Original Article

Comparative evaluation of remineralizing effect of fluoride and nonfluoride agents on artificially induced caries using different advanced imaging techniques

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Abstract

Aim: The aim of the study is to evaluate and compare the *in-vitro* remineralization efficacy of remineralizing agents, i.e., fluoride-free toothpaste, fluoride toothpaste, casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), CPP-amorphous calcium fluoride phosphate (CPP ACFP), and silver diamine fluoride (SDF) paste on dentine over time.

Methodology: Seventy-five extracted human permanent molars were selected. The buccal section of these samples was shaped into a slab. Artificial caries was induced by dipping the samples into the demineralizing solution (pH 4.5) for 21 days. They were then divided into five groups ($n = 15$). Each experimental group underwent one of the following treatments: Group 1 – Fluoride-free toothpaste (negative control), Group 2 – Fluoride toothpaste (positive control), Group 3 – CPP-ACP, Group 4 – CPP-Amorphous Calcium fluoride Phosphate and Group 5 – SDF. Postremineralizing treatment, Scanning electron microscope, Energy dispersive X-ray and Quantitative light induced fluorescence imaging were carried out to analyze the remineralizing efficacy. The data obtained was then subjected to statistical analysis using ANOVA and Paired *t*-tests.

Results: It was seen that SDF showed highest remineralization efficacy followed by CPP-amorphous calcium fluoride phosphate, CPP-ACP, fluoride toothpaste and fluoride-free toothpaste. The difference in mean value among the groups was statistically significant ($P < 0.001$).

Conclusion: SDF showed the highest remineralizing potential in scanning electron microscopy and energy dispersive X-ray, followed by CPP-ACFP, CPP-ACP, Fluoride toothpaste, and Fluoride-free toothpaste. Quantitative light fluorescence analysis showed more fluorescence changes in the CPP-ACFP followed by CPP-ACP, Fluoride toothpaste, and Fluoride-free toothpaste.

Keywords: Casein phosphopeptide-ACPF; casein phosphopeptide-amorphous calcium phosphate; fluoride; remineralization; silver diamine fluoride

INTRODUCTION

Dental caries is a multi-factorial disease. It has many risk factors associated with its progression, including environmental, biological, and socio-behavioral factors.[1]

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Generally, Crystals at the tooth surface regularly go through natural periods of mineral loss i.e., demineralization, and mineral gain i.e., remineralization. Under normal conditions, there is a balance of ions in saliva. If the pH falls below a critical level, which happens to be 5.5 for enamel and 6.2 for dentin, it causes the dissolution of tooth structure, resulting in dental caries.[2] There are two mechanisms of action which are responsible for the anti-caries activity:

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Firstly, inhibition of tooth demineralization by increasing the pH where remineralization is necessary, and secondly, enhancement of remineralization in early carious lesions by raising the calcium (Ca) and phosphate (P) ion concentrations.[3] Remineralization during the initial stage of dental caries would provide significant benefit to the patient. Fluoride has been known to be a standard remineralizing agent. Besides fluoride, there are some products that contain milk proteins like casein phosphopeptide (CPP). CPP-ACP has been believed to escalate the remineralization of early carious lesions.[4] Attiguppe *et al*. reported the combined effect of CPP-ACP and fluoride that resulted in the formation of CPP-stabilized amorphous calcium fluoride phosphate (CPP-ACFP), which helps in arresting caries.[5] Another material which has a high concentration of fluoride is silver diamine fluoride (SDF). SDF is easily available, affordable and safe to use.[6] SDF facilitates remineralization by inhibiting biofilm formation and caries prevention.[7] Only advanced lesions are detected by clinical and radiographic methods; early carious lesions are not easy to detect clinically.[8] Quantitative light fluorescence (QLF) is an imaging technique that utilizes optical properties to detect early caries and their progression and regression using principle of fluorescence. It also helps in the quantification of caries.[9]

Aim and objective

This study was conducted to evaluate the remineralization efficacy of SDF in comparison with other remineralizing agents like fluoride-free toothpaste, fluoride toothpaste, CPP-amorphous calcium phosphate (CPP-ACP) and CPP-ACP fluoride (CPP-ACPF) paste using scanning electron microscopy (SEM), energy dispersive X-ray (EDX) and QLF.

MATERIALS AND METHODS

The study was approved by the Institutional Research Ethical Committee (Ref. Code 90th ECM II B-Thesis/P31).

