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Original Article

Remineralization effects when using different methods to apply fluoride varnish *in vitro*

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Abstract *Background/purpose:* Remineralization efficacy for early caries lesion may change when fluoride varnish (FV) is applied directly or indirectly to the lesion. This *in vitro* study compared direct and indirect remineralization efficacies of FV on artificial caries lesions and evaluated acid-resistance of lesion remineralized by FV and artificial saliva.
Materials and methods: One hundred and twenty-six bovine demineralized specimens were allocated to four varnish groups (Duraphat®, EnamelPro®, MI™, and ClinproWhite™, $n = 28$ each) and a negative-control group ($n = 14$). Half of specimens from each varnish group had the FV applied and the other specimens didn't. The specimens treated and not treated with the FV were immersed together in 20 mL of artificial saliva at 37 °C for 24 h. Then the applied FV was removed carefully from the specimen, and immersion process was continued in fresh artificial saliva for 48 h. The negative-control group was immersed in artificial saliva for same time as in varnish groups. The acid resistance of remineralized specimens from varnish groups was compared to negative-control group. Vickers microhardness number (VHN) was measured to evaluate re-demineralization effect.
Results: The ΔVHN was significantly higher for indirect remineralization (134.4 ± 31.5, mean ± SD) than for direct remineralization (66.8 ± 27.9). All varnish groups showed significant differences between the direct and indirect application methods. The acid resistance of remineralized specimens was higher in the all FV groups than in the negative-control.
Conclusion: This *in vitro* study confirmed that the remineralization effect of fluoride varnishes would be higher in the vicinity than the underneath of the varnish treated surface.

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Introduction

Fluoride varnish (FV) is applied to teeth to form a thin film that remains on the teeth for a long time, and thereby provides sustained fluoride release.[1] It was first introduced in Europe in 1964, and numerous clinical studies performed over the past 25 years have identified it as a safe and effective method for applying fluoride.[2] FV has the advantage that a low concentration of fluoride is maintained in the oral cavity for 1–7 days after application, depending on the product, and it remains attached to the tooth for a relatively long time.

Several studies have evaluated the prevention of dental caries by FVs. The Cochran review performed by Marinho et al.[3] found that FV was effective in reducing dental caries in children and adolescents, including in permanent teeth, with permanent reduction rates of 46% and 33%, respectively. White-spot lesion (WSL) has been defined as subsurface enamel porosity produced by carious demineralization. Initial treatments for WSLs should adopt the most conservative approaches, such as applying topical fluoride (e.g., FV).[4]

The method used by clinicians to apply FV in patients with WSL is normally based on the manufacturer's recommendation. The FV is most commonly applied to the smooth surface of the tooth using an applicator brush. In particular, FV is normally applied directly above the site of dental caries to facilitate remineralization. However, the present authors questioned whether the varnish should be applied either over the surface of initial dental caries or around them so that fluoride ions are supplied but without blocking the contact of the tooth with saliva. The authors hypothesized that applying FV directly to an initial dental caries surface would prevent saliva contact in the oral cavity and thereby block the supply of minerals from the saliva.

Previous studies have evaluated the effect of the direct application of FV to dental caries on surface remineralization.[5,6] They assumed that calcium, phosphorus, and fluoride directly penetrate the surface through microdefect pores on the dentate surface due to caries. Such studies therefore did not take into account the possibility of the absorption of fluoride released from the FV applied adjacent to the tooth surface in the remineralization process. Castellano and Donly[5] compared the effect of applying FV at different locations on remineralization, and suggested that there was no significant difference between directly applying FV above the initial dental caries surface and applying it around the dental caries surface or in an adjacent area. In contrast, Lippert et al.[6] suggested that there was a significant difference between direct application and applying FV in the adjacent area. The discrepancies between these findings indicate the need for further studies comparing the efficacy of remineralization between indirect- and direct-application methods.

Most studies of dental erosion using an acid-challenge test have involved immersing the tooth in an acidic solution or beverage and comparing the degree of corrosion in sound enamel, with the results suggesting that FVs can prevent enamel loss.[7] No research has compared the resistance to acidic solutions or acid challenges after remineralizing a demineralized specimen using FV. It is expected that fluoride-remineralized teeth will have a high content of fluorapatite, and therefore also exhibit greater resistance to acid challenges.

