Atomic force microscopic comparison of remineralization with casein-phosphopeptide amorphous calcium phosphate paste, acidulated phosphate fluoride gel and iron supplement in primary and permanent teeth: An *in-vitro* study

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Abstract

Context: Demineralization of tooth by erosion is caused by frequent contact between the tooth surface and acids present in soft drinks. **Aim:** The present study objective was to evaluate the remineralization potential of casein-phosphopeptide-amorphous calcium phosphate (CPP-ACP) paste, 1.23% acidulated phosphate fluoride (APF) gel and iron supplement on dental erosion by soft drinks in human primary and permanent enamel using atomic force microscopy (AFM). **Materials and Methods:** Specimens were made from extracted 15 primary and 15 permanent teeth which were randomly divided into three treatment groups: CPP-ACP paste, APF gel and iron supplement. AFM was used for baseline readings followed by demineralization and remineralization cycle. **Results and Statistics:** Almost all group of samples showed remineralization that is a reduction in surface roughness which was higher with CPP-ACP paste. Statistical analysis was performed using by one-way ANOVA and Mann-Whitney U-test with $P < 0.05$. **Conclusions:** It can be concluded that the application of CPP-ACP paste is effective on preventing dental erosion from soft drinks.

Keywords: Atomic force microscope, dental erosion, remineralization

Introduction

Dental erosion is a destructive process that renders the tooth surface hypomineralized, leading to an irreversible loss and its progressive softening thereby increases its susceptibility toward the mechanical forces.[1,2] Epidemiological studies had shown the prevalence of dental erosion in children which varies widely, ranging from 2% to 57% respectively.[3-5]

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The sucralose soft drinks report published annually suggests that consumption of soft drinks had increased consistently over past 15 years.[6] Such drinks causes a drop in plaque pH and on prolonged exposures leads to dental erosion.[7] According to Jaeggi and Lussi distribution of erosion in the primary dentition is on occlusal surface of molars predominantly and scarcely on the facial surface of maxillary incisors although in the permanent dentition it is seen on palatal surface of maxillary incisors.[8,9] Besides that the susceptibility of teeth toward erosion also accounts on its mineral content, porosity and degree of enamel microcrystal arrangement as primary teeth been the susceptible one.[10] Eventually erosion is complete dissolution of hydroxy-apatite (HAP) that occurs layer by layer while carious lesion formation occur by partial dissolution of HAP.[11]

The substantial reduction in the intake of such beverages is imperative to restrain the process of erosion.[12] Certain bioactive agents for remineralization are also advocated including non-fluoridated agents like casein-phosphopeptide (CPP) with amorphous calcium phosphate (ACP) which forms a protective layer and inhibits demineralization.[13,14] Recently introduced iron supplement result in precipitation of ferric phosphate on the enamel, rendering it more resistant to erosion.[15,16] Correspondingly the iron remineralizes eroded enamel by apatite nucleation and substitution of calcium in apatite as well.[17] Moreover most widely used are fluoridated agent like acidulated phosphate fluoride (APF) gel which acts by deposition of calcium fluoride (CaF_2) predominantly on the enamel surface which successfully transformed into fluorapatite crystals.[18,19]

Dental erosion has been widely investigated through a number of macro-microscopic techniques. The novel one is atomic force microscope having images in three dimensions, at atomic resolution with minimal sample preparation.[20] On the contrary scanning electron microscope (SEM) has the ability to image very rough samples due to its large depth of field and lateral field of view.[21] Atomic force microscopy (AFM) is widely recommended to study the bio-minerals and also applied to measure the early stages of enamel loss.[22] But contemporary the remineralization of erosion cavities is not widely investigated using AFM.

Thus, this paper aim to evaluate the remineralization potential of CPP-ACP paste, 1.23% APF gel and iron supplement on dental erosion produced by soft drinks in human primary and permanent enamel using AFM.