Collection of samples

Seventy-five freshly extracted human mandibular first molars were collected. Morphologically intact noncarious teeth extracted for periodontal reasons were selected for this study. Teeth with developmental defects, caries and white spot lesion, fluorosis affected teeth, hypo\hyper mineralized teeth and those subjected to previous treatment were excluded. An ultrasonic scaler was used to remove the tissue debris. Samples were kept in 10% formaldehyde for 24 h to disinfect them. A double-faced diamond disc was used for the decoronating of the teeth, and each buccal section was shaped into a 5 mm × 4 mm × 2 mm size slab. 0.1% thymol solution was used to store all the specimens.

Demineralization of the tested specimens

The demineralizing solution was prepared by adding 0.1 M lactic acid to 0.1 M sodium hydroxide and the pH was adjusted to 4.5. Each of the specimens was immersed separately into 2 ml Eppendorf tubes containing the prepared demineralization solution for 1 week at room temperature. After demineralization, they were randomized into the five groups ($n = 15$).

Experimental materials

- Group I: Fluoride-free toothpaste (0 ppm F) (negative control). Colgate Kid fluoride-free toothpaste. (Fluoride-Palmolive company New York)
- Group II: Fluoride toothpaste (1000 ppm F) (positive control) (Fluoride-Palmolive company New York)
- Group III: 10% w/v CPP-ACP + Fluoride toothpaste (1000 ppm F) (GC tooth mousse, recaldent™, Europe)
- Group IV: 10% w/v CPP-ACP + 900 ppm F + Fluoride toothpaste (1000 ppm F).(0.2% w/w sodium fluoride) (GC tooth mousse, recaldent™, Europe)
- Group V: 38% SDF + Fluoride toothpaste (1000 ppm F). (FAgamin)

All the specimens were assessed for the formation of white opaque areas and analyzed under energy-dispersive X-ray (*EDX* or *EDS*).

Analysis after demineralization procedure

Quantitative light-induced fluorescence microscopy

All samples were air-dried and mounted in a customized positioner, which was colored black to avoid ambient light interfering with the analysis. The specimens and QLF-D were fixed at the same location and angle each time the images were acquired. QLF baseline analysis was done to compare the ΔF on untreated dentinal slabs and demineralized dentinal slabs The dentinal slab with the best result was selected for use in the study to detect the differences in ΔF posttreatment. After QLF baseline analysis, the same samples were subjected for EDX evaluation.

Energy-dispersive X-ray analysis

The specimens were placed in a dried environment and care was taken to avoid contact with air or moisture. Then they were dried in a series of ethanol concentrations. After drying, the demineralized specimens were mounted on a metal block for EDX analysis.

Formulation of experimental toothpaste slurries

Nonfluoridated and fluoridated toothpaste slurries were prepared by mixing it with artificial saliva in 1:4 ratio by weight, with the help of Whirl Mixer for 1 min. CPP-ACP CPP-ACFP and SDF slurries were prepared by mixing it with distilled water in 1:4 ratio. In the Whirli Mixer for 1 min.

The specimens were rinsed gently for 1 min with distilled water. Afterward, they were placed in the toothpaste slurry for 30 min. After 30 min, the specimens were rinsed for 1 min with distilled water and kept in daytime artificial

saliva for 60 min. The specimens were dipped in acetic acid solution (pH 4.8) and exposed to their first demineralization challenge for 5 min, and kept in daytime artificial saliva after rinsing with distilled water for 1 min. This procedure was continued until the dentinal slabs were submitted to five demineralization challenges to simulate five events of food consumption throughout the day. After the last acid exposure, the slabs were placed in the toothpaste slurry for 30 min.

For the nonfluoride and fluoride toothpaste groups, the specimens were kept in night time artificial saliva, while for the CPP-ACP, CPP-ACFP and SDF groups, the specimens were rinsed with distilled water and then kept in their respective groups' solution for 30 min and then in artificial saliva at night.

Analysis after remineralization procedure

Quantitative light-induced fluorescence

After creating subsurface lesions on slabs, QLF (QLF-D Biluminator™) measurements were obtained after comparing the chosen demineralized slab with the remineralized dentinal slab at the end of the 21-day experiment period under controlled conditions. All the slabs were examined in a dark room using the following settings: A shutter speed of 1/10 s, the aperture value of 16 and ISO speed of 1600. All fluorescence images were examined using analyzing software. The analyses were performed with the help of a single trained examiner.

