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Comparative Evaluation of Ion Release and Uptake of Fluoride Varnish Containing Dicalcium Phosphate Dihydrate and Casein Phosphopeptide-Amorphous Calcium Phosphate – An Experimental Approach.

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ABSTRACT

The effectiveness of fluoride varnish may be restricted due to inadequate bioavailability of phosphate and calcium ions in the oral cavity. Dicalcium phosphate dihydrate and Casein phosphopeptide–amorphous calcium phosphate have exhibited ability to enhance absorption of calcium and fluoride. The present study aims to compare the remineralization potential of fluoride varnishes containing DCPD and CPP-ACP.

Materials and Method:

The ion release, precipitation and surface microhardness of enamel blocks were compared following topical application of various Floride varnishes. Thirty enamel blocks were divided into 3 groups following randomization that included: NaF varnish (Group A), CPP-ACP NaF varnish (Group B) and DCPD- NaF varnish (Group C). Pre- and post-treatment microhardness was evaluated using Vickers Microhardness Tester. UV-Vis Spectroscopy assessed the fluoride release at 2 hrs, 4 hrs and 6 hrs. The uptake of fluoride, calcium and phosphates were determined using SEM-EDX. The statistical significance was tested with One-way ANOVA variance for normality followed by post-hoc tests ($p \leq 0.05$).

Results:

All the groups showed statistically significant increase in microhardness post-treatment (Group A: p=0.03, Group B, C: p=0.00). The mean increase in VHN was Group B (53.67) > Group C (34.75) > Group A (27.40). No significant difference in the F release was noted over time (p=0.00). The fluoride release over all three time intervals was Group C > Group B > Group C. Fluoride, Calcium and Phosphates showed highly significant differences between groups (p=0.00) with Group C > Group A> Group B.

Conclusion:

The novel NaF-DCPD Varnish displayed a promising remineralization potential, however it cannot be conclusively considered to be superior to the commercially available NaF-CPP ACP Varnish. Further *in-vivo* investigations need to be undertaken to determine the efficacy of NaF-DCPD varnish to determine its feasibility in clinical practice.

INTRODUCTION

Dental caries is widely recognized as one of the most common microbial disorders worldwide. An estimate by *'World Health Organization' (WHO)* states that, dental caries have been encountered by about *60% to 90%* young children.[1]

Dental caries is a complex illness which leads to loss of minerals in the structure of teeth. The primary causative elements consist of the existence of endogenous cariogenic bacteria, regular intake of fermentable sugars, and most importantly a vulnerable host.[1] If left untreated, dental caries can result in cavitation of the tooth. However, it is possible to prevent, reverse, or stop the progression of dental caries in its initial stages. Enamel undergoes multiple cycles of demineralization and remineralization throughout the day. The carious lesions might undergo progression, stabilization, or reversal based on the equilibrium between remineralization and demineralization processes. The dissemination of calcium and phosphate ions into the oral cavity, results in the demineralization of the enamel. During the remineralization process, phosphate and calcium ions permeate the enamel surface to later combine and create

fluorapatite which exhibits resistance to demineralization, and promotes remineralization.[2]

In 1964, a scientific publication documented a varnish designed with the intention to prolong the duration of contact between fluoride and enamel; named 'fluoride varnish'.[3] Currently, there are around thirty commercially available varnishes with a wide range of delivery techniques and formulations. Fluoride varnishes are a secure and efficient method for administering and preserving fluoride on the surface of teeth. In addition, by stimulating the process of remineralization on the tooth surface and inhibiting demineralization, they effectively decelerate the advancement of dental decay. Varnishes provide a useful addition for caries control in patients with specific requirements, including those with developmental disabilities, children receiving head and neck radiation, and patients using long-term oral medications as well as those undergoing orthodontic therapy. Due to its relative safety and ease, it is considered the optimal method of preventive in community-based dentistry initiatives.[4]

The effectiveness of fluoride varnish may be restricted due to the inadequate presence of bioavailable phosphate and calcium ions inside the mouth. A stable form of calcium phosphate, known as amorphous calcium phosphate (ACP), may be utilized for targeting ACP present in dental plaque. This aids in regulating the activity of calcium phosphate and calcium ions by using a phosphopeptide derived from casein, also known as CPP. Another option is to utilize Dicalcium Phosphate Dihydrate (DCPD) [$CaHPO_4.2H_2O$], which has a notable solubility and can offer readily absorbable calcium and phosphate. Consequently, it maintains enamel in a state of supersaturation and facilitates remineralization by inhibiting demineralization.[5]

