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THE EFFECT OF A SINGLE APPLICATION OF DIFFERENT FLUORIDE VARNISHES ON ENAMEL SUBSURFACE LESIONS *IN VITRO*

Pinar Kulan Yildiz,[a] Betul Sen Yavuz,[b] Muesser Ahu Yilmaz,[b] Nihal Sehkar Oktay,[c] Betul Kargul[b]

Istanbul, Turkey

ABSTRACT: This *in vitro* study aimed to evaluate the therapeutic effect of different fluoride ion (F) varnish formulations for controlling the carious development of enamel subsurface lesions and the F release into artificial saliva for 2 hr, 24 hr, 48 hr, and 7 days. Artificial enamel carious lesions were created and divided into 6 groups (5 varnish groups and a control group). Varnishes were applied to enamel specimens and then the specimens were incubated in artificial saliva, with the artificial saliva replenished daily. Varnish was removed and lesions were remineralized in artificial saliva for 24 hr. Surface microhardness was measured three times: (i) initially, (ii) after creating the artificial enamel lesions, and (iii) after applying the varnishes. The F release was analyzed after 2 hr, 24 hr, 48 hr, and 7 days of exposure using an ion-selective electrode. Data were analyzed using a One-way Analysis of Variance with the Tukey-Kramer Multiple Comparisons test and the Kruskal-Wallis test with the Dunns Multiple Comparisons test. The highest percentage surface microhardness recovery was found for the treatment with the MI Varnish. According to the percentage surface microhardness recovery results, a statistically significant difference was found between the varnishes and the control group ($p<0.05$ and <0.001). All varnishes released measurable levels of fluoride ions. However, the release of F was the highest in the MI Varnish group ($p<0.01$). Duraphat, Enamel Pro Varnish, and MI Varnish released the most F into artificial saliva. Calcium phosphate-based F varnishes improve the capacity of the enamel surface re-hardening. CPP containing F varnish had the highest release of F as compared to the other F releasing varnishes. Further *in vivo* investigations are also required to prove the clinical applications of the different ingredients containing varnishes.

Keywords: CPP-ACP; Demineralization; Fluoride release; Fluoride varnishes; Microhardness; Remineralization; Tricalcium phosphate.

INTRODUCTION

Over recent years, the fluoride ion (F) has proven to be an effective vehicle in the fight against dental caries. F shows its effect against dental caries by being constantly present in the oral cavity and contributing to the demineralization-remineralization process. There are many over the counter and professionally applied products which deliver F to the mouth.[1,2]

F varnishes have been developed for many years to increase the effect of the enamel on caries control by extending the F intake time.[3] F varnishes typically contain five percent sodium fluoride (NaF) which aids in the formation of long-lasting intraoral F reservoirs.[2] The American Dental Association was suggested that "Fluoride varnish applied every six months is effective in preventing dental caries in the primary and permanent dentition of children and adolescents" and this encourages paediatric dentists to use F varnishes more.[4] Post-application F varnish, F is released from the varnish and is taken up by the tooth enamel, plaque, and saliva.

[a]Private Practice, Istanbul, Turkey; [b]Department of Pediatric Dentistry, School of Dentistry, Marmara University, Istanbul, Turkey; [c]Department of Basic Science Biochemistry, School of Dentistry, Marmara University, Istanbul, Turkey. For correspondence: MA Yilmaz, Marmara University, Faculty of Dentistry, Department of Paediatric Dentistry, Maltepe, Basibuyuk Saglik Kampusu, 34854, Istanbul, Turkey; E-mail: ahu.durhan@marmara.edu.tr

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The ionic F in solutions around the tooth inhibits demineralization and promotes remineralization, thereby reversing the development of early carious lesions.[5]

F varnishes with an improved content are available which contain functionalized tricalcium phosphate (fTCP) consisting of tricalcium phosphate modified by fumaric acid, amorphous calcium phosphate (ACP), and casein phosphopeptide-amorphous calcium phosphate (CPP-ACP).[6,7] Although *in vitro* F release from varnishes has been investigated for a long time, as far as we know no studies have examined the F release and therapeutic effects of varnishes containing calcium phosphate and F.

This study aimed to evaluate the therapeutic effect of different varnish formulations for controlling the carious development of enamel subsurface lesions and the *in vitro* F release into artificial saliva from F varnish. Two null hypotheses were established in this study; (1) there was no difference between the therapeutic effects of the varnishes, and (2) there was no difference between the F release of the varnishes.

