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

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ABSTRACT: This in vitro study aimed to evaluate the therapeutic effect of different fluoride (F) varnish formulations for controlling the carious development of enamel subsurface lesions and the F release into artificial saliva for 2h, 24h, 48h, 7 days. Artificial enamel carious lesions were created and divided into 6 groups (5 varnishes and control group). Varnishes were applied to enamel specimens then specimens were incubated in artificial saliva, with artificial saliva replenished daily. Varnish was removed and lesions were remineralized in artificial saliva for 24 hours. Surface microhardness was measured three times: at initial, after creating artificial enamel lesions, and applying varnishes. The F release was analyzed after 2h, 24h, 48h, and 7 days of exposure using an ion-selective electrode. Data were analyzed using a One-way Analysis of Variance with Tukey-Kramer Multiple Comparisons test and Kruskal-Wallis test with Dunns Multiple Comparisons test. The highest percentage surface microhardness recovery was found for the treatment with MI Varnish. According to the percentage surface microhardness recovery results, a statistically significant difference was found between varnishes and the control group (p<0.05). All varnishes released measurable F ion however on the release of F was the highest in 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 for the clinical applications of different ingredients containing varnishes.

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

Over the last years, fluoride (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 application products which deliver F to the mouth.\textsuperscript{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.\textsuperscript{3} F varnish typically contain five percent sodium fluoride (NaF) which aids in the formation of long-lasting intraoral F reservoirs.\textsuperscript{2} The American Dental Association was suggested that "Fluoride varnishes applied every six months is effective in preventing dental caries in the primary and permanent dentition of children and adolescent", this encourages paediatric dentists to use F varnishes more.\textsuperscript{4} Post-application F varnish, F is released from the varnish and is taken up by the tooth enamel, plaque, and saliva. The ionic F in solutions around the tooth inhibits demineralization and promotes remineralization, thereby reversing the development of early carious lesions.\textsuperscript{5}

The following F varnish with improved content are available: containing functionalized tricalcium phosphate (fTCP) consisting of tricalcium phosphate modified by fumaric acid, amorphous calcium phosphate (ACP) and casein phosphopeptide-amorphous calcium phosphate (CPP-ACP).\textsuperscript{6,7} Although in vitro F release from varnishes has been investigated for a long time, as far as we know no studies are examining 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

\textit{Preparation of enamel specimen}

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 4x4x3 specimens mm$^3$ (length × width × depth) were prepared and polished using silicon carbide paper from 400 to 1200 grit to create a flat surface.

*Artificial carious lesion*

Specimens were immersed into a solution of Na$_2$HPO$_4$·2H$_2$O (2.0 mmol/L), Ca(NO$_3$)$_2$·4H$_2$O (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 specimen were randomly distributed in six groups. The treatment groups were: 5 varnishes 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 = (IL$_{remin}$ - IL$_{lesion}$) / (IL$_{sound}$-IL$_{lesion}$) ×100.
Artificial saliva composition

The artificial saliva used in the study had the following composition: Na$_3$PO$_4$·12H$_2$O (3.9 mM), KCl (17.98 mM), NaCl (4.29 mM), CaCl$_2$ (1.1 mM), MgCl$_2$·6H$_2$O (0.08 mM), H$_2$SO$_4$ (0.5 mM), NaHCO$_3$ (3.27 mM). The final pH was adjusted to pH 7.2.  

F release from the varnishes

F varnish was applied according to 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 37°C and incubated 2, 24, 48 hours, and 7 days. Every day, the artificial saliva was renewed. At the end of 7 days; after the 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 hours. F release was measured by an ion-sensitive electrode (Thermo Scientific Orion 4-Star Plus pH/Conductivity Meter, USA) buffering in TISAB III.

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 Tukey-Kramer Multiple Comparisons test applied. For the F release, the data were not normally distributed and 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 also evaluated and the means and standard deviations for %SMHR were presented in Table 1. MI Varnish showed the highest %SMHR value. According to %SMHR results, a statistically significant difference was found between F varnishes and the control group (p<0.01). However, there were
no statistically significant differences in %SMHR between other F varnish, except Fluor Protector. MI Varnish resulted in a statistically significantly higher %SMHR than Fluor Protector (p<0.01) (Table 2).

The F releases of the varnishes in artificial saliva

The mean F releases of the tested F varnishes and corresponding statistical analyses were shown in Table 3. Duraphat, Enamel Pro Varnish, and MI Varnish released the most F into artificial saliva, but all varnishes release patterns were different. The F release for Enamel Pro, Duraphat and MI Varnish exhibited an initial burst and then gradually declined, the other Fluoride varnishes exhibited 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.

The release rate of F is graphically shown in Figure 1. Our data have shown that the release of F was the highest in MI Varnish group (p<0.01). The release rate of F by MI increased to the highest level in 24 hours. In Fluoride varnishes, 60% of F release occurred in the first 24 hours, then the rate of release slowed down. However, Fluor Protector and Clinpro White released less than other varnishes.