Combining dicalcium phosphate dihydrate and Casein phosphopeptide–amorphous calcium phosphate with fluoride varnishes separately, have exhibited their ability to enhance the absorption of calcium and fluoride. Nevertheless, there has been no investigation into the comparative effectiveness of these methods in promoting the remineralization of carious lesions. The existence of abundant calcium and fluoride ions in the mouth can promote the process of enamel remineralization. Patients with a high risk of developing tooth decay can benefit from the application of fluoride varnish that releases a significant amount of fluoride and calcium at initial application.[2]

Thus, this study aimed to evaluate and compare the change in microhardness, the release of fluoride ions and the uptake of fluoride, calcium and phosphate ions from fluoride varnishes containing CPP-ACP or DCPD with a positive control of 5% fluoride varnish.

MATERIALS AND METHODS:

The study was primarily conducted in the Department of Pediatric and Preventive Dentistry of the Institute. The other centres that partnered the research requirements included the Department of Nanosciences, Rajagiri Institute of Engineering and Technology and Unibiosys Biotech Research Labs.

Due approval was obtained from the Institutional Ethics Committee [Ref No: ECASM-AIMS-2024-099].

STUDY SAMPLES

The samples included thirty sound extracted premolars and were divided into three groups (n=10).

Thirty premolars freshly extracted for orthodontic purposes at the Department of Pediatric and Preventive Dentistry were collected. It was ensured that the collected teeth had no evidence of carious lesions, fractures or other morphological changes in enamel. The collected teeth were cleaned with a hand scaler and pumice slurry and stored in a sealed container with deionized water and was labelled as *Biohazard.*[6]

SAMPLE PREPARATION

A high-speed diamond disc and with cooling was used to cut each tooth sample at the cementum-enamel junction (CEJ) level. Sections of enamel were removed from the residual coronal tooth structure mesiodistally, splitting it into the lingual and buccal regions. The sectioned samples from each tooth (n=30) were then embedded in acrylic blocks with dimensions of 4×4×3 cm each to form 'Enamel Blocks'. Samples were assigned into three groups, with 10 specimens per group (Figure 1).

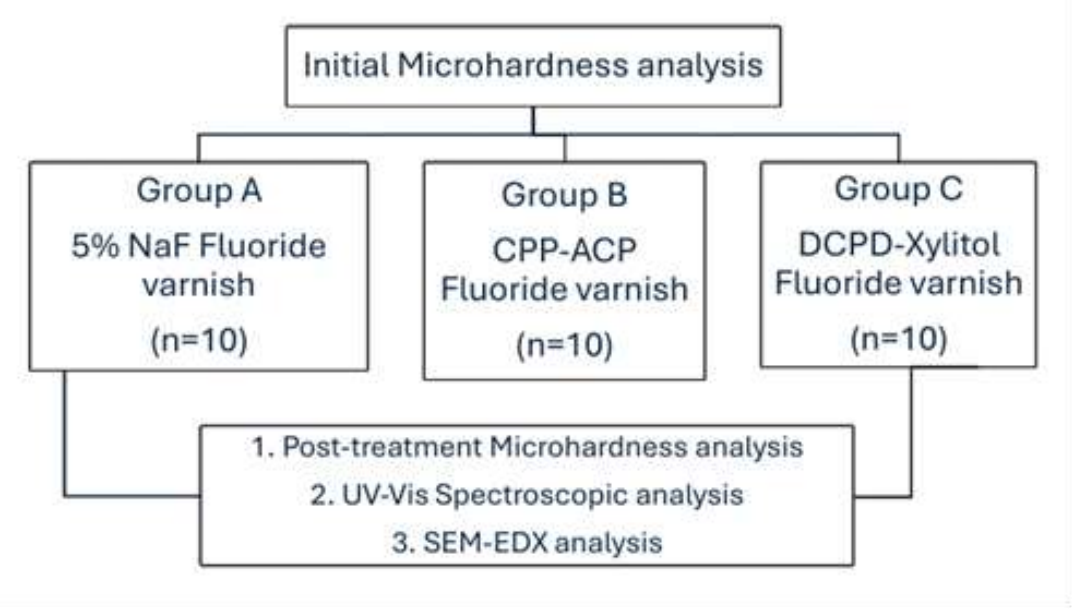


Figure 1: Study Design

- Group A (n=10): 5% NaF Varnish
- Group B (n=10): 5% NaF- CPP ACP Varnish
- Group C (n=10): 5% NaF-DCPD Varnish

STUDY INTERVENTION

Group A and Group B received applications of 5% sodium fluoride varnish (Profluorid varnish, VOCO, Germany) and 5% sodium fluoride varnish with CPP-ACP (MI Varnish, GC India), respectively.