The purpose of the present study was to compare the remineralization effects on demineralized enamel between the direct application of FV (on the initial dental caries surface) and indirect application (around the dental caries surface or in the adjacent area). We also determined whether any variations in the remineralization rate with application method differed between FV products. Finally, we compared the resistance of remineralized enamel to acid using FV and artificial saliva.

Materials and methods

Bovine specimen preparation and lesion formation

One hundred and twenty-six enamel specimens (3 mm in diameter) were drilled from extracted bovine teeth. Each specimens was mounted on a polyacrylic rod using self-cured direct acrylic resin. Then specimens were sequentially ground and polished using 600-, 1200-, 2000-, 2400-, and 4000-grit abrasive paper, followed by polishing with a 1-μm gamma alumina abrasive suspension. Specimens with Vickers microhardness numbers (VHNs) from 305 to 360 (317.9 ± 27.6, mean $\pm$ SD) were used in this study.

The enamel specimens were immersed in 13 mL of demineralizing gels[8] (0.1 M lactic acid, 0.2% Carbopol ETD 2050, and 50% saturated hydroxyapatites [calcium phosphate]; pH 5.0) at 37 °C for 24 h. They were then carefully washed under tap water to remove any excess gel. Only specimens with average VHNs of 60–100 were used as demineralized specimens.

Fluoride varnish applications

The 126 demineralized specimens were then divided into the four treatment groups ($n = 28$ each) and a negative-control group ($n = 14$) according to their hardness values. The following four types of fluoride varnishes containing 5% NaF as the main component were used in this study: (1) Duraphat® (DP, Colgate-Palmolive Co., Guildford, Surrey, UK), (2) Enamel Pro® (EP, Premier Dental, Plymouth Meeting, PA, USA), (3) MI™ (MI, GC corp., Tokyo, Japan), and (4) Clinpro™ (CP, 3M ESPE, ST. Paul, MN, USA). All of the FV products were stored at room temperature for 24 h prior to the start of the experiment. The products were packaged in disposable quantities and fully mixed before being applied in accordance with the manufacturers' recommendations.

The four treatment groups were directly and indirectly treated with each fluoride varnish. Half of specimens from each treatment group had the FV applied (FV-direct group) and the other specimens did not applied FV but later exposed indirectly to fluoride ion released from specimens applied with FV (FV-indirect group). FV (0.01 mL) was applied evenly to the specimens in the FV-direct group. One piece of the varnish-applied specimen and one piece of the unapplied specimen were paired, and both specimens immersed into the same container with 20 mL of artificial saliva at 37 °C for 24 h to induce remineralization of the lesion. The artificial saliva was not exchanged during this period. After immersion for 24 h, the FV that had been applied in the FV-direct group was rubbed off using cotton balls soaked in acetone three times. All of the specimens were then washed in distilled water and finally immersed again in artificial saliva for 48 h at 37 °C. A pair of specimens from the negative-control group were only immersed in artificial saliva for 24 h and 48 h continuously. After 24 h, the artificial saliva was replaced with fresh artificial saliva as in the treatment groups. The composition of the artificial saliva was as follows; 1.45 mM $CaCl\cdot 2.2H_2O$, 5.40 mM KH_2PO_4, 14.90 mM KCl, 28.40 mM NaCl, and 2.20 g/L mucin; adjusted to pH 6.8 with 5 M NaOH.[9]

The specimens were then rinsed in distilled water and stored in frozen form until the hardness was measured.

Acid resistance of remineralized specimens

The acid resistance of the remineralized specimens with and without fluoride was evaluated. The specimens with similar VHNs were collected from the FV-indirect group and the negative-control group. The specimens from the FV-direct group was excluded since this group (the mean of VHN_{remin}: 137.5) showed significantly lower VHNs than the FV-indirect (205.3) and the negative-control (190.2) group (Table 1). Several specimens of FV-indirect group and negative-control group were also ruled out since they had VHN of surface less than 170 and more 250 or relatively severe multiple surface crack problem due to indentation load of microhardness tester. Finally, 40 specimens from the FV-indirect group and 10 specimens from the negative-control group were used for evaluation of acid resistance.