DISCUSSION

Preventive dentistry practices are extremely important in preventing initial carious lesions by non-invasive methods and maintaining oral health.\textsuperscript{10,11} For this reason, many researches are carried out in dentistry to detect initial carious lesions with current diagnostic methods at an early stage and treat them with effective remineralization agents and 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 regarding to enamels fine microstructure, non-homogenous and brittle nature.\textsuperscript{12}

In this study, the enamel specimens treated with F varnishes have a higher SMHR than the control group. The present study can tell that F varnishes can reduce the progression of the carious lesion on the enamel and showed that the highest %SMHR was achieved with MI Varnish. Due to differences in "adhesive properties" of the varnishes between some groups (NaF,
TCP, and CPP, ACP), small amounts of the varnish could be observed. This described effect would vary 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 compared to NaF was observed. Bahrololoomi et al. suggested that the application of products with calcium, phosphate, and F are in increasing 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.\(^{16}\) There might be reasons regarding to be used bovine enamel specimens for their study. Bovine enamel structure shows differences therefore artificial carious lesion creation and structure were 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 varnish (MI Varnish and Enamel Pro Varnish) delivered statistically significant more F ions into saliva than other varnishes. Since demineralized enamel structure presents higher number of pores with potential F retention sites it is possible and expected higher ions concentration.\(^{18}\) Therefore, the F uptake into the demineralized enamel specimens prevented dissolution and promote remineralization using F from artificial saliva. This study indicated that the ACP containing varnish formulations delivered statistically significant more F to both released and demineralized enamel than other F varnishes. The results of this study as well as of the previous studies indicate CPP seems to be, especially when compared to NaF. It can, thus, be speculated that varnishes containing CPP-ACP form 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 showed in the 3 weeks and then slowed down.\(^{23}\) But 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 F varnish application.\textsuperscript{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.\textsuperscript{26} To promote remineralization, tricalcium phosphate (TCP) interacts with demineralized enamel.\textsuperscript{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.\textsuperscript{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.\textsuperscript{18}

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

The different F release patterns have been reported from different varnishes. In the present study, it was found that after varnish application, the rate of F ions in 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. MI Varnish showed the highest rate of F release in the first 24 hours, among the products tested. 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 F ions into the storage medium.\textsuperscript{31}

Clinpro White Varnish and Fluor protector 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 \textit{et al.}\textsuperscript{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.\textsuperscript{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 P et al.\textsuperscript{32} concluded that although all varnishes contain 5% NAF, the rate of F release was various in the short term.

Overall, the MI varnish, Enamel Pro varnish, and Duraphat have better performance in terms of F release over all time points compared to other F varnishes. In accordance with our study results, Schemehorn et al.\textsuperscript{24} concluded that the varnish containing ACP increased significantly more F deposition on both sound and demineralized enamel than the TCP containing varnish. Virupaxi et al.\textsuperscript{23} found that Fluor Protector Varnish is the lowest F release among 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.\textsuperscript{33}

Within the limitation of this study, the two null hypotheses of the current study were rejected. Although \textit{in vitro} studies mimic the oral cavity using human teeth and artificial solutions with pH similar to that of saliva, \textit{in vitro} results may not be accurately representative of \textit{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.

**CONCLUSION**

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

\textbf{Statement of Ethics:} All procedures used were in accordance with the guidelines of the Helsinki Declaration on Human Experimentation.

\textbf{Disclosure Statement:} None of the authors declared a conflict of interest.
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REFERENCES


| 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 |

| Table 2. The Pairwise comparison 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           | >0.05             | <0.001   |
| Duraphat         | >0.05             | >0.05     | >0.05           | >0.05             | <0.001   |
| Fluor Protector  | >0.05             | >0.05     | >0.05           | <0.05             | <0.001   |
| Enamel Pro Varnish | >0.05             | >0.05     | >0.05           | <0.05             | <0.001   |

Statistically significant differences between fluoride varnishes are highlighted in bold (p<0.05).
Table 3. Mean ± standard deviation of fluoride release (ppm) from the varnishes in artificial saliva at each time point

<table>
<thead>
<tr>
<th>Varnish</th>
<th>2h</th>
<th>24h</th>
<th>48h</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>MI Varnish</td>
<td>6.72(3.44)</td>
<td>22.66(6.79)</td>
<td>0.76(0.26)</td>
<td>0.084(0.11)</td>
</tr>
<tr>
<td>Clinpro White Varnish</td>
<td>0.62(0.27)</td>
<td>5.07(3.87)</td>
<td>2.22(0.85)</td>
<td>0.48(0.16)</td>
</tr>
<tr>
<td>Duraphat</td>
<td>2.3(0.54)</td>
<td>12.81(4.85)</td>
<td>2.70(0.90)</td>
<td>0.69(0.31)</td>
</tr>
<tr>
<td>Fluor Protector</td>
<td>0.37(0.096)</td>
<td>0.42(0.15)</td>
<td>0.05(0.03)</td>
<td>0.05(0.03)</td>
</tr>
<tr>
<td>Enamel Pro Varnish</td>
<td>2.33(0.94)</td>
<td>12.30(5.10)</td>
<td>5.52(2.64)</td>
<td>0.62(0.31)</td>
</tr>
</tbody>
</table>

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

Figure 1. The in vitro release of fluoride from fluoride varnishes into artificial saliva according to the different periods.