sound (Fig 4A) and fluorosed (fig 4B) dentine. Random distribution of fibrils could be revealed with an interwoven network-like structure. In contrast to the overlapping and closely packed fibrils seen with normal dentin, fluorosed dentin structure shows a loose arrangement of fibrils. Therefore with DFM observation the dentin collagen network structure appeared to be a less complex lay out in the severe fluorosed dentine.

DISCUSSION

Dentine is a hydrated biological composite which is the most abundant mineralized tissue in the tooth. Type 1 collagen forms a fibrous three-dimensional network of the matrix[15]. Glycoproteins and proteoglycans covering the collagen fibrils are associated with the inorganic

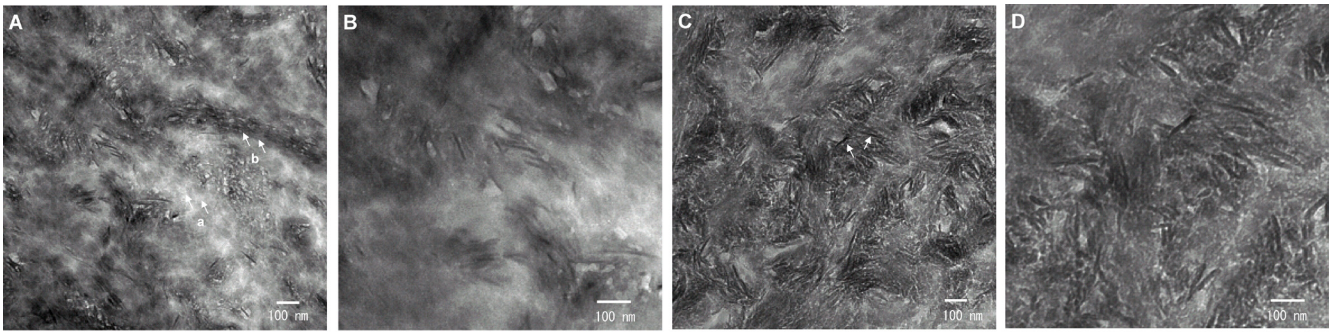

Figure 1 : (A) TEM images of the nondemineralized normal dentine. Mineral crystallites orientation with their c-axes along the collagen fibril is shown with [b] arrows. Less mineral dense areas are pointed with [a] arrows (Magnification x100000).
(B) High magnification of (A) with slender and acicular crystallites (Magnification x200000).
(C) Low magnification TEM image of non demineralized fluorosed dentin showing haphazardly arranged mineral crystallites (arrows).
(D) High magnification of(C) showing 'obese' mineral crystallites.

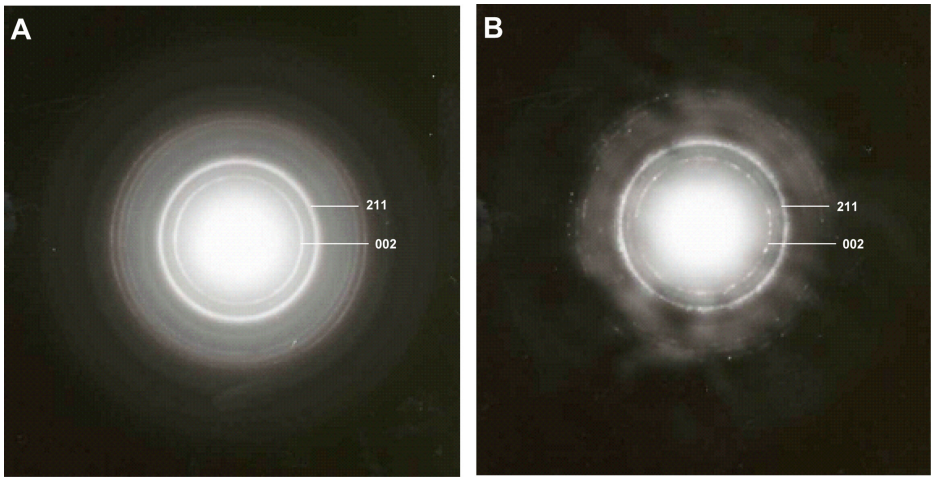

Figure 2 : (A) SAED pattern for the minerals in the normal dentine. Presence of (002) and (300/112 and 211) rings confirmed apatite
(B) SAED pattern obtained for the intertubular mineral apatite in fluorosed dentine; the spots are more clearly delineated.