Materials and Methods

A total of 30 teeth specimens were used in the study, of which 15 were primary teeth and the remaining 15 permanent teeth. After extraction, teeth were cleaned of soft-tissue debris by ultrasonic scaler, inspected for cracks, hypoplasia or any white spot lesion under light microscope 10X (iNEA Olympus Pvt. Ltd., New Delhi). Moreover disinfected in 5% sodium hypochlorite for 1 h and stored in de-ionized water (pH = 7.413). The specimens were sectioned transversely in the cervical area to separate crown from the root and sliced longitudinally for the labial portion using diamond disc (Dentorium, Germany) with a water spray. The labial segments of tooth specimens were placed in die stone mold measuring 10 mm × 8mm × 2mm in dimension and embedded in acrylic resin (Dentsply, New Delhi). The labial surface of each enamel specimen was grounded using silicon carbide papers (3M products) with consecutive grades of 600, 1000 and 1200 under water irrigation to remove 50-100 μm for producing a flat surface.[23]

The pH of soft drink was determined using digital pH meter (U-Tech, Singapore). The primary and permanent teeth specimen were randomly divided into three groups (with $n = 5$). Then specimens were subjected to a demineralization cycle by immersing in 6 ml of soft drink for 2 min stirred at constant speed of 120 rpms[24,25] at room temperature at four consecutive intervals of 6 h imitating the meal time. The application of remineralizing agents were carried out at 0, 8, 24 and 36 h intervals[23] according to manufacturer's instruction and stored for further assessment. In between the experiment the specimen were stored in de-ionized water. The AFM observations were made after each phase i.e. baseline, demineralization and remineralization.

Group I: CPP-ACP paste-applied with applicator tip/brush for 3 min[23]

Group II: APF gel-applied with applicator tip/brush for 4 min[26]

Group III: Iron supplement-freshly prepared 6 ml 10 mM/L ferrous sulfate solution/specimen for 3 min.[27]

Atomic force microscope AutoProbe CP 100 (Themormicroscopes, Veeco, USA) used for the assessment of surface roughness specimens equipped with a piezoelectric scanner of 100 mm × 100 mm with a range of 7 mm in the z-direction. The root-mean-square roughness (R_{rms}) was obtained from the AFM investigations with a film area of 30 μm × 30 μm and resolution of 256 × 256 pixels. The labial surface of each sample was analyzed for at least 10 different sites.[23] Differences in the mean values among the groups were analyzed by one way ANOVA and Mann-Whitney U-test with $P < 0.05$.

Results

The pH of soft drink was 2.27 ± 0.04 at 25°C. The mean R_{rms} of enamel surface at each phases of treatment were shown in Table 1. The R_{rms} values of un-exposed (baseline) samples were in the range of 103.287 ± 5.4-129.406 ± 64.7 nm and 127.9 ± 61.4-170.776 ± 80.7 nm in primary and permanent teeth respectively. After demineralization, all groups showed an increase in R_{rms} while on application of remineralizing agent a marked reduction in R_{rms} was present in Group I (CPP-ACP paste) with 74.709 ± 19.01 and 125.17 ± 27.4 nm followed by Group II (APF gel) with 66.357 ± 25.1 and 116.259 ± 30.7 and Group III (Iron) with 146.589 ± 29.6 nm in permanent teeth while an increase in R_{rms} was seen with 152.696 ± 98.5 nm in primary teeth. The change in Δ R_{rms} i.e. Δ UNEXP-DEMIN, Δ UNEXP-REMIN, Δ REMIN-DEMIN was statistically not significant in CPP, APF and Iron Group (intra-group) for primary teeth and permanent ($P > 0.05$ ANOVA) as shown in Table 1.

When the changes in R_{rms} were compared between the groups (inter-group: CPP, APF and Iron Group), differences were statistically not significant for both primary and permanent teeth as shown in Figure 1. When primary and permanent teeth were compared, there were no significant differences in Δ R_{rms} for different groups ($P > 0.05$ Mann-Whitney U-test).

In all the groups, unexposed samples was quite smooth except for some scratches. All the demineralized samples showed "honey-comb" appearance which on remineralization undergoes surface deposition as shown in Figures 2 and 3 of primary and permanent teeth respectively.