The fluoride varnish with 2% DCPD was formulated following the composition described by Eriwati *et al.*[5]. A digital precision weighing scale was used to dispense 0.16 gm of DCPD powder which was added to the 8 ml NaF varnish and stirred continuously for 1 hr until no residue was left behind. This formulation was then applied to enamel blocks in Group C.

I. SURFACE MICROHARDNESS TESTING

The microhardness of each enamel block was tested with a Vickers Microhardness Tester (Mitutoyo, MVK- H11). A load of 100 g was applied on the surface of each enamel block for 10 s. The measurements were made prior to the experiment and post-treatment with the respective varnishes used in the three groups, 6 hrs after the application.[7]

II. UV-VIS SPECTROSCOPY

Once the microhardness values were obtained, all the enamel blocks were placed in artificial saliva medium at room temperature to evaluate the amount of fluoride ions leached into the medium. The ion release in *ppm* was examined at 2 hrs, 4 hrs and 6 hrs for each sample using UV- Vis Spectroscopy. The APHA 24th Ed. 4500 F^- method by Lipps *et al*. [8] was adopted for the analysis.

III. SEM- EDX ANALYSIS

The observation under scanning electron microscopy (SEM) was conducted by a trained and standardized observer. The examination was conducted using a Field Emission Scanning Electron Microscope (JSM-7800F, JEOL, Japan) at a magnification of 2500x to appreciate the formation of CaF_2 globules on the enamel surfaces.[9]

Energy Dispersive X-Ray Spectroscopy analysis was performed to determine the elemental composition and chemical analysis of the tooth surfaces. The APEX EDAX software (Amtek Inc. USA) was used to analyse the data obtained from the spectroscopy. The atomic percentages of Calcium, Fluoride and Phosphate were calculated for each specimen.

STATISTICAL ANALYSIS

The IBM® SPSS® Statistics Software version 20.0 was used to perform the statistical analysis. Using Mean, Standard Deviation, and Median (IQR), microhardness, Fluoride release and Fluoride, Calcium and Phosphate uptake were analysed. To test the statistical significance of the comparison of the various properties of NaF varnish (Group A), NaF- CPP ACP varnish (Group B) and NaF-DCPD varnish (Group C), one-way ANOVA variance test was used for normality followed by post hoc test. The significance was considered at $p \leq 0.05$ for all tests.

RESULTS

I. SURFACE MICROHARDNESS

The highest post-treatment mean VHN value was observed in Group B containing NaF-CPP ACP (291.04 ± 26.28). Similarly Group B also had the highest increase in microhardness post-treatment (53.67 ± 12.57). The least increase in microhardness was seen in group A containing NaF (27.40 ± 0.36) (Table 1). The difference in mean increase in microhardness was found to be statistically significant for Group B and C (p=0.00) and Group A (p=0.03) (Table 1).

Table 1: Mean Pre and Post- Treatment VHN values with paired mean differences.

Groups	*Pre-Treatment (Mean)*	*Post- Treatment (Mean)*	*Post - Pre Treatment Paired differences (Mean)*	*SD*	*Sig.*
A	238.81 ± 20.71	266.21 ± 28.82	27.40	21.59	**.003***
B	237.37 ± 18.31	291.04 ± 26.28	53.67	12.57	**.000***
C	230.64 ± 22.78	265.39 ± 22.88	34.75	18.33	**.000***

**The mean difference is significant at the 0.05 level.*

I. FLUORIDE RELEASE

The highest fluoride release among all three time intervals was observed in group C containing NaF-DCPD and lowest with Group A. The difference in mean fluoride release was found to be statistically significant between the three groups at all the time intervals (p=0.00) (Table 2). However, on intragroup comparison no significant difference in the F release over time for all the groups (p>0.05) was seen (Figure 2) (Table 3).

Table 2: Mean Fluoride release in ppm at different time intervals and inter-group comparison.