MATERIALS AND METHODS

Preparation of enamel specimens:

A total of 48 caries-free permanent human molars with no visible white spot lesions, or any other kind of restorations were selected. Written informed consent was obtained from the patients and their parents after receiving approval by the Ethics Committee of the Health Sciences of Marmara University (ref no: 24.12.2014-2), Turkey. Enamel specimens with a size of 4×4×3 mm (length × width × depth), were prepared and polished using silicon carbide paper from 400 to 1200 grit to create a flat surface.

Artificial carious lesions:

Specimens were immersed in a solution of $Na_2HPO_4 \cdot 2H_2O$ (2.0 mmol/L), $Ca(NO_3)_2 \cdot 4H_2O$ (2.0 mmol/L), acetate buffer (75 mmol/L), 0.04 mg/L F, and adjusted to pH 4.7. Artificial caries lesions on the enamels were produced by incubating the samples at 37ºC for four days (96 hours).[8]

Treatment:

Forty-eight enamel specimens were randomly distributed in six groups. The treatment groups were: 5 varnish groups and a no-treatment group (control) with eight specimens per treatment group.

Specimens were classified as follows:

Group 1: MI Varnish® (CPP-ACP and 5% NaF) (GC, America, USA)

Group 2: Clinpro® White Varnish (TCP and 5% NaF) (3M ESPE, MN, USA)

Group 3: Duraphat® Varnish (2.26% F) (Colgate-Palmolive, NSW, Australia)

Group 4**:** Fluor Protector® (0,1% F) (Ivoclar Vivadent, NY, USA)

Group 5: Enamel Pro® Varnish (ACP and 5% NaF) (Premier Dental, PA, USA)

Group 6: Negative control

Surface microhardness analysis:

Initially, the surface microhardness of the sound enamel specimens was measured using a microhardness indenter (Vickers microhardness testing machine; Buehler Micromet Microhardness Tester, Germany) at a load of 50 g for 11 seconds. Varnish was applied to lesions, which were then incubated in artificial saliva. (Saliva samples were retained for F analysis.) Three indentations were made on each sample in order to calculate the mean hardness as Vickers hardness number. The percentage surface microhardness recovery (%SMHR) was calculated for each specimen using the formula:

$$\%SMHR = \frac{IL_{remin} - IL_{lesion}}{IL_{sound} - IL_{lesion}} \times 100.$$

Where:

%SMHR	=	Surface microhardness recovery (%)
IL_{remin}	=	Indenter load for remineralized enamel specimen
IL_{lesion}	=	Indenter load for specimen with an artificially induced caries lesion of the enamel
IL_{sound}	=	Indenter load for specimen of sound tooth with no caries lesion of the enamel

Artificial saliva composition:

The artificial saliva used in the study had the following composition:

$Na_3PO_4 \cdot 12H_2O$ (3.9 mM), KCl (17.98 mM), NaCl (4.29 mM), $CaCl_2$ (1.1 mM), $MgCl_2 \cdot 6H_2O$ (0.08 mM), H_2SO_4 (0.5 mM), and $NaHCO_3$ (3.27 mM). The final pH was adjusted to pH 7.2.[9]

F release from the varnishes:

F varnish was applied according to the manufacturer's instructions and approximately ten plus or minus one mg of F varnish were applied to each specimen for all varnish treatment groups. Immediately after the F varnish application, enamel specimens were placed into artificial saliva (10 mL for each specimen). The specimens were placed into an incubator set at 37ºC and incubated for 2, 24, and 48 hours, and for 7 days. Every day, the artificial saliva was renewed. At the end of 7 days, after rinsing with running deionized water for approximately 30 seconds, the applied F varnish was then carefully removed using chloroform-moistened cotton swabs. All specimens were incubated into fresh artificial saliva again for 24 hr. F release was measured by an ion-sensitive electrode (Thermo Scientific Orion 4-Star Plus pH/Conductivity Meter, USA) with buffering in TISAB III.

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Statistical analysis:

The data collected were analyzed using SPSS 16.0 (Statistical Package for the Social Sciences). For the %SMHR, the data were normally distributed and analyzed using One-way Analysis of Variance (ANOVA) with the Tukey-Kramer Multiple Comparisons test applied. For the F release, the data were not normally distributed and were analyzed using Kruskal-Wallis test with Dunns Multiple Comparisons test. The level of significance set at $p<0.05$.

