Effect of Remineralization Potential of ACP–CPP with Fluoride and ACP–CPP on Enamel Subsurface Lesion in Primary and Young Permanent Teeth: *In situ* Study

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ABSTRACT

Purpose: To compare the remineralization potential of amorphous calcium phosphate-casein phosphopeptide (ACP–CPP), ACP–CPP with fluoride and placebo group in an *in situ* model concerning enamel subsurface lesion in primary and young permanent teeth.

Materials and methods: This randomized double-blind crossover *in situ* study was conducted for 21 days. Fifteen healthy children in the age group of 12–15 years with a mean age of 13.5 years wore acrylic palatal appliances having two rows of four demineralized enamel slabs. The left row consisted of the enamel slabs of primary teeth, and the right row consisted of enamel slabs of young permanent teeth. Changes in the enamel microhardness and surface roughness were assessed using surface microhardness assessment analysis and Scanning electron microscopy in three different groups (group I ACP–CPP with fluoride, group II ACP–CPP, and group III Nonfluoridated toothpaste).

Results: Mean change in microhardness was higher in enamel slabs of primary teeth as compared to enamel slabs of young permanent teeth. Group I (ACP–CPP with fluoride) showed maximum microhardness (p 0.579). Statistically, the insignificant difference was found between group II (ACP–CPP) (p 0.739) and group III (non-fluoridated toothpaste) (p 0.011). SEM evaluation revealed mean scores were minimum at baseline and maximum after demineralization. After remineralization, mean scores were minimum in group I (ACP–CPP with fluoride) (3.94) and maximum in group III (nonfluoridated toothpaste) (6.94).

Conclusions: ACP–CPP with fluoride showed maximum remineralization potential between primary and permanent enamel groups as compared to ACP–CPP alone and nonfluoridated toothpaste.

Keywords: Amorphous calcium phosphate-casein phosphopeptide, Primary teeth, Remineralization, Surface microhardness, Young permanent tooth.

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INTRODUCTION

Prevention of dental caries is always the first line of treatment but once the demineralization has set in, the first basic step is the remineralization of early carious lesions, advocating a biological approach rather than the traditional radical treatment for early subsurface lesions. One of the key elements of a biological approach is the usage and application of remineralizing agents to the tooth^{1,2}. Prevention is the most important tool to manage at caries risk tooth surfaces and incipient caries. These lesions should be treated by noninvasive approach remineralization can be achieved effectively with bioavailable calcium, phosphate, and fluoride ions to restore the strength and esthetic appearance of the lesion^{3,4}. Therefore, the present study aimed to compare the remineralization potential of CPP–ACP, CPP–ACP with fluoride and placebo group in an *in situ* model concerning enamel subsurface lesion in primary and young permanent teeth.

MATERIALS AND METHODS

Preparation of Enamel Slabs

This randomized double-blind crossover *in situ* study was conducted after obtaining clearance from the university ethical committee. Blinding was done by masking the exterior look of the tubes by painting them and labeling A, B, and C respectively to all the samples. Sample A was (ACP–CPP with fluoride tooth mousse for local application), sample B was (ACP–CPP tooth mousse for local application), sample C (nonfluoridated toothpaste as a tooth mousse) (Flow chart 1).

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Preparation of Enamel Slabs

Enamel specimens of dimensions 4 mm x 3 mm x 2 mm were obtained using the water-cooled diamond disk from the cervical third of the labial/buccal surface of sound human premolars extracted for orthodontic reasons and primary mandibular incisor teeth and were mounted in the acrylic slab. Baseline surface microhardness (SMH) and scanning electron microscope (SEM) analysis of the slabs for the baseline data were done (Fig. 1).

Flow chart 1: Consort Flowchart of study

Figs 1A and B: (A) Palatal appliance having left a row of deciduous teeth enamel slab and the right row of young permanent teeth enamel slab; (B) Enamel specimens were mounted on acrylic resin blocks

Creation of Artificial Subsurface Lesion

Enamel slabs were kept in the plastic vials containing demineralizing solution; SMH and SEM reading for demineralized enamel slabs were noted. Sixty enamel slabs in subjects were randomly divided into three groups in which each group had 20 slabs.

Fabrication of Intraoral Appliances

Acrylic palatal appliances containing two rows of 4 demineralized enamel specimens (left row: primary teeth; right row: young permanent teeth) (Fig. 1B).

