Evaluation of External Surface Structure, Roughness, and Absolute Depth Profile of Fluorotic Enamel Compared to Healthy Enamel Using Atomic Force Microscope: An *In Vitro* Study

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ABSTRACT

Fluoride has been previously shown to alter the morphology of hydroxyapatite crystals at various levels leading to altered enamel surface.

Aim: To characterize the external surface, roughness, and abs.

Keywords: Absolute depth profile, Fluorotic enamel, Healthy enamel, Surface roughness, Thylstrup–Frejeskov index.

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INTRODUCTION

Enamel development process, called amelogenesis, can be segregated into four phases in healthy state, namely, presecretory, secretory, transition, and maturation. It is in the final phase that an accentuated developmental process is exhibited by ameloblasts, and the secretory protein properties of these cells are subsequently lost. In this stage, any interruption results in enamel pathologies, such as enamel hypoplasia, amelogenesis imperfecta, and dental fluorosis (DF). If dental enamel gets exposed to over-optimal fluoride levels during stages of amelogenesis, it can lead to enamel's mineralization defect known as enamel fluorosis.¹

The process of mineralization is hindered when induced by fluoride. This interference however relies on many factors such as dosage and the duration of fluoride dissemination. Concentration of fluoride in the blood plasma draws a parallel with disturbances of enamel mineralization. Such disturbances in the formation of enamel are induced in most species inclusive of humans by the levels of chronic plasma fluoride intake ranging from 2 to 12 µmol/L obtained by consuming drinking water containing fluoride over prolonged periods.²

From initial stages of subsurface hypomineralization, culminating in severe conditions such as posteruption staining, enamel changes that happen differ from pale chalk-like or white impervious areas to pits and grooves. Fluoride intake as little as 0.03 mg F/kg body weight give rise to enamel fluorosis in children of young age, and this has a visible positive proportionate association between dosage of fluoride and episode of enamel fluorosis, irrespective of fluoride intake from potable water, complimentary sources, or from other external resources. The suggested methods of development of DF include a fluoride effect systemically on calcium homeostasis, deranged protein secretion, compromised biosynthesis of the matrix, direct impact on extracellular proteins, and enzymes such as proteinases besides effects specifically on functioning and metabolic activity of cells.³

In healthy enamel, crystals appear lengthened and the potential of crystal growth including crystal sizes and their shapes are

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regulated well by extracellular matrix proteins in the process of amelogenesis.⁴

The current spotlight of dental materials research is on the physical aspects of structure, surface roughness, and mechanical properties of dentine substrate. The researchers' challenge is to scrutinize the materials' structure–property linkages even on small scales. While other techniques can be used, the uniqueness of the atomic force microscope (AFM) is the need for minimum sample preparation, maintaining the originality of materials and their characteristics. Although a lot of studies have been reported on fluorosis epidemiology, the volume of dental literature on characterization and comparison, especially of external structure healthy as well as fluorosis affected enamel, is minimal.

The purpose of this study is to assess and evaluate the surface roughness as well as the absolute depth profile of fluorotic enamel with healthy enamel through the contact mode of AFM.

PARTICIPANTS AND SAMPLE PREPARATION

This study was carried out in the Department of Pediatric and Preventive Dentistry at Mamata Dental College and Hospital, Khammam, Telangana, India, in collaboration with Centre for Cellular and Molecular Biology, Hyderabad, Telangana, India.

Patients visiting hospitals and private clinics were advised to contribute their third molar teeth that were indicated for extraction after obtaining informed written consent. In total, 20 extracted third molars were collected from the district of Nalgonda and surrounding areas that were considered endemically fluorosed areas (with fluoride concentration ranging from 0.5 to 12 ppm) in the state of Telangana.

The samples were thoroughly cleaned and subjected to strict disinfection via immersion in an ultrasonic cleaner and subsequently cleansed in flowing water and then dried. Through visual observation, they were analyzed to assess the severity of fluorosis following the Thylstrup–Fejerskov index (TFI), which is a 10-point scale that shows zero as nonaffected and 9 as most severely affected.⁵ Random classification of selected molars was done in this line: Group C (TFI = 0), Group MI (TFI = 1–3), Group MO (TFI = 4–5), and Group S (TFI = 6–9). The first observer was blinded while diagnosing conditions of clinical fluorosis in order to avoid bias, following which second observer conducted the valuation of AFM images. The molars chosen for the study were separated into four categories and stored in 0.02% sodium azide till the time experimental procedures were through. Sectioning was done along the buccal surface of each molar having pathologic fluorosis at right angle to the long axis of the teeth. It was done using water-cooled, low-speed diamond to obtain samples with width in the range of 3 mm. Then, the samples were mounted on acrylic blocks, and polishing was performed using a rubber cup.