RESULTS

The percentage surface microhardness recovery (SMHR%) of the varnishes:

The effect of F varnish application on enamel surface microhardness was evaluated and the means and standard deviations for the %SMHR were presented in Table 1. MI Varnish showed the highest %SMHR value. According to the %SMHR results, statistically significant differences were found between the F varnishes and the control group ($p<0.05$ and $p<0.001$).

Table 1. Mean and standard deviations (SD) of %SMHR for all groups

%SMHR	Mean	SD
MI Varnish	60.32†	16.69
Clinpro White Varnish	39.20†	19.79
Duraphat	54.53†	20.42
Fluor Protector	30.90*	26.35
Enamel Pro Varnish	54.85†	56.73
Negative control	4.62	7.94

*: Compared to negative control group $p<0.05$
†: Compared to negative control group $p<0.001$

However, there were no statistically significant differences in the %SMHR between the F varnishes, except for the MI Varnish being significantly better than Fluor Protector ($p<0.01$) (Table 2).

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Table 2. The pairwise comparison of %SMHR of fluoride varnishes

%SMHR	Clinpro White Varnish	Duraphat	Fluor Protector	Enamel Pro Varnish	Control
MI Varnish	>0.05	>0.05	<0.01*	>0.05	<0.001
Clinpro White Varnish		>0.05	>0.05	>0.05	<0.001
Duraphat			>0.05	>0.05	<0.001
Fluor Protector		>0.05		>0.05	<0.05
Enamel Pro Varnish		>0.05	>0.05		<0.001

*: Compared to Fluor Protector, MI Varnish had a significantly higher %SMHR value, $p<0.01$.

The F release of the varnishes in artificial saliva:

The mean F releases of the tested F varnishes and corresponding statistical analyses are shown in Table 3. Duraphat, Enamel Pro Varnish, and MI Varnish released the most F into the artificial saliva, but all the varnishes release patterns were different. The F release for Enamel Pro, Duraphat, and MI Varnish exhibited an initial burst and then gradually declined, while the other fluoride varnishes exhibited a very low F release overall, with the majority of F being released within hours. The highest F release occurred in the first 24 hours of application.

Table 3. Mean ± standard deviation of fluoride release (ppm) from the varnishes in the artificial saliva at each time point

Varnish	Fluoride release (mean ± standard deviation in ppm) from the varnishes in the artificial saliva at each time point			
	2 hr	24 hr	48 hr	7 days
MI Varnish	6.72(3.44) ^{aA}	22.66(6.79) ^{aB}	0.76(0.26) ^{aC}	0.084(0.11) ^{aC}
Clinpro White Varnish	0.62(0.27) ^{bB}	5.07(3.87) ^{bA}	2.22(0.85) ^{acB}	0.48(0.16) ^{abB}
Duraphat	2.3(0.54) ^{bB}	12.81(4.85) ^{abA}	2.70(0.90) ^{acB}	0.69(0.31) ^{bbB}
Fluor Protector	0.37(0.096) ^{bA}	0.42(0.15) ^{baA}	0.05(0.03) ^{abB}	0.05(0.03) ^{acB}
Enamel Pro Varnish	2.33(0.94) ^{bB}	12.30(5.10) ^{abA}	5.52(2.64) ^{bBC}	0.62(0.31) ^{bBC}

*Different lowercase letters show significant differences among the treatments for each time point (comparison per column), whereas different uppercase (capital) letters show significant differences among the treatments for each time point (comparison per line) ($p<0.05$).

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The release rate of F is shown graphically in the Figure. Our data show that the release of F was the highest in the MI Varnish group ($p<0.01$). The release rate of F by MI increased to the highest level in 24 hours. In the fluoride varnishes, 60% of F release occurred in the first 24 hours after which the rate of release slowed down. However, Fluor Protector and Clinpro White released less than other varnishes.