Citric acid and orange juice have generally been used for acid-resistance tests of tooth enamel.[10] In the present study the specimens were immersed in 13 mL of commercially available orange juice (pH 2.3) (Minute Maid®, Coca-Cola Korea, Seoul, Korea) at 37 °C for 2 h.

Pre and post-treatment lesion analyses

The treatment effect of each group was evaluated by measuring the VHN of enamel surface with a Vickers microhardness tester (Mitutoyo HM-122®, Mitutoyo, Japan). Four indentation were made on each specimen before and after each treatment using a single load of 100 g for 10s. The average hardness value of the specimens was determined from four indentations. Following measurements of VHN after treatment were indented at the place adjacent to first measured.

The difference (ΔVHN) between VHN_{demin} and VHN_{remin} was calculated to evaluate remineralization effect by fluoride varnish application mode. The difference (ΔVHN) between VHN_{remin} and VHN_{acid} was calculated to evaluate acid resistance of remineralized specimens. The change ratio was calculated as a percentage according to change ratio (%) $= (VHN_{after} - VHN_{before})/VHN_{before} \times 100$.

Statistical analysis

One-way ANOVA was used to compare the differences in the VHNs of the specimens after remineralization among the FV-direct, FV-indirect groups and negative-control group and the differences in remineralization according to FV products. Duncan's multiple-comparison method was used for post-hoc analysis. Differences in the VHNs of the specimens after acid challenges between the FV-indirect and negative-control groups were compared using the independent *t*-test. A value of $P < 0.05$ was considered to indicate a statistical significance. All of the statistical analysis was performed using the IBM SPSS statistics program (IBM SPSS 21.0, IBM, Armonk, NY, USA).

Results

VHN_{demin} did not differ significantly between the FV-direct, FV-indirect, and negative-control groups. After 72 h of remineralization, the hardness increased significantly in the FV-indirect and negative-control groups compared to the

Table 1 Change in enamel hardness induced by remineralization.

Groups	*N*	VHN_{demin}	VHN_{remin} [a]	ΔVHN[a]	Change (%)[a]
FV-direct group	56	70.6 ± 13.9	137.5 ± 33.4^a	66.8 ± 27.9^a	97.0 ± 41.1^a
FV-indirect group	56	70.9 ± 12.6	205.3 ± 32.9^b	134.4 ± 31.5^b	196.1 ± 56.4^b
Negative-control group	14	70.1 ± 8.0	190.2 ± 42.6^b	120.1 ± 38.4^b	170.8 ± 53.2^b
Total	126	70.7 ± 12.7	173.5 ± 47.1	102.8 ± 44.6	149.3 ± 68.5

FV = Fluoride varnish.
Data are mean ± SD values.
Change (%) $= (VHN_{remin} - VHN_{demin})/VHN_{demin} \times 100$.
[a] Different superscript letters indicate significant differences in columns using ANOVA and post-hoc Duncan's multiple-comparison method.

FV-direct group. VHN_{remin} was 205.3 ± 32.9 in the FV-indirect group and 190.2 ± 42.5 in the negative-control group (Table 1).

Table 2 presents the ΔVHNs according to FV application methods and products. ΔVHN was highest for DP ($\Delta VHN = 88.8 \pm 22.3$) and MI ($\Delta VHN = 79.6 \pm 26.1$) and lowest for EP ($\Delta VHN = 40.2 \pm 18.7$) ($p < 0.05$) in the FV-direct group, while it was highest for EP ($\Delta VHN = 153.9 \pm 22.3$) and MI ($\Delta VHN = 146.1 \pm 20.3$) and lowest for DP ($\Delta VHN = 111.6 \pm 39.1$) ($p < 0.05$) in the FV-indirect group. The mean remineralization increases for all products were significantly higher in the FV-indirect group than in the FV-direct group ($p < 0.001$). EP showed the greatest difference in remineralization between the two application methods, being $61.2 \pm 33.2\%$ in the FV-direct group and $228.4 \pm 45.5\%$ in the FV-indirect group, while DP showed the lowest difference of less than 25% between the two application methods. DP was remineralized more in the FV-direct group. For EP, the remineralization rate was relatively low in the FV-direct group but remained high in the FV-indirect group. In other words, the remineralization rate varied with the type of FV and the application method ($p < 0.001$).