The samples were then studied in the contact mode under AFM (Multiview 1000, Nanonics Imaging Ltd. Manhat Technology Park, Malcha Jerusalem, Israel), which was considered appropriate for measuring the surface roughness from firm, solid tissues such as dental enamel. Cantilever, 300 μm long, was mounted to a tuning fork having resonance of 36 kHz frequency and spring constant 2,600 $\mu\text{N}/\mu\text{m}$. Tuning fork's oscillation was set in motion, and then changes in amplitude, frequency, quality factor, and phase were monitored consequently. A Cr-coated glass probe with a tip of 10 nm radius was connected to the cantilever (AFM's Super Sensor Probes). A sample area of $40 \times 40 \mu\text{m}$ having an image size 256×256 pixels was scanned for imaging at the rate of 8 ms per point. Using inbuilt WsxM 5.0 Develop 1.1 software, two-dimensional and three-dimensional images depicting surface roughness and distance vs height profiles were produced.

Using S_a and S_y parameters, enamel surface roughness (ESR) was assessed. S_a (average of roughness) represented the arithmetical mean of the scanned surface profile's absolute values, and S_y (peak–valley-height) represented the absolute measure of average of height of five highest peaks and depths of five deepest valleys within the sample length profile on the scanned surface area. Peak-to-valley height is generally termed as S_y .

RESULTS

Mean and standard deviation of ESR presented in nanometers are Group C: 90 ± 25 , Group MI: 170 ± 45 , Group MO: 235 ± 163 , and Group S: 541 ± 121 . Mean ADP of enamel in nm were Group C: $1,559 \pm 359$, Group MI: $2,265 \pm 985$, Group MO: $2,527 \pm 1,719$, Group S: $6,010 \pm 3,439$ as presented in Table 1. It establishes that ESR and ADP raise in tandem with severity of fluorosis. The test Mann–Whitney U test revealed substantial variation in p' . Statistical significance was set at a p value < 0.05 as presented in Table 2. All four groups are shown as three-dimensional images in Figure 1, with the ESR are

presented in Figure 2, and with depth profiles in absolute terms of all four groups given in Figure 3.

DISCUSSION

Fluorosis occurs during odontogenesis due to unwarranted fluoride intake, causing not just alterations in structure but the composition of enamel as well. According to Fejerskov and coworkers and Myhrberg and Sundstrom, the obstruction of the maturation phase of the ameloblasts at an early stage in amelogenesis are the main pathology behind the development of dental fluorosis. Den Besten et al. viewed that regulation of ameloblasts including both morphologies of cells, that is the smooth as well as ruffle-ended, are affected by fluoride in the period of enamel maturation. Besides observing the reduction in smooth, along with ruffle-ended remodeling of ameloblasts, prolonged intake of surplus fluoride impacts the morphology of dental organ as stated by Smith and coworkers in 1993.⁶

Of late, technological advances have enabled in-depth studies of protein sequencing and its conformity vis-a-vis the task of such molecules in regulating crystal growth. Nevertheless, the inorganic partner's role has received less attention, probably because of the activity of candidate proteins being mediated through the interactions between protein species and the defects of a specific crystal lattice. High-resolution scanning electron microscopy (HR-SEM) and high-resolution transmission electron microscopy (HR-TEM) have been studied previously to take images of potential defects and/or growth sites. For HR-TEM, fixed and dehydrated samples are mandatory as they provide only two-dimension images, while HR-SEM generates three-dimensional images, but the latter is noncomputable in Z-axis. Also, plating the samples with metals in order to generate images is almost obligatory in HR-TEM. With the advent of AFM, most of these bottlenecks have been resolved, providing significant breakthrough in high-resolution imaging using a biological approach in many of its applications.⁷

AFM can help investigate the relationship of mechanical properties and its implications at the microstructural platforms at submicron level with the noninvasive treatment of the sample surface. Complementary to other methods, AFM, in fact, is the only recognized microscopy that can offer nanometer resolution on dentine samples within its native conditions, where quantitative measurements such as surface roughness could be used. It has been observed as an analytical apparatus that is not only versatile but more effectual for a scientist of dental materials.⁸ The commonly used methodology in dental research is AFM's contact mode, in which case probe remains in contact with the surface being investigated with the resultant image being a topographical map of the sample surface used in this study.