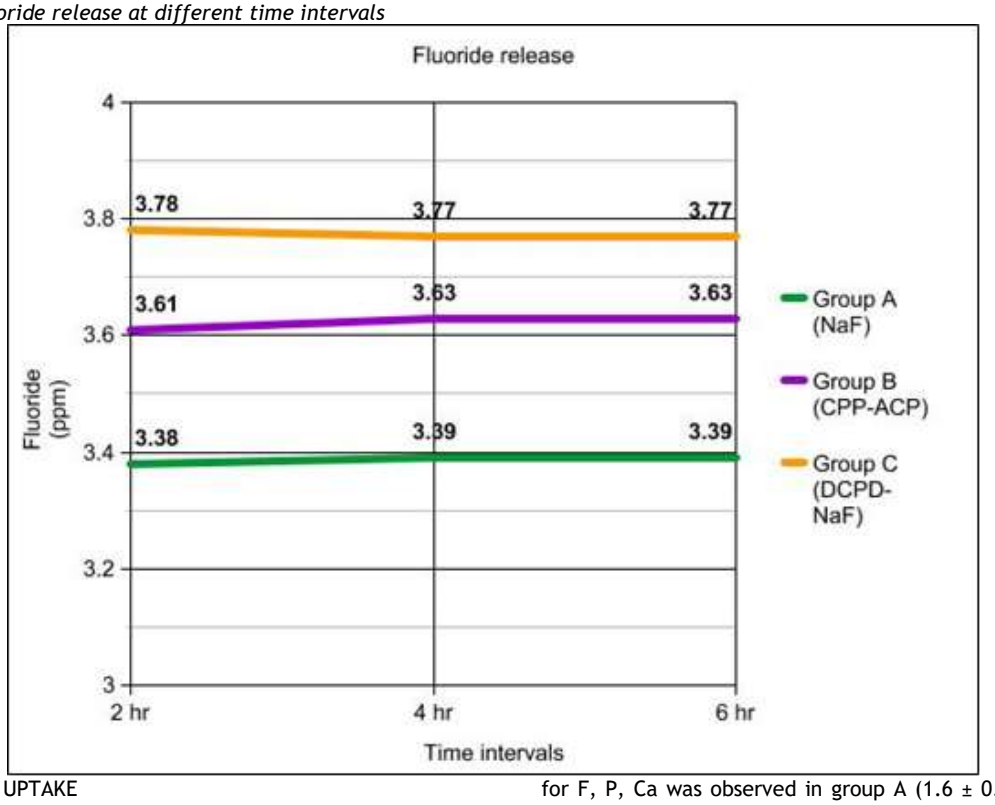
F^- release		*N*	*Mean*	*SD*	*Sum of Squares*	*df*	*Mean Square*	*Sig.*
2 Hrs	***A***	10	3.38	.01	.722	2	.361	.000
	B	10	3.61	.01				
	C	10	3.76	.01				
4 Hrs	***A***	10	3.39	.00	.724	2	.362	.000
	B	10	3.63	.01				
	C	10	3.77	.00				
6 Hrs	***A***	10	3.39	.00	.727	2	.364	.000
	B	10	3.63	.00				
	C	10	3.77	.00				

**The mean difference is significant at the 0.05 level.*

Table 3: Change in Fluoride release between 2 hrs to 4 hrs, 2 hrs to 6 hrs and 4 hrs to 6 hrs.

Change in F release	*Groups*	*Mean*	*Sig.*
2 hr to 4 hr	A	0.01	0.21
	B	0.02	
	C	0.01	
2 hr to 6 hr	A	0.01	0.61
	B	0.02	
	C	0.01	
4 hr to 6 hr	A	0	0.21
	B	0	
	C	0	

Figure 2: Mean fluoride release at different time intervals



I. MINERAL UPTAKE

Under a scanning electron microscope (SEM), Calcium Fluoride formations were visible as tiny globules on the tooth surface after varnish application. Group A showed minimal globule formation while Groups B and C showed considerable amount of uniform CaF_2 globules (Figure 3). The highest mean atomic wt % for F, P, Ca was observed in group A (1.6 ± 0.34, 11.26 ± 1.29, 21.57 ± 2.01) respectively and lowest with Group B (0.09 ± 0.1, 0.24 ± 0.06, 1.08 ± 0.34) respectively (Figure 4) (Table 4). The post hoc intergroup comparison (Table 5) showed a high statistical significance (p=0.00).