Scanning electron microscopy

Surface analysis of the remineralized specimens was done using SEM at ×1000 magnification. The images were digitally recorded, and classified into four categories of scores adapted from Kato *et al.*, as follows, score 1-open dentinal tubules, without debris; score 2-open dentinal tubules, with debris covering <50% of the area; score 3-open dentinal tubules, with debris covering more than 50% of the area; and score 4-covered dentinal tubules and debris in 100% of the area examined. After the analysis all the specimens were subjected to statistical analysis.

Statistical analysis

Descriptive statistics were calculated. Statistical analysis was done using the Paired *t*-test and ANOVA test.

OBSERVATIONS AND RESULTS

Energy dispersive X-ray

On comparing the Ca/P ration, the mean value of SDF group [Figure 1e] was more than the fluoride-free [Figure 1a], fluoride [Figure 1b], CPP-ACP [Figure 1c], CPP-ACFP [Figure 1d] group. The difference in mean value among the groups was statistically significant ($P < 0.001$). After the treatment, all the five groups showed increase in Ca/P ratios. The ratio of Calcium and phosphate after remineralization observed in different study groups were in the following order [Table 1].

Group V > Group IV > Group III > Group II > Group I

Quantitative light fluorescence result

On comparing the F change with DM (ΔF) for the groups, it was found that the minimum mean ΔF was observed in DM to Group CPP-ACFP which further concludes maximum mineralization while the maximum change in DM to the fluoride-free group which further concludes minimum mineralization [Figure 2a-c]. The difference among the groups was statistically significant ($P < 0.001$) [Table 2].

Table 1: Intergroup comparison of Ca/P ratios among the study groups

Group	C/P ratio		*F*	*P*
	Mean	SD		
F - free	1.58	0.05	74.49	<0.001
Fluoride	1.74	0.12		
CPP-ACP	1.84	0.21		
CPP-ACFP	1.91	0.06		
SDF Group	2	0.07		

ACFP: Amorphous calcium fluoride phosphate, ACP: Amorphous calcium phosphate, CPP: Casein phosphopeptide, SD: Standard deviation, SDF: Silver diamine fluoride

Table 2: Comparison of F change with DM from various study groups and scanning electron microscopy scores among various study groups

Change	ΔF		Mineralization	*F*	*P*
	Mean	SD			
DM	−23.92	4.74	-	73.89	<0.001
DM to F – Free	−22.65	1.9	1.27		
DM to Fluoride	−17.97	1.65	5.95		
DM to CPP-ACP	−12.79	1.57	11.13		
DM to CPP-ACFP	−8.38	1.32	15.54		

Comparison of SEM scores among various study groups

SEM score	Group	Mean	SD	*F*	*P*
Score 1	F – free	58.2	16.28	59.82	<0.001
	Fluoride	26.67	5.38		
	CPP-ACP	25.93	11.57		
	CPP-ACFP	16.93	3.95		
	SDF	7.13	4.16		
Score 2	F – free	5.87	1.68	47.69	<0.001
	Fluoride	18.87	5.62		
	CPP-ACP	20.07	4.91		
	CPP-ACFP	25.4	5.47		
	SDF	10.53	2.85		
Score 3	F – free	4.53	3.64	36.49	<0.001
	Fluoride	13	5.28		
	CPP-ACP	20.73	5.48		
	CPP-ACFP	20.2	3.28		
	SDF	13.73	2.69		
Score 4	F – free	2.13	2.1	168.75	<0.001
	Fluoride	12.47	5.19		
	CPP-ACP	16.8	4.83		
	CPP-ACFP	22.8	6.7		
	SDF	54	8.43		

ACFP: Amorphous calcium fluoride phosphate, ACP: Amorphous calcium phosphate, CPP: Casein phosphopeptide, SD: Standard deviation, SEM: Scanning electron microscope, SDF: Silver diamine fluoride, DM: Demineralisation