Owing to a shortage of dental literature in characterizing and comparing the external configuration of both healthy and fluorotic enamel, this study using AFM contact mode was carried out to assess the surface roughness and fluorotic enamel's absolute depth profile with that of healthy controlled enamel.

This study has used a single type tooth to avoid problems of discrepancies in the concentration of fluoride from different tooth types within an individual. Third molars not erupted and hence not disclosed to the niches of oral cavity are assembled owing to their possible characteristic of accumulating high fluoride reserves. Some evidence is available to show that concentration of fluoride is more in teeth, wherein time span from the entire

Table 1: Mean external surface roughness and absolute depth profile of all the four groups

Group	Parameter					
	ESR			ADP		
	Mean	SD	Range	Mean	SD	Range
C	90	25	55–125	1,559	359	535–1,645
MI	170	45	110–280	2,265	985	1,110–503
MO	235	163	125–375	2,527	1,719	1,035–8,925
S	541	121	337–739*	6,010	3,439	1,997–12,671

Table 2: Comparison of *p* values of ESR and ADP between groups

Groups	ESR	ADP
C vs MI	0.0001	0.0001
C vs MO	0.0001	0.0001
C vs S	0.0001	0.0001
MI vs MO	0.0110	0.9349
MI vs S	0.0001	0.0001
MO vs S	0.0001	0.0001

Figs 1A to D: 3D images of the surface roughness of all the four groups. (A) Group C; (B) Group MI; (C) Group MO; (D) Group S

formation of enamel to eruption of tooth is increased to which the cases of third molars could be an example. Here, mineralization occurs comparatively belatedly and stay frequently nonerupted for prolonged durations. One can expect a 6-years-long period at an average from completion of enamel formation to the eruption of a tooth.⁹

The present study uses TFI as the index to owing to its property to clinically relate the fluorotic appearance of the enamel with pathological changes, the same being the index of preference for assessing various severity of fluorosis. On a scale of 10 points, zero stands for the unaltered tooth and 9 represents the tooth most rigorously affected. Mean ESR and standard deviation in nanometers were: Group C: 90 ± 25 , Group MI: 170 ± 45 , Group MO: 235 ± 163 , Group S: 541 ± 121 . Mean ADP of enamel in nanometers was as follows: Group C: $1,559 \pm 359$, Group MI: $2,265 \pm 985$,

Group MO: $2,527 \pm 1,719$, Group S: $6,010 \pm 3,439$. This establishes that ADP and ESR increase in line with fluorosis intensity.

These results show the mean and standard deviation of the ESR and the enamel's absolute depth profile observed under AFM in the four groups referred to earlier. Also, in the ESR values, substantial statistical differences were seen in all groups. Nevertheless, ADP values among MI and MO groups were statistically nonsignificant. As for the diameter of crystals on enamel, fluorotic enamel crystals are higher than that of the usual healthy enamel crystal as confirmed by HR-electron microscope (60–70 nm for crystals afflicted by fluorosis vis-a-vis 40–50 nm for crystals that remain unaltered), using technique of X-ray diffraction on enamel samples that were pulverized (mean values, being 21 nm width for crystals with fluorosis and 16 nm for unaffected crystals) or using a scanning microscope on fragmented samples of enamel from the inner aspect (mean values for crystals with fluorosis was 10 nm and for unaltered healthy crystals, it was about 4 nm wide).¹⁰