Discussion

The aprisamtic outermost layer of enamel had a dense arrangement, less permeable because of its high mineral

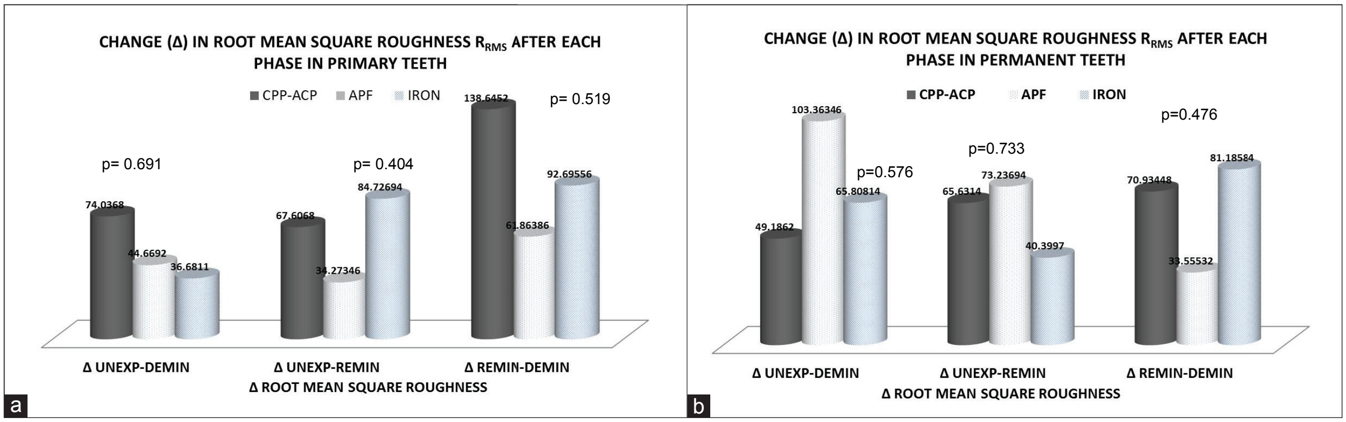

Figure 1: Change in root mean square roughness after each phase (unexposed, demineralization and remineralization) within all the treatment groups of (a) promary and (b) permanent teeth