Table 3 presents the changes in hardness after the acid challenges using orange juice. The mean difference (ΔVHN) after the test was -130.5 ± 21.3 in the negative-control group and -77.8 ± 21.5 in the FV treatment group ($p < 0.001$). The ΔVHN and the percentage change after the test differed significantly according to the FV product ($p < 0.001$). The percentage change of MI and EP were -30.4 ± 9.6 and -33.4 ± 9.5, respectively, and were significantly higher for DP (-43.0 ± 10.4) and CP (-43.3 ± 6.8) (both $p < 0.05$).

Discussion

Many laboratory and clinical research studies have demonstrated the effectiveness of FVs.[11–15] However, knowledge of the differences in initial carious remineralization according to FV application method is necessary when investigating methods for improving the remineralization efficacy. The purpose of this study was to determine the difference in remineralization between the direct and indirect application of FV on demineralized specimens by measuring changes in the surface microhardness *in vitro*.

This study found that hardness increased more in the FV-indirect and negative-control groups than in the FV-direct group after 72 h of remineralization treatment. It is considered that components of FV such as a resin base and rosin inhibited the contact between the artificial saliva and the demineralization specimen during the initial remineralization period, thereby interfering with the infiltration of inorganic ions and fluoride.

It is known that the mechanism underlying remineralization by fluoride ions also promotes the remineralization of a caries lesion. In contrast, this study found no statistically significant difference in the increase of hardness after remineralization between the FV-indirect and negative-control groups. That is, in the present *in vitro* experimental model, the presence of fluoride in the immersion solution due to the application of FV did not significant affect the increase in hardness. These results can be attributed to the composition of the artificial saliva used for the remineralization process. The artificial saliva used in many studies to reproduce real oral conditions is rich in minerals, which results in it being effective for remineralization.[16] Artificial saliva has also been used as a remineralization solution in the pH-cycling model.[17,18] The artificial saliva used in the present experiment was supersaturated with calcium and phosphorus, which can be assumed to underlie why there was no significant difference between the FV-indirect and negative-control groups.

The increase in hardness of the initial caries lesion was greater in the FV-indirect group than in the FV-direct group for all four products tested in this study: the increase was 3.73-fold for EP, 2.02-fold for CP, 1.99-fold for MI, and 1.20-fold for DP. Lippert et al.[6] reported that the hardness of the FV as measured using the Knoop surface microhardness

Table 2 Increase in enamel hardness according to FV product.

Groups	Varnish products	N	VHN_{demin}	VHN_{remin}*	ΔVHN*	Change (%)*
FV-direct group	DP	14	73.3 ± 17.9	162.1 ± 36.6^{a}	88.8 ± 22.3^{a}	124.1 ± 27.1^{a}
	EP	14	69.8 ± 16.3	110.0 ± 23.5^{b}	40.2 ± 18.7^{c}	61.2 ± 33.2^{c}
	MI	14	71.2 ± 8.7	150.8 ± 27.0^{a}	79.6 ± 26.1^{a}	113.7 ± 41.7^{ab}
	CP	14	68.3 ± 11.8	127.0 ± 18.1^{b}	58.7 ± 15.7^{b}	89.3 ± 32.3^{b}
	Total	56	70.6 ± 13.9	137.5 ± 33.4	66.8 ± 27.9	97.0 ± 41.1
FV-indirect group	DP	14	77.4 ± 14.2	189.0 ± 41.0^{b}	111.6 ± 39.1^{c}	149.1 ± 52.0^{a}
	EP	14	69.0 ± 12.4	223.0 ± 26.6^{a}	153.9 ± 22.3^{a}	228.4 ± 45.5^{b}
	MI	14	66.6 ± 11.6	212.7 ± 23.4^{ab}	146.1 ± 20.3^{ab}	226.4 ± 52.2^{b}
	CP	14	70.6 ± 10.4	196.4 ± 29.8^{b}	125.8 ± 23.7^{bc}	180.3 ± 34.7^{a}
	Total	56	70.9 ± 12.6	205.3 ± 32.9	134.4 ± 31.5	196.1 ± 56.4