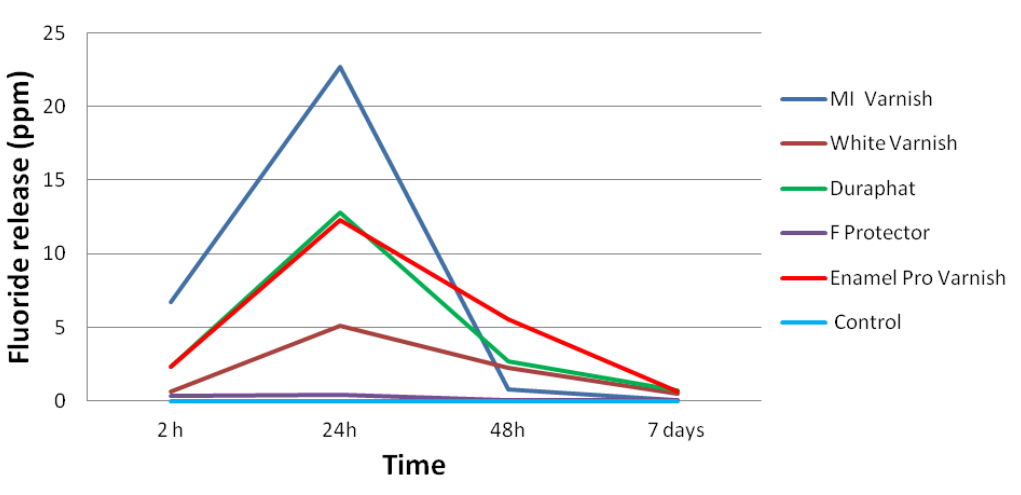

Figure. The *in vitro* release of fluoride from the fluoride varnishes into the artificial saliva according to the different time periods.

DISCUSSION

Preventive dentistry practices are extremely important in preventing initial carious lesions by non-invasive methods and maintaining oral health.[10,11] For this reason, much research is carried out in dentistry to detect initial carious lesions with current diagnostic methods at an early stage and to treat them with effective remineralization agents in order to prevent tooth hard tissue loss. The present *in vitro* study compared the effects of fluoride varnishes on artificial enamel caries lesions.

Microhardness indentation provides a relatively simple, rapid, and non-destructive method in demineralization and remineralization studies It is a proper test for enamel having regard for the fine microstructure of enamel and its non-homogenous and brittle nature.[12]

In this study, the enamel specimens treated with F varnishes had a higher SMHR than the control group. The present study was able to tell that F varnishes can reduce the progression of a carious lesion on the enamel and showed that the highest %SMHR was achieved with MI Varnish. Due to differences in the "adhesive properties" of the varnishes between some groups (NaF, TCP, and CPP, ACP), small amounts of the varnish could be observed. This described effect varied between the groups. CPP-ACP and NaF are supposed to be incorporated into the biofilm and onto the tooth surface. By buffering the biofilm pH, free Ca^{2+} and PO_4^{3-} dissociate.[13] Thus, it not only acts as a reservoir for storing bioavailable Ca^{2+} and PO_4^{3-}, but also maintains a state of supersaturation with respect to the tooth mineral. Hence, enamel demineralization is prevented and remineralization is promoted.[14] Regarding CPP, a significantly higher SMHR was observed, compared to NaF. Bahrololoomi et al. suggested that the application of products with calcium, phosphate, and F increase the microhardness of the enamel.[15] Contrary to our study, Al Dehailan et al. showed

the application of Enamel Pro Varnish provides more enamel re-hardening compared to MI Varnish.[16] A reasons for this may be the use of bovine enamel specimens for their study. Bovine enamel structure shows differences from human enamel and therefore the artificial carious lesion creation and structure would be different.

Similar to our findings, Zhou et al.[17] concluded that the combined use of CPP-ACP and F is more effective for remineralization than NaF. In this study, the CPP and ACP containing varnishes (MI Varnish and Enamel Pro Varnish) delivered statistically significant more F ions into the saliva than the other varnishes. Since demineralized enamel structure presents a higher number of pores with potential F retention sites, it is possible this resulted in a higher than expected fluoride ion concentration.[18] Therefore, the F uptake into the demineralized enamel specimens prevented dissolution and promoted remineralization using F from artificial saliva. This study indicated that, compared to the other F varnishes, the ACP containing varnish formulation both released and delivered to demineralized enamel, statistically significantly more F. The results of this study, as well as of the previous studies, indicate that CPP seems to be, especially effective when compared to NaF. It can, thus, be speculated that varnishes containing CPP-ACP form a reservoir of bioavailable Ca^{2+} and PO_4^{3-}, especially.

The F ion produces fluorapatite, which is more resistant to demineralization when calcium and phosphate are released from saliva, dental plaque, or enamel demineralization.[19,20] The remineralization of enamel subsurface lesions can be achieved by increasing of CaF_2 reservoirs and releasing F ions by applying F varnish.[21,22]

The highest release rate of F from varnishes occurs in the first 3 weeks and then slows down.[23] It has been thought that the varnishes remain in place for only 24 hours.[24] Arends and Schuthof[25] showed, by micro-hardness analysis and microradiography, that 24-hour contact of F varnish was sufficient to completely inhibit demineralization. Manufacturers typically recommend children to eat only soft food during the first two hours after a F varnish application.[2] Therefore, the two major F release points determined in this study were within the first 24 hours.