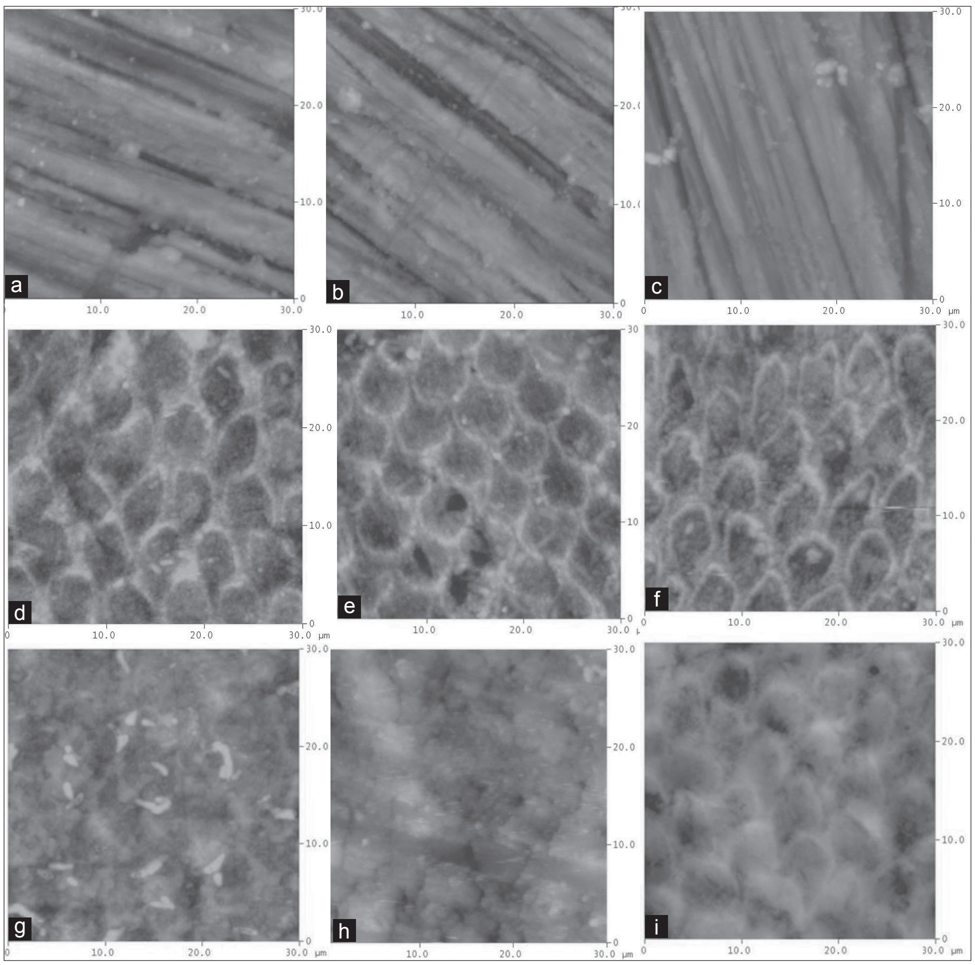

Figure 2: Primry tyeeth; (a,b,c) unexposed sample of group I, II,III; (d,e,f) demineralized sample of group I,II,III; (g,h,i) remineralized sample of group I,II,III respectively

content and gradually wears off.[23,28,29] To standardize the specimens were grounded flat and polished, eliminating natural variations from enamel surface.[10,23,30] Further specimens were stored in de-ionized water which is in accordance to the technical report of the ISO (1991).[31] The complexity of the oral environment was not stimulate as the artificial saliva enhances the action of CPP-ACP paste by interacting with hydrogen ions and forms calcium hydrogen phosphate lead to enamel mineralization.[32] Remineralization cycle was carried out at an interval of 0, 8, 24 and 36 h so as to stimulate the application of any topically applied remineralizing agent.[23]

Table 1: Mean surface roughness values (nm) and within group comparison using one way ANOVA

Group (*n*=5)	Mean (VHN)±SD			ANOVA
	Unexposed	Demineralization	Remineralization	
Primary				
CPP-ACP	111.521±14.1	138.916±28.38	74.709±19.01	0.593*
APF gel	103.287±5.4	111.592±23.9	66.357±25.1	0.622*
Iron	129.406±64.7	130.227±42.4	152.696±98.5	0.264*
Permanent				
CPP-ACP	170.776±80.7	175.476±44.8	125.17±27.4	0.802*
APF gel	127.9±61.4	132.712±42.2	116.259±30.7	0.527*
Iron	137.735±20.9	158.582±36.9	146.589±29.6	0.546*

*Not significant $P<0.05$. VHN: Vickers hardness number; CPP: Casein-phosphopeptide; ACP: Amorphous calcium phosphate; SD: Standard deviation; APF: Acidulated phosphate fluoride