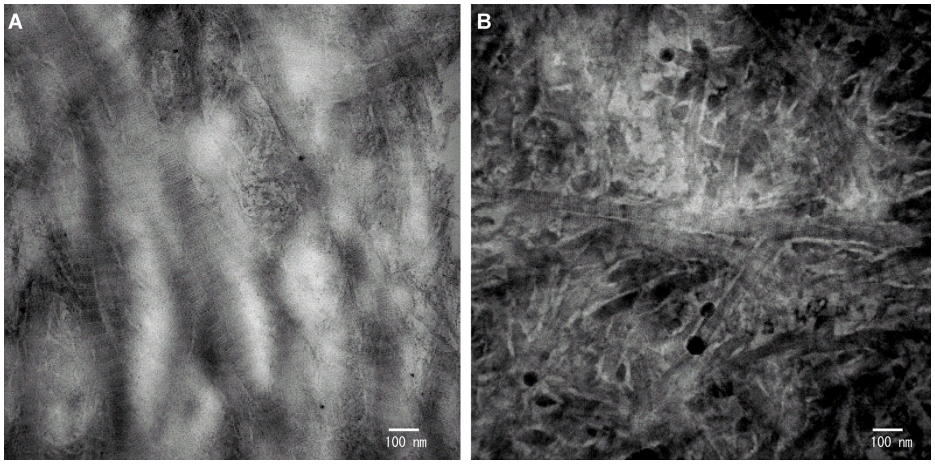

Figure 3 : (A) Transmission electron micrograph of stained, demineralized normal dentine showing the ultrastructure of collagen fibrils with the characteristic axial periodicity 67nm.
(B) TEM image of the fluorosed dentine showing the ultrastructure of collagen fibrils with the characteristic axial periodicity

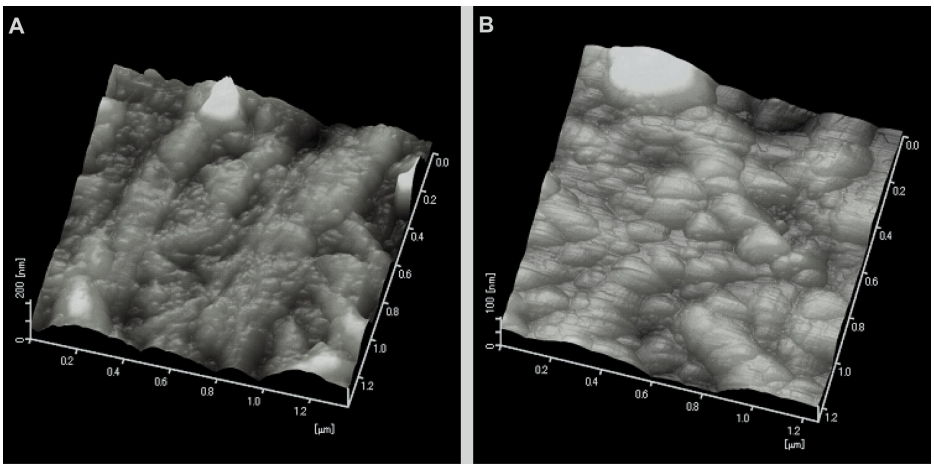

Figure 4 : (A) High magnification tapping mode dynamic force microscopy (DFM) image of the dentine collagen fibrils on intact normal dentine showing the complex arrangement.
(B) High magnification tapping mode DFM image of fluorosed dentine collagen arrangement. In contrast to the overlapping and closely packed fibrils seen with normal dentin fluorosed dentin structures shows a loose arrangement of fibrils.

Table 1. Mean values and standard deviations of the apatite crystallite length, width and collagen fibril axial periodicity of dentine (Nanometers).

	Normal dentine	Fluorosed dentine
Length of crystallites	114.8 ± 31.5	129.4 ± 21.1
Width of crystallites	9.9 ± 3[a]	21.4 ± 3.9[a]
Axial periodicity of collagen fibrils	67 ± 4.4	67.1 ± 3.9

(n = 12; Same superscripts indicate the statistical difference with in each row)

phase. The apatite crystallites in dentine are located either within the gap zones between collagen molecules as intrafibrillar crystallites, or along the surface of the collagen fibrils as interfibrillar crystallites[16].

This study focused mainly on the superficial intertubular dentine of molar teeth where the lesions are characterized as severe fluorosis based on the TFI index. Peritubular dentin was not observed due to its hypermineralized nature and different crystallite diamentions[17]. Normal molar teeth of the subjects who were residing in the same geographical location were used as controls.