After the Duraphat Varnish application, F ions form a loose bond with the enamel.[26] To promote remineralization, tricalcium phosphate (TCP) interacts with demineralized enamel.[27] The F varnish containing TCP (Clinpro White Varnish) has shown better efficacy regarding the treatment of enamel demineralization. However, the reason for the low inorganic phosphate and calcium ion release from Clinpro White Varnish may be thought to be its low solubility.[24,27] Although the F contents are very similar in percentage and type, the additional component might cause the different patterns of ion release. Calcium phosphate-based varnishes are an ion source for bioavailable calcium, phosphate, and F.[18]

CPP is similar to TCP and is more reactive than calcium phosphates.[28] The combined use of F with CPP-ACP is more effective than using F only.[29] It is claimed that adding calcium, phosphate, and F in bioavailable forms to varnish increases the remineralization capacity compaered to products containing only F.[14,30]

Different F release patterns have been reported from different varnishes. In the present study, it was found that after the varnish application, the concentration of F

ions in the artificial saliva was statistically higher than in the control group for all groups. The rate of F release from F varnishes showed a peak at 24 hours. For the products tested, the MI Varnish showed the highest rate of F release in the first 24 hours, Our results showed that varnishes with 5% NaF with CPP-ACP had the highest F ion release in comparison with the 5% NaF varnishes. This could be explained due to the higher ability of CPP-ACP to dissolve in the aqueous solution increasing the passage of F ions into the storage medium.[31]

Clinpro White Varnish and Fluor Protector Varnish released less F than Duraphat, MI, and Enamel Pro Varnish at 2 and 24 hours. The rate of release of F by Enamel Pro Varnish was also high up to 48 hours. Al Dehailan et al.[16] showed the highest release from the varnishes examined was within the first 15 minutes to 1 hr of application. They showed that initially both Enamel Pro and MI Varnish presented similar F releases; then at the 3 hours, MI Varnish released more F than Enamel Pro. But, in the last 3 hours of the experimental period, Enamel Pro exhibited a more gradual decrease and released more F. The ion release pattern of each varnish containing F is different. The carrier in the varnish might be the component that appears to make the difference in the F release.[23] Fluor Protector Varnish had a high content of solvent ingredients (ethyl acetate, isoamyl propionate) that would have resulted in an underestimation of F ion release. Ritwik et al.[32] concluded that although all varnishes contain 5% NAF, the rate of F release varied in the short term.

Overall, the MI varnish, Enamel Pro varnish, and Duraphat have better performance in terms of F release over all the time points compared to the other F varnishes. In accordance with our study results, Schemehorn et al.[24] concluded that the varnish containing ACP increased significantly the F deposition on both sound and demineralized enamel than the TCP containing varnish. Virupaxi et al.[23] found that Fluor Protector Varnish is the lowest F release among the F varnishes evaluated.

The reaction of the F in varnishes with enamel is time-dependent. The insoluble F in the formulation plays a significant role in slowing this process, in comparison with the soluble fluoride concentration, which is responsible for chemically forming fluoride calcium-like reservoirs on the enamel, over a short period of time.[33]

Within the limitations of this study, the two null hypotheses of the current study were rejected. Although *in vitro* studies mimic the oral cavity using human teeth and artificial solutions with a pH similar to that of saliva, *in vitro* results may not be accurately representative of *in vivo* results. In this study, F ion release was evaluated from enamel specimens immersed in artificial saliva, and that may not take into account the dynamic nature of conditions in the oral cavity.

CONCLUSIONS

In conclusion, under the conditions chosen, this *in vitro* study suggests that all F varnishes showed the potential to increase F concentration in artificial saliva. Calcium phosphate-based F varnishes improve the capacity of the enamel surface re-hardening. CPP varnish was more effective in both reducng enamel surface demineralization and in releasing F than the other F varnishes. Further *in vivo* investigations are also required to prove for the clinical applications of different ingredients containing varnishes.

STATEMENT OF ETHICS

All procedures used were in accordance with the guidelines of the Helsinki Declaration on Human Experimentation.

DISCLOSURE STATEMENT

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