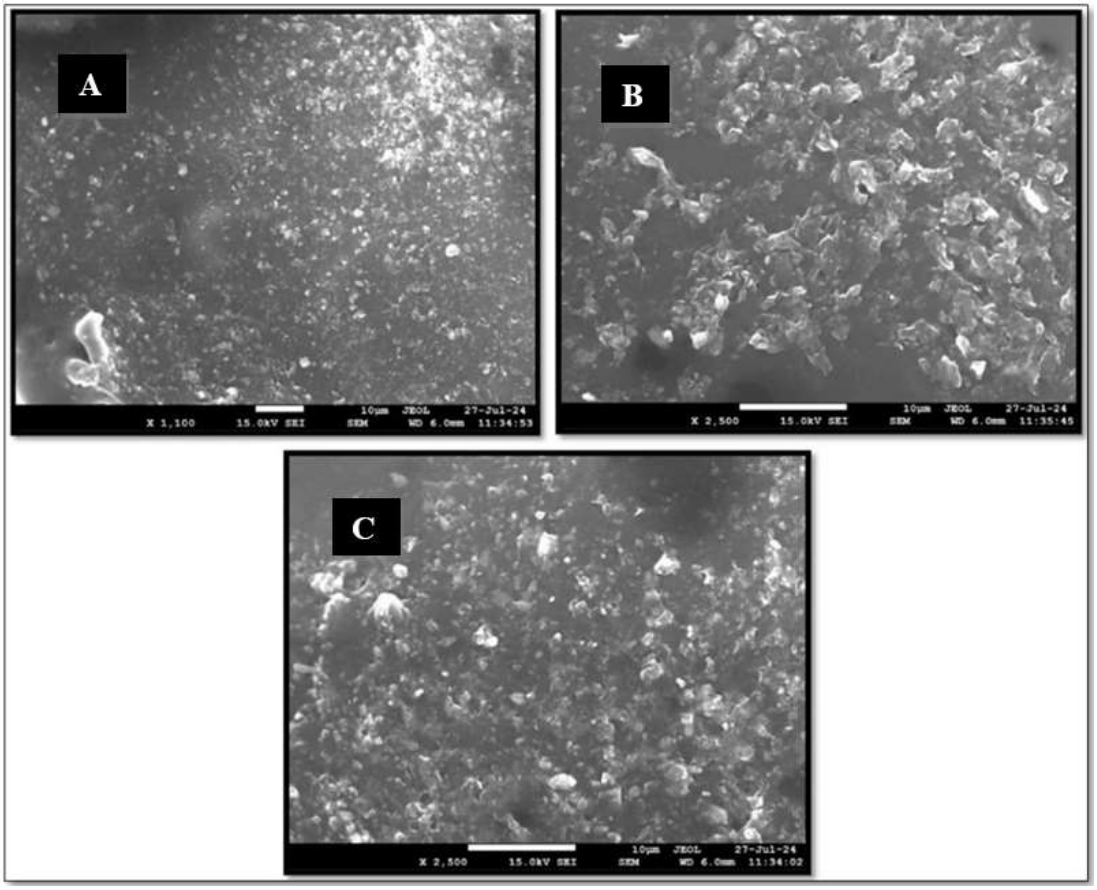


Figure 3: SEM EDX images post-treatment for Groups A, B and C

Table 4: Descriptive analysis of atomic % of minerals along with inter-group comparison.

Elements		*Mean*	*Std. Deviation*	*Sig.*
F	*A*	1.16	.34	0.00*
	B	.09	.01	
	C	.79	.46	
P	*A*	11.26	1.29	0.00*
	B	.24	.06	
	C	10.99	1.42	
Ca	*A*	21.57	2.01	0.00*
	B	1.08	.34	
	C	20.82	2.06	

**The mean difference is significant at the 0.05 level*

Table 5: Inter-group pair-wise comparison of atomic % of F, P and Ca

Elements	*Groups*			*Sig.*
F	A		B	**.000***
			C	.061
	B		A	.000*
			C	**.000***
	C		A	.061
			B	.000*
P	A		B	**.000***
			C	1.000
	B		A	.000*
			C	**.000***
	C		A	1.000
			B	.000*
Ca	A		B	**.000***
			C	*.988*
	B		A	.000*
			C	**.000***
	C		A	.988
			B	.000*