FV = Fluoride varnish; DP = Duraphat®; EP = EnamelPro®; MI = MI Varnish™; CP = Clinpro™ White Varnish.
Data are mean ± SD values.
Change (%) = $(VHN_{remin} - VHN_{demin})/VHN_{demin} \times 100$.
* Different superscript letters indicate significant differences in columns using ANOVA and post-hoc Duncan's multiple-comparison method.

Table 3 Differences in enamel hardness after immersion in orange juice for 2 h.

Group		N	VHN_{remin}	VHN_{acid}	ΔVHN	Change (%)
Negative-control group		10	212.9 ± 19.0	82.5 ± 21.1	−130.5 ± 21.3	−61.4 ± 8.9
Fluoride treatment group		40	211.1 ± 22.2	133.3 ± 28.4	−77.8 ± 21.5	−37.1 ± 10.6
Fluoride Treatment group	DP	10	204.8 ± 26.8	117.0 ± 26.3[a]	−87.8 ± 22.5[bc]	−43.0 ± 10.4[a]
	EP	9	213.4 ± 18.4	142.7 ± 27.5[b]	−70.7 ± 17.7[ab]	−33.4 ± 9.5[b]
	MI	12	216.3 ± 18.8	150.2 ± 23.0[b]	−66.1 ± 22.7[a]	−30.4 ± 9.6[b]
	CP	9	209.0 ± 25.8	119.4 ± 24.5[a]	−89.6 ± 11.2[c]	−43.3 ± 6.8[a]

DP = Duraphat®; EP = EnamelPro®; MI = MI Varnish™; CP = Clinpro™ White Varnish.
Date are mean ± SD values.
No significant intergroup differences in VHN_{remin}.
Change (%) = $(VHN_{acid} - VHN_{remin})/VHN_{remin} \times 100$.
Independent sample t-test showed signifacant intergroup difference in VHN_{acid}, ΔVHN, Change (%).
Different superscript letters indicate significant differences in columns using ANOVA and post-hoc Duncan's multiple-comparison method.

test after remineralization treatment increased significantly when the FV was applied indirectly to the adjacent tooth surface compared to applying FV directly to the demineralized tooth surface. That study used four types of FV: CS, EP, MI, and Prevident (Colgate ®), and the hardness increase was 1.1- to 2.0-fold higher for indirect application than for direct application, depending on the product. However, those authors focused on remineralization according to the application method of FV and the product used, and they did not compare differences in the remineralization effects with the negative-controls in which specimens were immersed into artificial saliva and an acid-challenge test was performed, which represent substantial differences from the present study.

The difference in remineralization according to the application method found in this study is assumed to be attributable to a masking effect of varnish and the contact time between artificial saliva and the demineralization specimen. In the FV-direct group, it is presumed that the contact of the artificial saliva during the initial 24 h was interrupted by the FV, and that remineralization by the artificial saliva only occurred after the FV was removed. In the FV-direct group, fluoride and inorganic ions (which are components of the FV) were absorbed into the initial dental caries surface immediately after application for 24 h. However, since the hardness was not measured at 24 h, this could not be concluded definitively. In the FV-indirect group, remineralization is first achieved by artificial saliva, and remineralization may have been promoted due to fluoride and inorganic ions liberated therefrom. For this reason, fluoride and organic ions were more likely to reach the initial dental caries surface in the FV-indirect group than in the FV-direct group, resulting in them being able to promote remineralization.