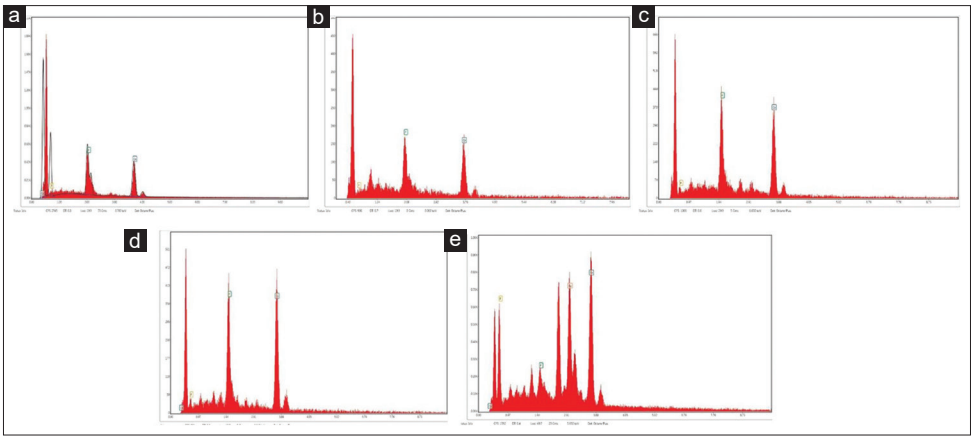

Figure 1: EDX analysis of Group 1 (Fluoride – Free) (a) Group 2 (Fluoride) (b) Group 3 (CPP-ACP) (c) Group 4 (CPP-ACFP) (d) Group 4 (SDF) (e) showing peaks of calcium and phosphate ions after remineralization

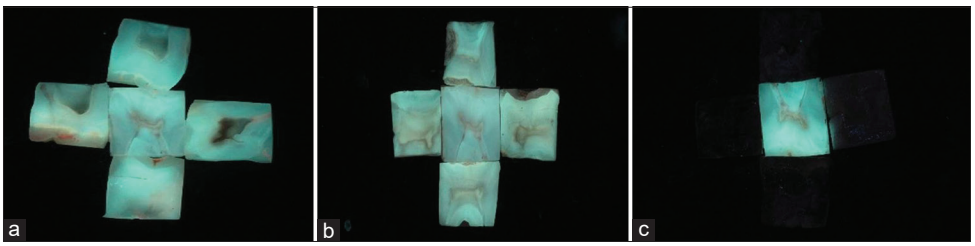

Figure 2: (a) Depicts comparison of demineralizing sample in center with the Group 3 (CPP-ACP) and showing difference in fluorescence. (b) Depicts comparison of demineralizing sample in centre with the Group 4 (CPP-ACFP) and showing difference in fluorescence. (c) Depicts comparison of demineralizing sample in center with the Group 5 (SDF) and No fluorescence seen in Group 5

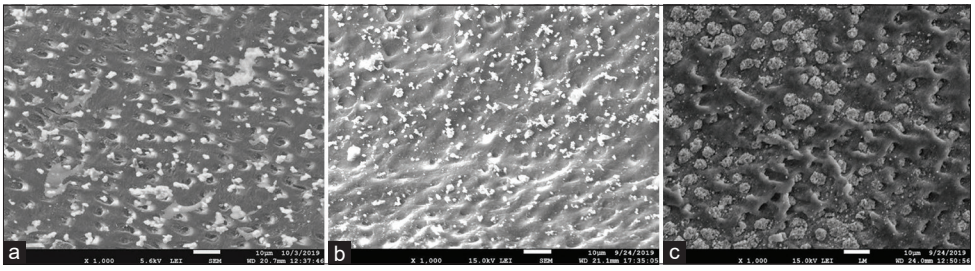

Figure 3: (a) SEM micrograph (×1000) of Group – 3; CPP-ACP. Image depicts moderate open and occluded dentinal tubule. (b) SEM micrograph (×1000) of Group – 4; CPP-ACFP. Image depicts more occluded and minimal open dentinal tubule. (c) SEM micrograph (×1000) of Group – 5; SDF. Image depict moderate open and more occluded and lesser open dentinal tubule

Scanning electron microscopy result

The difference in the mean number of tubules among the groups was statistically significant ($P = 0.001$) [Table 2 and Figure 3a-c]. The occlusion of dentinal tubules which was the result of remineralization was evaluated by SEM.