A positive correlation has been observed between dental fluorosis severity and the dentine fluoride concentration[18]. Fluoride has been shown to alter the adsorption of proteoglycans and glycosaminoglycans or the non collagenous proteins to hydroxyapatite[19]. This may affect the inhibition of the growth of crystals in various directions that decides the shape[20,21,22]. Thus, we speculate that the increased fluoride level in fluorosed dentine may have altered the composition of the crystal growth-regulating proteins[23,24]. and resulted in an alteration of the crystallite growth regulation giving rise to the significantly increased width of apatite crystallites and uncontrolled orientating of the c-axes of crystals that was observed in severe fluorosed dentine. Along with these the diffraction faded spots which formed the ring diffraction patterns indicate the lower density of crystals in the area[25]. In contrast, the continuous rings of the diffraction pattern in normal dentine did reveal the high density of crytallites in the large selected area with their c-axes perpendicular to the electron beam, a preferred orientation of crystallites probably their c-axes along the collagen fibrils. Therefore the sparsely arranged large crystallites in fluorosed tissue are easily exposed to acid attack than the small crytallites incorporated in to collagen fibrils in normal dentine. These abbreviations in crystals and their arrangement may play a role to trigger the acid attack in fluorosed tissue. Biological apatite minerals in normal human dentine are reported to be in the form of calcium deficient and carbonate-rich hydroxyapatite[20]. Lower calcium content and high carbonate content leads to higher acidic solubility of the dentin apatite crystals[26]. Fluoroapatite is reported to be present in the enamel[27]. Fluoroapatite has a very similar atomic

structure and belongs to the same space group as hydroxyapatite. Although high resolution-TEM (HR-TEM) was not performed in the present study to examine the lattice structure of the mineral phase, indexing of the principal rings of the SAED patterns enabled us to confirm that the crystallites in fluorosed dentine were in apatite form. Carbonated apatite is reported to be much more spherical[28] in shape rather than the plate-like and irregular morphology that is reported with fluoroapatite[29] Therefore with the morphology of crystallites in this study it can be speculated that fluorosed dentine might be made of apatite that is much more towards carbonated apatite rather than the normal dentine and not pure hydroxyl apatite or fluoro-hydroxy apatite which are less soluble in acid. This feature makes it more vulnerable to artificial caries attack than the normal dentine tissue[7].

It has been reported that there is no difference in the dentine crystallite dimensions among three communities with different dentine fluoride concentrations[30]. Also the size of crystallites in normal dentin shows a difference with our results[30]. This may be explained by the different ethnicity of the populations, since the contribution of a genetic component in the pathogenesis of dental fluorosis is well recognized[31]. Lack of a control and the difference in implemented methodology may have also contributed to the results.

Type 1 collagen forms a fibrous three dimensional network which constitutes the bulk of the dentine organic matrix. Altering the conditions under which the assembly of collagen molecules occur may result in the formation of different structures[32]. Fluoride alters the expression and post-translational modifications of extracellular matrix proteins in dentine, acting as an inhibitor of type 1 collagen formation when it is present in high concentrations[33]. In this study, we observed the 67 nm type 1 fibril axial periodicity[34]. In collagen fibrils derived from both normal and the severely fluorosed dentine. But the difference in the complex arrangement structure may have made them a suitable candidate for the action of the released metalloproteinase and other collagenolitic enzymes which are released during the caries process. Further immunohistochemical analysis is necessary for clarification of structure and in understanding the properties of collagen in dental fluorosis.

The caries vulnerable dentin which is mostly exposed to the external environment by chipping off of the enamel in moderate and severe fluorosed dentin reveals the necessity of immediate and durable restorative treatments. Most of the current dentin adhesive systems rely on hybridization with dentin forming a polymerized structure in partially demineralized tissue. Therefore the deviated morphology and arrangement of apatite crystallites and collagen fibrils of fluorosed dentin may not produce the expected bonding durability with the routine dental materials and new appropriate dental materials are necessary for better treatment approaches for fluorosed teeth. This suggests the necessity of further studies on the dentin structure and dental materials for regular treatment of caries in patients with chronic severe endemic dental fluorosis.

In conclusion, chronic endemic severe dental fluorosis caused a significant enlargement and a reduction in density of the apatite crystallites with a random orientation in the dentine of human teeth in comparison with the normal dentine tissue.

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