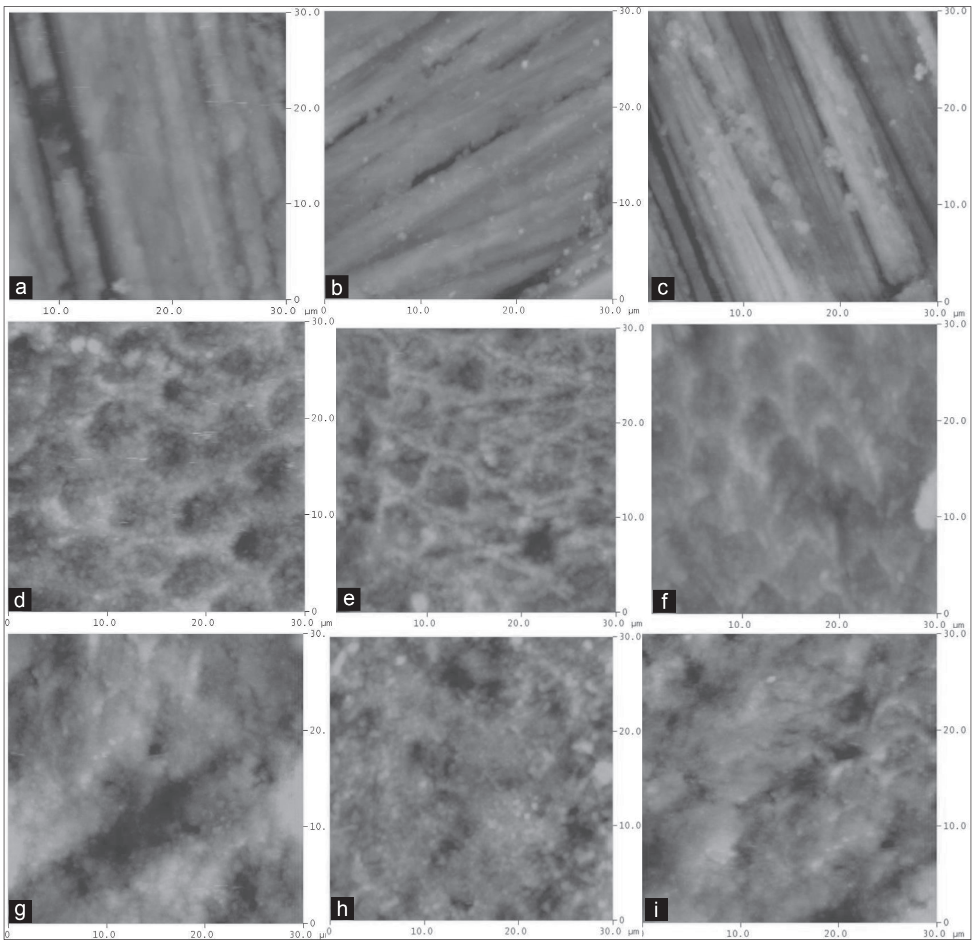

Figure 3: Permanent teeth; (a,b,c) unexposed sample of group I, II,III; (d,e,f) demineralized sample of group I,II,III; (g,h,i)remineralized sample of group I,II,III respectively

The baseline R_{rms} of the present study was higher than the previous studies; with a range of 49-56 nm by Murakami *et al*. but was almost similar according to Quartarone *et al*. with a range of 50-120 nm.[20,33] This variation in surface roughness accounts on anatomical topography of tooth varying with geographical location, age, oral environment present. Following demineralization no significant difference was present in mean surface roughness of primary and permanent teeth respectively. Previous study had similar mean surface roughness with a range of 140-290 nm after demineralization.[23] A marked reduction in surface roughness was present in Group I of primary teeth when compared with permanent teeth but was not statistically significant. The present result elucidated the post-eruptive enamel maturation advocating that the "older" tooth exposed to the oral environment for longer duration encounter acid and fluoride exposure rendering it more resistant. Also increased porosity and removal of aprismatic layer results primary enamel more reactive and enhance surface deposition.[28]

A considerable decrease in R_{rms} was seen after remineralization with CPP-ACP paste (Group I) of both primary and permanent teeth but was not statistically significant which was in accordance to Poggio *et al*. and Quartarone *et al*.[20,23] CCP-ACP paste results in formation of a layer that fills interprism cavities caused by erosion.[19] While due to its acidic nature APF gel further results in an increase in erosion cavities. Moreover, the presence of iron results in substitution of a phosphate group by carbonate thus confronts the negative influence of iron on enamel remineralization[34] which is confirmed by Alves *et al*.[17] Iron supplement showed increased in surface roughness instead which was explained by Weiss *et al*. as mature enamel had a greater affinity toward iron.[35]

Present *in-vitro* study was designed to assess remineralizing agent on eroded enamel surface using AFM which is not addressed in literature till now. AFM was used in the present study to verify protective effect of remineralizing agent on eroded enamel surface with images of high contrast and resolution.[23] According to Poggio *et al*., AFM analyzes topographical aspects of the tooth surface qualities like when enamel was treated with CPP-ACP paste, a collagen network with precipitated crystals was evident which was further confirmed by SEM.[36] Similarly Quartarone *et al*. showed that the CPP-ACP paste significantly reduces the erosion cavities depth. This was assessed by AFM data analysis through scaling concept and continuum stochastic equations which revealed the remineralizing mechanism of calcium phosphate paste.[20]

Thus, CPP-ACP paste showed a reduction in surface roughness than APF gel followed by iron supplement both in primary and permanent teeth but was not statistically significant. Moreover, CPP-ACP paste can be self-applied and expected to be effective intraorally as saliva will enhance the action of CPP-ACP according to the manufacturers. Hence been a very promising remineralizing material.

Conclusions

The following conclusions were derived:

1. All the samples showed an increase in surface roughness after demineralization and primary teeth shows greater demineralization but were not statistically significant
2. Reduction in surface roughness after remineralization seen in all samples except iron supplement group of primary teeth
3. Greater remineralization was present in samples of CPP-ACP paste than APF gel and iron supplement but was not statistically significant.

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