Total number of tubules – Group I < Group II < Group III < Group V < Group IV

Score 1 – Group I > Group II > Group III > Group IV > Group V

Score 2 – Group IV > Group III > Group II > Group V > Group I

Score 3 – Group III > Group IV >Group V > Group II > Group I

Score 4 – Group V > Group IV > Group III > Group II > Group I

DISCUSSION

For this study, human permanent first mandibular molars were selected because more carious teeth were observed on the mandible (66.7%) than the maxilla, and the first molars (66.7%) showed significantly more prevalence than the second molars. The buccal section of the mandibular molar was shaped into one dentinal slab

that was approximately 5 mm × 4 mm × 2 mm in size to standardize the size of samples. Remineralization of carious dentin is also important to increase the durability of bonding between dentin and resin materials in clinical practice, therefore dentinal slabs were chosen to mimic the clinical situation.[10]

Artificial saliva was prepared by using McKnight Hane and Whitford formula[11] in this study toothpaste slurries of experimental materials was used to imitate the dilution of the paste with the saliva in the mouth.[12] Artificial saliva was changed every 24 h during the remineralization regimen to ensure ionic balance and maintenance of pH.[13]

After the five cycles of demineralization, the dentinal slabs were kept in CPP-ACP, CPP-ACFP, and SDF in its respective group for half an hour The remineralization rate increases with the duration of time the paste is in contact with the tooth surface.[14]

An evaluation of the remineralizing effect of the experiment was assessed using EDX, SEM, and QLF. EDX has been used for elemental analysis at the ultrastructural level. The scanning electron microscope is used to determine and compare the morphological variations between the demineralized and remineralized samples.[15] The amount of mineral content is directly correlated with the fluorescence. Loss in mineral will show a decrease in fluorescence allowing for quantification of the mineral loss.[16] In comparison to other diagnostic aids, QLF exhibits greater sensitivity in detecting early carious lesion.[17] The intergroup comparison of the calcium (wt%) and phosphate (wt%) ratio between the demineralizing and remineralizing groups under EDX [Table 1] it was found that minimum remineralization has occurred in the fluoride-free toothpaste (Group I). Since the group I toothpaste does not contain any fluoride, this remineralization may be due to the artificial saliva that was used in the study. Some clinical studies have demonstrated that early caries lesions could be remineralized by saliva when fluoride application was incorporated.[18]

Although saliva has some remineralization potential, it cannot raise the amount of calcium and phosphate excretion by itself. As a basic mechanism of remineralization, an acid-resistant, hyper mineralized, fluorapatite-like layer is formed on the remnants of the existing crystal which acts as a remineralization nucleus. This fluorapatite layer is formed via seeding of calcium and phosphate ions from saliva and other topical sources.[19] Enough concentration of calcium and phosphate ions should be present to penetrate the surface layer and mineral deposition to occur within the body of the lesion,[20] which fluoride-free toothpaste does not contain.

Group-IV CPP-ACFP showed more remineralization than the fluoride-free toothpaste and fluoride-containing toothpaste and CPP-ACP. This may be because seeding of fluoride with CPP-ACP can give a combined effect and increase remineralization than CPP-ACP.[21]

The results were in favor with the other studies which stated that CPP-ACFP paste was better as a remineralizing agent when compared to sodium fluoride varnish (NaF) and CPP-ACP gel for the remineralization of erosive lesions which were artificially induced in both primary and permanent dentition.[22]

In this study SDF demonstrated maximum changes in calcium and phosphorus ratio after remineralization in comparison to all the groups. 38% SDF contains 44,800 ppm fluoride. This is the highest concentration among the fluoride agents available for clinical use.

In SEM, SDF showed the maximum number of occlusions of dentinal tubules followed by CPP-ACFP. CPP-ACP, fluoride-containing toothpaste, and fluoride-free toothpaste [Table 2].

In QLF, CPP-ACFP showed more significant changes with the highest amount of mineral gain quantitatively, followed by CPP-ACP, Fluoride containing and fluoride-free toothpastes. It also showed a maximum change in fluorescence than the other groups.

SDF didn't show any fluorescence under QLF; this may be attributed to the chemical reactions that occur between hydroxyapatite of teeth and SDF, which leads to the formation of silver phosphate and calcium fluoride. The silver chloride (8.9 9 10_4 g/100 ml) is less soluble in comparison to silver phosphate (6.4 9 10_3 g/100 ml), therefore silver phosphate could react with alkali chlorides in remineralization solutions to form silver chloride. Incorporation of metallic silver particles into the hydroxyapatite crystals may cause interference in the fluorescence of QLF.

CONCLUSION

To summarize, the results of the present study deduced that remineralization potential was maximum for the SDF, followed by CPP-ACFP, CPP-ACP, and minimum in the control group.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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