**The mean difference is significant at the 0.05 level*

DISCUSSION

Topically applied fluoride medicaments have been in clinical use for many decades since now. Fluoride varnishes have a long history of acceptance from both clinicians and patients alike. However, when used alone, fluoride varnish does not provide a total remedy for caries prevention. This is because, the minimal bioavailability of Ca and P ions limits the fluoride reservoirs creation and the remineralization capacity of saliva. According to Dai *et al.*,[10] every two fluoride ions require ten calcium ions and six phosphate ions to form one fluorapatite molecule. Hence, it has been attempted to improve the reserve of fluoride as well as calcium ions in the oral cavity by including Ca and P ions with the varnishes.[11] This has resulted in development of Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), a milk product that facilitates remineralization, inhibits demineralization, and prevents caries by providing a reservoir of calcium-phosphate.[12]

Another such attempt was made by Eriwati *et al.*[5] by the addition of 'Dicalcium phosphate dihydrate (DCPD)' which is a highly soluble Ca-P compound that can provide freely available calcium and phosphate to fluoride varnish. Tschoppe *et al.*[13] have demonstrated that saliva solutions containing excessive amounts of DCPD promote the process of dentinal remineralisation. Rirattanapong *et al.*[14] demonstrated that Ca ions from DCPD also functioned as a fluoride carrier. This carrier increased the absorption of fluoride in artificial caries in bovine teeth, thereby enhancing remineralisation. However, the mechanism of this ion release from DCPD in fluoride varnish is yet to be investigated. The current study was undertaken to assess the effect of adding two types of modifiers – CPP-ACP and DCPD, to the traditional sodium fluoride varnish on various properties exhibited by these varnishes which are indicative of their remineralization potential.

Microhardness is the measurement used to determine the material's ability to resist indentation or penetration. Microhardness may be used to directly indicate the gain or loss of minerals due to demineralisation and remineralisation. This approach has been demonstrated to be reliable and practical in evaluating changes in enamel surface demineralisation.[15]

The comparison of mean microhardness among the three groups in the present study showed highest post-treatment VHN for NaF CPP-ACP group followed by NaF group and the lowest VHN was for NaF- DCPD group of 265.39. However, on comparison of mean increase in the microhardness after the respective interventions, Na-CPP ACP showed the maximum increase in followed by NaF-DCPD and the least microhardness increase was shown by NaF.

Parallel to our findings, Tuloglu *et al.* [16] found that when primary teeth were subjected to pH cycling after application of various varnishes, MI Varnish (CPP ACP FV) had the least reduction in the surface microhardness followed by Clinpro White (FV with fTCP) and the poorest performance in preserving the microhardness was by Duraphat (NaF Varnish). They concluded that fluoride varnish with CPP-ACP presented the highest resistance against acid challenge of deciduous teeth when compared to other fluoride varnishes. Kooshki *et al.*[17] also reported similar observations while comparing MI varnish with Duraphat wherein, MI Varnish which is enhanced with CPP-ACP showed more increase in microhardness.

When CPP-ACP is combined with fluoride, it causes Ca and P ions to be concentrated with F on the surface of the enamel. CPP-ACP offers the benefit of combining the three ions into a single product.[17] Divyapriya *et al.* [18] stated that "The CPP molecule has the capacity to attach to a maximum of 25 calcium ions, 15 phosphate ions, and 5 fluoride ions". The calcium phosphate incorporated in these complexes can be readily used for the process of remineralisation of sub-surface lesions. The current findings suggest that the increased capacity for remineralisation observed with CPP-ACP may be attributed to the reservoir of calcium phosphate on the surface of enamel.

In contrast, Subramaniam *et al.*[19] found no significant variation in the SMH of enamel between CPP-ACP fluoride varnish and fluoride only varnish. This may be due to the enamel specimens undergoing polishing and flattening to accurately evaluate the surface microhardness. Therefore, the region exposed to the SMH did not correspond to the original experimental exposure.

Another indication of the remineralization ability of a varnish is the rate of release of fluoride from an agent into the oral environment as well as the amount of ions that get retained by the enamel from the varnish. The structural stability of the enamel depends on the oral fluids being either saturated or supersaturated in relation to tooth material. Thus, it is crucial to have enough amounts of freely available calcium, phosphate, and hydroxide or fluoride ions in the medium that surrounds the teeth. Since fluoride has been chiefly responsible for the caries preventive efficacy of the varnish, it is crucial that the incorporation of Ca and P ions does not decrease the bioavailability of fluoride ions and in turn, the ion release.[20] Therefore, it is necessary to assess how the addition of CPP–ACP affects the fluoride varnish's ability to release fluoride.