In an in-depth study of severe human enamel fluorosis, the densely hypermineralized surface seemed to contain flat hexagonal crystals (3,050 nm thick and 60–100 nm wide) besides numerous minute unevenly formed crystals that were habitually not as much of, width of 10 nm and thickness of 10 nm as stated by Yanagisawa and coworkers in 1989. Images depicting higher resolution showed exceptionally petite crystals attached and emerging on the large crystal's surfaces. In the hypomineralized subsurface, crystals were quite less, majority of which were bigger of about 22 nm of thickness and 70 nm of width, with hardly any minute crystals being noticed. Of those crystals with larger dimensions, some showed discontinuity approximating a gap formation on the central aspect, large in size, alongside the *c*-axis, while many of the smaller crystals were partially liquefied (in line with the length of their *a*- and *b*-axes), indicative of dental caries.¹¹

Viewed by AFM at nanoscale, however, fluorotic crystals were rough in surface more than that of the nonfluorotic crystals. The roughness of crystal surface likely to be seen extremely small on the surface of large crystals became pronounced, as the fluoride levels of drinking water increased. Thus, small persistent fluoride level within a range of micro-moles that show the initial quantifiable indication of fluorosis does not seemingly alter the size of crystal at macro-scales, although they impact the structural properties of crystal at nano-metric level.¹² Considering the mean of surface enamel ADP and ESR, least values for Group C and higher values for Group S were seen agreeing to the findings of veronica et al. These are also in agreement with the studies reported hitherto at nanoscale, observed in AFM wherein fluorotic crystals had high surface roughness than unaffected crystals.⁷

Between ESR and ADP among various groups, obvious differences were noticed. Although there was a resemblance in the structure amid MI and MO groups in ADP, ESR was proven to be more liable to externally scrutinize structural alterations in

Figs 2A to D: Absolute depth profile of all the four groups. (A) Group C: 1559 nm; (B) Group MI: 2265 nm; (C) Group S: 6010 nm; (D) Group MO: 2527 nm

enamel surface affected by fluorosis. The roughness of crystals, more so the small crystals observed at larger crystal surfaces (Yanagisawa et al., 1989), varies through the fluoride concentration in potable water (Chen et al., 2006). Thus, persistent low fluoride levels, especially in range of micro-moles, cause the earliest quantifiable indication of fluorosis, which fail to contribute to changing the crystal size but the impact the surface features of crystals at nano-levels, thus paving the way for the first clinical sign of enamel fluorosis.¹³

According to Veronica et al., AFM's three-dimensional images for different groups differ further in topography, thereby supporting a direct relationship between the parameters under consideration, i.e., S_a and S_y representing surface roughness and depth respectively with that of fluorosis severity. Fluorotic enamel, similar to the normal enamel, is characterized by the concentration gradient of fluoride, which is higher near the surface compared to its middle. Increased fluoride concentration can be observed near dentin–enamel junction too. The more severe the fluorosis, greater the fluoride accumulation on the enamel, but it still maintains a density titer along the surface of enamel¹⁴ (Figs 1 to 3).

Despite using adhesive techniques and materials, some of the treatments do not prove effective due to mishandling of material or etched enamel's inability to sufficiently form retentive

mechanisms. The adhesive method used in dentistry depends on the irregularities of the surface caused by the conditioning of phosphoric acid.¹⁵ The ability to get a suitably etched enamel surface is inhibited by fluorosis. Healthy enamel alone can act against phosphoric acid being etched to raise the surface energy, and it happens by creating micro-porosities, which leads to the mechanical interlocking required for adhesion that does not work on fluorotic enamels. The outer layer of fluorotic enamel is highly mineralized and resists acid dissolution with subsurface porosity and for improving adhesive properties of restorative materials deproteinized by sodium hypochlorite¹⁶ which is followed by acid etching and microabrasion of fluorotic enamel surface is advocated.¹⁷

The present study confirms clinical observations at nano-metric level about the enamel in health and that afflicted by varying gradations of fluorosis. In order to provide details of quantitative topology in three dimensions for similarly affected enamel in developmental disorders such as amelogenesis imperfect, enamel hypoplasia, etc., further studies are warranted, especially on parameters such as ESR and ADP of crystallized enamels at various stages of amelogenesis. It is because AFM may be an appropriate accessory device in determining mechanisms like crystal size control and/or skeletal tissue morphology.

Figs 3A to D: External surface roughness of all the four groups. (A) Group C: 90 nm; (B) Group MI: 170 nm; (C) Group MO: 235 nm; (D) Group S: 541 nm

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