Did the remineralization characteristics differ according to the product? Among FV-direct group, DP showed a significant hardness increase compared to the other FV products. This suggests that the action of fluoride on the surface of the artificial carious lesion to which DP was applied was greater than that of other products, or that the artificial saliva passed through the FV and acted on the surface of the artificial caries. Cruz et al.[19] reported that the application of DP results in the formation of a relatively large amount of loosely bound fluoride ions among the dental caries in the enamel, and that these surface fluoride ions can improve remineralization when FV is applied directly. Castellano and Donly[5] found no significant difference in remineralization according to the FV application method when using Duraflor (Pharmascience, Canada). This might be due to minerals passing through the resin base, or the varnish dissolving mostly in the solution during the experiment or not adhering to the specimen. In other words, the resin component of DP would have passed more easily through the FV compared to the other products, or the time for which the artificial saliva was kept from the specimen was shorter than that for the other products.

In the FV-indirect group, EP showed a significant hardness increase compared to the other FV products. EP releases fluoride and mineral ions and seems to have a great remineralization effect. MI and EP exhibited higher efficacy when they were applied in the vicinity rather than directly to the lesion, compared to fluoride uptake in the enamel.[6] The significant difference in the increase in VHN depending on the product in the indirect-application group could be due to the difference in the concentration of fluoride dissolved in the solution. A previous study found that the fluoride concentration differed significantly among five FV products,[20] with EP and MI contributing significantly more fluoride ions after 96 h of immersion compared to the other three products. In the present study, EP and MI produced larger hardness increases after remineralization among the FV-indirect group, and the difference in remineralization among those groups may be due to differences in the amount of released fluoride ions.

The presence of fluoride ions is reported to improve the acidic dissolution resistance of the enamel and protect the enamel surface.[9,20,21] It is well known that fluorapatite is more resistant to acid than is hydroxyapatite.[22] In the acid-challenge test involving orange juice, the loss of enamel hardness (i.e., ΔVHN) was significantly greater in the control group than in the FV-indirect group. That is, there was no significant difference in remineralization depending on the presence or absence of FV, but there was a significant difference in the loss of hardness in the acid-resistance test. The remineralized specimens in artificial saliva contained hydroxyapatite damaged by demineralization due to the penetration of calcium and phosphorus ions in the artificial saliva into the microdefect structure (pore) of the demineralized specimen surface, while the FV-indirect group formed fluorapatite using the fluoride contained in the varnish and the minerals in the artificial saliva acting together. It is considered that hydroxyapatite and fluorapatite formed in each group were responsible for the differences in acid solution resistance according to the presence or absence of FV.

Several limitations of this study should be discussed. Artificial saliva contains more minerals than actual human saliva but does not contain proteins and enzymes, which could have resulted in remineralization occurring more easily than in the real oral environment.[23] In addition, the acid challenge test is an experimental model that reproduces the phenomenon of acid challenges mainly *in vitro*, and the phenomenon that occurs on the enamel surface differs from demineralization. Demineralization is caused by the surface layer being protected and the internal minerals disappearing, while the acid challenges investigate corroding of the surface enamel induced by an acid.[24] The present study evaluated the degree of surface erosion based on changes in the microhardness of the enamel surface. Further investigations using methods that directly measure the degree of surface erosion are needed for accurately testing the resistance to acid solutions.

The results of this study suggest that the remineralization effect is primarily due to the presence of calcium and phosphorus in the artificial saliva, followed by fluoride and minerals liberated from indirectly applied FV. However, some questions remain regarding how to apply this remineralization process in the real oral cavity. The application of FV results in a sharp increase in the concentration of fluoride ions in the oral cavity, but this subsequently decreases to the pre-application state after about 24 h.[25] Therefore, remineralization by FV is considered a short-term effect due only to the action of liberated ions. In addition, the present experiments did not reproduce salivary circulation in the oral cavity since only a simple immersion method was employed. Future studies should perform remineralization experiments after varnish application using human saliva and a pH-cycling model, or use an *in situ* model to demonstrate remineralization. The results of this study suggest that the FV-indirect application method results in a higher remineralization efficacy than the direct application of FV to carious lesions. It is considered that the contact with saliva and the presence of fluoride ions are involved in remineralization of initial dental caries. Further *in vivo* research will be required before the results obtained in this study can be applied clinically.

Conflicts of interest

The authors declare that they have no competing interests.

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