Subject Recruitment

Fifteen healthy children in the age group of 12–15 years (mean age–13.5 years) took part in 21 days study period. The children wore acrylic palatal appliances and changes in the enamel microhardness and surface roughness were assessed using SMH analysis and SEM.

The inclusion criteria for the study were the absence of any clinically detectable carious lesions, children with good general health and those who agreed to brush their teeth with the assigned study products twice a day.

Surface Microhardness Assessment and Demineralization

Enamel specimens were mounted on acrylic resin blocks, and their outer surfaces were polished flat with 320,420,600 grades of silicon carbide abrasive papers and the final polishing was completed using 1 μ alumina paste at low speed before obtaining baseline SMH values (Fig. 1B). Vicker's hardness number (VHN) was determined by making an indentation in each specimen using a square-based diamond pyramid Vicker's indenter under a load of 500 g for 15 s (Fig. 2). All specimens were then immersed into demineralized water (pH 2.8) for 5 days and incubated at 37°C followed by a second SMH assessment (Fig. 2).

Fig. 2: Vicker's hardness number (VHN) was determined by making an indentation in each specimen using a square-based diamond pyramid

In Situ Remineralization of Demineralized Enamel Specimens

Pre-reading for VHN for demineralized enamel slabs was noted. For 7 days initial washout period; all the children included in the study were given a nonfluoridated toothpaste for brushing twice per day under supervised conditions with the help of parents. At the end of the washout period, the children were delivered palatal appliance with the demineralized enamel slabs mounted on the palatal surface. Children were given instructions regarding wearing them full day along with the cleaning instructions. During the study period, parents were contacted telephonically to check whether children were following instructions regularly. All testing samples ($n = 60$) were coded and blinded for the investigator and volunteer. Samples were randomly divided via chit method into three groups ($n = 20$ in each group); group I—used sample A for local application, group II—sample B and group III—nonfluoridated toothpaste as mousse. During the study period of three weeks, children brushed their teeth for 2 minutes with appliances in the mouth with the non-fluoridated toothpaste (half the length of toothbrush head). After brushing pea size of respective tooth mousse samples (A, B and C) was applied on enamel slabs with the help of dry finger/cotton tip and instructed to be left undisturbed for 3 minutes and then spread throughout the mouth; to expectorate and avoid rinsing. At the end of the study period (21 days), the appliances were taken from children and enamel slabs were removed with the help of acrylic burs, washed with deionized water, and stored in moist conditions in duly labeled plastic vials for SMH and SEM analysis (Fig. 3).

Statistical Analysis

The microhardness values of different groups were compared using the Kruskal–Wallis test. The micrographs were graded according to 8 parameters that were flatness and smoothness of surface, homogeneous of enamel crystals, crystal irregularity, porosity, micro defects, crystal outline, clogging and fusion of crystals, amorphous shape and disorderly arrangement of crystals on a scale 0–9 (0–baseline value; 9–lowest value) regarding quality of that parameter; studied by two blinded examiners and interexaminer reliability was observed.

RESULTS

The percentage changes in surface microhardness values in baseline to demineralization showed no statistically significant

difference between enamel slabs of deciduous and young permanent teeth ($p > 0.05$) (Table 1 and Graph 1). Percentage changes in demineralization to remineralization; mean change was higher in enamel slabs of deciduous teeth as compared to enamel slabs of young permanent teeth for overall and group I (ACP–CPP with fluoride).

All enamel slabs in group I (ACP–CPP with fluoride) showed maximum SMH after remineralization (VHN) 10.36 ± 14.49 followed by group I 3.02 ± 2.53 and group III 1.37 ± 1.47 ($p = 0.149$). Both ACP–CPP and ACP–CPP with fluoride tooth mousse were able to remineralize statistically significantly better than placebo tooth mousse ($p = 0.001$). Deciduous enamel slabs in group I showed maximum SMH after remineralization (VHN) (14.90 ± 19.13) and (5.81 ± 5.56) followed by group II (3.18 ± 3.16) and group III 0.67 ± 0.55 (p value 0.436). In young permanent teeth enamel group I more remineralization was seen followed by groups II and III with percentage change 5.81 ± 5.56 , 2.86 ± 1.86 , 2.07 ± 1.77 respectively. But the difference between them was not statistically significant. SEM evaluation revealed mean SEM scores to be minimum at baseline and maximum after demineralization. After remineralization, mean scores were minimum in group I (3.94) and maximum in group III (6.94). Statistically, intergroup differences were significant ($p < 0.001$)** (Table 2 and Graph 2).