In the present study, the NaF- DCPD group has shown the maximum release of fluoride ions after 2 hrs, 4 hrs and 6 hrs followed by NaF-CPP ACP group, and the least amount of fluoride was released by the NaF group. On analysing the release of fluoride by each group at 2 hrs, 4 hrs and 6 hrs, all groups showed a sustained release over the period of 6 hrs with no statistically significant change in the amount of ions leached over time. This could be indicative of the varnish's ability to maintain a steady reservoir of fluoride for a long time period, highlighting their effectiveness as remineralizing agents. The rapid ions release from MI varnish seen under the examined circumstances can be related to the significant solubility of CPP-ACP, as confirmed in this investigation.

Similar outcomes were seen by Cochrane *et al.*[21], Soares-Yoshikawa *et al.*[9] and Attiguppe *et al.*[22] who also evaluated fluoride release at various time points and found MI Varnish to have the maximum fluoride release and Duraphat with the lowest values. Additionally, Cochrane *et al.*[20] examined the release of Calcium and Phosphate ions and MI Varnish showed maximum leaching of these two ions as well.

Piesiak-Panczyszyn *et al.*[23] also stated that MI Varnish released the maximum fluoride and all varnishes had much greater release in an acidic environment than in a neutral setting, with maximum release occurring within the first two hours.

Previous studies have incorporated SEM-EDX technique to investigate surface changes due to demineralization-remineralization.[24, 25] The EDX analysis of our study revealed that the NaF group had the greatest atomic % of all the minerals followed by the NaF-DCPD group and the NaF- CPP ACP group having the least atomic % of the three. These findings are conflicting with the other findings of this study, wherein NaF-CPP ACP has shown the greatest potential for remineralization based on the other parameters tested. This can be attributed to the different pH values of fluoride varnishes used in the study.

In contrast to our findings, Shen *et al.*[26] have stated that the F uptake for MI Varnish is greater than that of Duraphat along with wt% of Ca and P. MI Varnish released a greater amount of free calcium, phosphate and fluoride than Duraphat composed of only fluoride. It was hence pronounced to be superior in promoting F precipitation and remineralization of incipient lesions.

Post-analysing the properties of microhardness & F release, it can be deduced that the remineralization ability of NaF-CPP ACP Varnish is superior than the other varnishes studied. The conflicting results seen with regards to the F uptake need to be further investigated to determine the relationship with the pH of the agent and other confounding factors. The microhardness and F release between NaF-CPP ACP Varnish and the novel NaF-DCPD varnish are comparable, suggesting the possibility of considering this agent in *in-vivo* settings, to assess its performance in a more detailed and predictable manner.

CONCLUSION

With respect to the surface microhardness, a higher remineralization potential of the NaF-CPP ACP varnish was seen when compared to the novel NaF-DCPD varnish. At all the three time intervals, NaF-DCPD varnish showed the maximum release of fluoride followed by NaF-CPP ACP varnish, with 5% NaF showing the least amount of fluoride release. The ion release between different time points- from 2 hrs to 4 hrs, 4 hrs to 6hrs and 2 hrs to 6 hrs for each group were comparable, indicating a steady release of fluoride from the three agents over the study period. The results for mineral uptake results suggested that NaF-CPP ACP has the lowest ion retention capacity amongst the three, which could be indicative of a poor remineralization efficacy.

CLINICAL SIGNIFICANCE

Within the limits of this *in-vitro* study, it can be concluded that the novel NaF-DCPD Varnish displays a promising remineralization potential, however it cannot be conclusively considered to be superior to the commercially available NaF-CPP ACP Varnish. Further *in-vivo* investigations need to be undertaken to determine the efficacy of NaF-DCPD varnish to determine its feasibility in clinical practice.

LIST OF ABBREVIATIONS:

CPP-ACP: Casein Phosphopeptide–Amorphous Calcium Phosphate
DCPD: Dicalcium Phosphate Dihydrate
NaF: Sodium Fluoride
SEM: Scanning Electron Microscope
EDX: Energy Dispersive X-Ray
VHN: Vickers Hardness Number
ppm: Parts per million
fTCP: Functional Tricalcium Phospate
SMH: Surface Microhardness

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