Graph 1: Group wise comparison of % change in SMH between enamel slabs of deciduous and young permanent teeth at different stages

Fig. 3: SEM micrographs of enamel surfaces of deciduous and young permanent teeth

Graph 2: Comparison of SEM scores between enamel slabs of deciduous and enamel slabs of young permanent teeth

DISCUSSION

Dental caries is a complex form of hard tissue pathology controlled by multiple factors. Cariogenic bacterial count, the status of normal healthy flora, protective salivary factors, dietary sources of fermentable carbohydrates and individual ionic ecosystem of the

oral cavity are few of many factors responsible for caries. Subsurface enamel demineralization is the result of complex physical and chemical interaction between the enamel surface and salivary oral environment⁵⁻⁷. The main factors known to control the stability of enamel hydroxyapatite are the pH status and availability of free active calcium, phosphate and fluoride concentrations in oral fluids^{8,9}.

Over the last few years, interest has been evoked in preservation and restoration of minerals of dental tissue by using products that can enhance the bioavailability of free active calcium, phosphate, and fluoride ion concentrations in the oral environment^{10,11}.

Fluoride is a proven key element in noninvasive management of early noncavitated carious lesions by remineralization and prevention of caries. The ability of fluoride to promote net remineralization is largely dependent on the availability of calcium and phosphate ions¹². Biomimetic approaches to stabilize available calcium, phosphate, and fluoride ions are therapeutic for non-cavitated caries lesions for controlled healing by remineralization. This process is the basis of noninvasive management of initial early carious lesions¹.

Recently, a range of novel calcium-phosphate-based remineralization delivery systems has been developed for clinical application. CPP–ACP slows the progression of caries as it has an ability to increase the incorporation of fluoride into plaque and to promote enamel remineralization *in situ*¹². Many studies on demineralization/remineralization have been conducted in

Table 1: Group wise comparison of the percentage change in SMH between enamel slabs of deciduous and young permanent teeth at different stages

S. No.	Time gap	Deciduous		Young permanent		Significance of difference (Mann–Whitney U test)	
		Mean	SD	Mean	SD	Z	p
Baseline to demineralization							
1.	Overall	–22.85	28.75	–9.28	7.47	1.375	0.169
Demineralization to remineralization							
1.	Overall	6.25	12.52	3.58	3.78	0.961	0.337
2.	Group I (ACP–CPP with fluoride)	14.90	19.13	5.81	5.56	0.605	0.579
3.	Group II (ACP–CPP)	3.18	3.16	2.86	1.86	0.378	0.739
4.	Group III (Placebo)	0.67	0.55	2.07	1.77	2.495	0.011

Table 2: Comparison of SEM scores between enamel slabs of deciduous and young permanent teeth

Sl. No.	Condition	Deciduous (n = 9)		Young Permanent (n = 9)		Significance of difference (Mann–Whitney U test)	
		Mean	SD	Mean	SD	z	p
1.	Baseline	0	0	2.56	0.53	3.890	<0.001
2.	Demineralization	8.89	0.33	7.67	0.71	3.218	0.001
3.	Group I (ACP–CPP with fluoride)	4.89	0.78	3.00	0.71	3.404	0.001
4.	Group II (ACP–CPP)	5.89	0.78	4.11	0.78	3.277	0.001
5.	Group III (Placebo)	7.56	0.73	6.33	0.87	2.612	0.011
6.	Total (n = 45)	5.44	3.14	4.73	2.10	1.921	0.055

young permanent teeth and rarely in deciduous teeth^{13,14}. As a pediatric dentist we have to deal more with caries lesions in young permanent and deciduous teeth enamel, so an *in situ* study was planned to compare the remineralizing potential of CPP–ACP with fluoride tooth mousse CPP–ACP tooth mousse and a placebo tooth mousse in artificially demineralized deciduous and young permanent enamel.

Surface microhardness value and scanning electron microscopy examination to assess changes at an ultrastructural level were selected as a method to assess mineralization of enamel at baseline, after demineralization, and after remineralization. SMH is an indirect technique; however, SEM required the gold coating in which samples once used could not be evaluated again, and hence baseline, demineralization and different type of remineralization studies could be done in separate samples only^{15,16}. The clinical part of the study ascertained that the specimens were obtained from a homogeneous population with similar dietary and oral healthcare; as the effect of dietary habits and oral healthcare on demineralization has been reported¹⁷.

In the present study, after artificial demineralization, mean percentage (VHN) loss of mineralized tissue from primary enamel was more than from young permanent enamel ($p = 0.169$). As deciduous enamel have greater susceptibility to dissolution during an initial linear stage: $1.5 \pm 0.5 \times 10^{-10}$ mol mm^{–2} min^{–1} compared with $2.6 \pm 0.5 \times 10^{-11}$ mol mm^{–2} min^{–1} for young permanent enamel¹³. Also the lower level of Ca and P, thinner thickness and higher numeral density of rods in deciduous enamel as compared to permanent teeth¹⁸. The mineral content for deciduous enamel is 81.3–94.2 wt%, while it is 97% in permanent enamel which is close to pure synthetic apatite^{19,20}. All this along with more accessible pore volume in partially demineralized

deciduous enamel contributes to the subsequent mineral loss which is more in deciduous enamel²¹.

In the present study, group I showed maximum SMH after remineralization followed by groups II and III; which shows that CPP–ACP with fluoride had more potential to remineralize the artificially demineralized tooth enamel ($p = 0.149$). The reason for remineralization in young permanent teeth even in case of control is that there are many different agents within saliva and plaque that serve to protect the tooth surface against caries development. Whereas saliva only could not remineralize excessively demineralized deciduous enamel; may be the localization of remineralizing sites were lost beyond repair and it needs more supply of therapeutic agents for remineralization²².

In SEM micrographs visual examination analysis of present study, deciduous enamel showed lowest (highly mineralized) baseline grade (0) value but subsequently for every step showed a higher mean value (indicating higher demineralization) as compared to young permanent enamel, thus indicating that demineralization affects deciduous enamel more severely than young permanent enamel and remineralization is less effective in deciduous enamel as compared to young permanent enamel.

Present study results show the requirement of calcium and phosphate ions for effective remineralization during therapeutic fluorides application. CPP–ACP containing fluoride dentifrices help the localization process of calcium and phosphate ions with fluoride ions at the tooth surface indicating the formation of some CPP–ACP/F nano-complexes like structures²³. High availability of calcium, phosphate, and fluoride ions at the tooth–oral fluid interaction surface junction drives positive diffusion of these ions into the enamel, leading to increased the activity of ions in the subsurface lesion fluid. This may result in more stable remineralized

fluoride containing crystal lattice formation at the site of action in the mineral phase. Hence, the ability to deliver calcium, phosphate, and fluoride ions in the correct molar ratio deep into the subsurface lesion may be attributable to the ability of the CPP to localize and stabilize the ions at the tooth surface in the correct molar ratio (Ca:PO₄:F = 5:3:1)²³. In the presence of a small quantity of fluoride (0.03–0.08 ppm) within biofilms, saliva, and artificial calcifying fluids, remineralization is favored over demineralization²⁴.

The physical characteristics of saliva like flow rate, buffering capacity, antimicrobial activity, microorganism aggregation and clearance from the oral cavity, immune system, and calcium phosphate binding proteins act and sum up collectively to inhibit or reverse demineralization of exposed tooth surfaces²⁵. Excessive demineralization in deciduous teeth causes diffusion of rods and easy invasion of bacteria through increased pores size as observed in SEM micrographs. With the opening of pathways, bacteria can invade deep, and further biofilm invasion causes demineralization; also remineralization is not possible after its nucleation sites are lost completely. Furthermore, to create conditions for saturation/supersaturation, there is a requirement of the supply of a significant amount of minerals from saliva. Therefore, the role of CPP–ACP with fluoride becomes a must as remineralization is not possible only with plaque control^{26,27}.

Remineralization is less effective in deciduous teeth as compared to young permanent teeth due to unfavorable mineral content ratios like a large number of elements like carbonate, water, and magnesium making it more porous²⁸. Incorporation of fluoride and calcium in surface enamel along with the alternate process of demineralization and remineralization, the new improved more resistant and less permeable remineralized enamel surface form is achieved which has less water with more favorable mineral content. This favorable incorporation of minerals in crystal lattice leads to more caries-resistant and less soluble enamel form²⁹.

In conclusion deciduous enamel loose more mineralized tissue during artificial demineralization. *In situ* results of the present study suggest that supervised twice daily use of “CPP–ACP plus fluoride” tooth Mousse may be superior to “CPP–ACP” alone tooth mousse when compared as remineralizing agent for artificially demineralized deciduous and young permanent tooth enamel while using nonfluoridated toothpaste.

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