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An *In Vitro* Investigation of Anticaries Efficacy of Fluoride Varnishes

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Clinical Relevance

This study provides clinicians with insight into the *in vitro* differences in the performance of commercially available fluoride varnishes. More clinical research is needed to equip dentists with evidence that will help them make better treatment choices.

SUMMARY

Most currently marketed fluoride varnishes (FVs) have not been evaluated for their effectiveness in preventing dental caries. The objective of this study was to investigate the anticaries efficacy, measured as fluoride release into artificial saliva (AS); change in surface microhardness of early enamel caries lesions; and enamel fluoride uptake (EFU) of 14 commercially available FVs and two control groups. Bovine enamel specimens (5×5 mm) were prepared and assigned to 18 groups (n=12). Early caries lesions were created in the specimens and characterized using Vickers microhardness (VHN_{lesion}). FV was applied to each group of specimens. Immediately afterward, specimens were incubated in 4 mL of AS for 18 hours, which were collected and renewed every hour for the first six hours. AS samples were analyzed for fluoride using an ion-specific electrode. Specimens were then brushed for 20 seconds with toothpaste slurry and subjected to pH cycling consisting of a four-hour/day acid challenge and one-minute treatments with 1100 ppm F dentifrice for five days. Microhardness was measured following pH cycling (VHN_{post}). EFU was determined using microbiopsy. Acid resistance (eight-hour demin challenge) was performed after pH cycling, and microhardness was measured (VHN_{art}) and compared with baseline values to test the FV impact after pH cycling. One-way analysis of variance was used for data analysis (α=0.05). FVs differed in their release characteristics (mean ± SD ranged from 14.97 ± 2.38 µg/mL to 0.50 ± 0.15 µg/mL), rehardening capability (mean ± SD ranged from 24.3 ± 15.1 to 11.7 ± 12.7), and ability to deliver fluoride to demineralized lesions (mean ± SD ranged from

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3303 ± 789 μg/cm^3 to 707 ± 238 μg/cm^3). Statistically significant but weak linear associations were found between $\Delta VHN_{(post\ -\ lesion)}$, EFU, and fluoride release (correlations 0.21-0.36). The results of this study demonstrated that differences in FV composition can affect their efficacy in *in vitro* conditions.

INTRODUCTION

The effects of topically applied fluoride treatments have been well known since the 1930s and have resulted in beneficial outcomes in caries reduction.[1-5] Several studies have confirmed the effectiveness of fluoride varnishes (FVs) in reducing caries incidence and prevalence with the added benefits of ease of use and, most importantly, virtually no side effects.[6-8]

In vitro models are very commonly used in cariology research. Most established models are designed to mimic clinical conditions as closely as possible. *In vitro* models have controlled scientific settings that limit variability and increase sensitivity compared with clinical models. However, they come with their own limitations in their inability to fully simulate the complex intraoral environment, especially circumstances leading to formation of dental caries.[9-11]

Most of the available *in vitro* studies that investigate the efficacy of FVs vary in their experimental design and testing conditions.[12,13] This creates heterogeneity, making it difficult to find benchmark tests that accurately assess efficacy. Therefore, we have selected to investigate different research variables that are of importance when studying fluoride efficacy in an attempt to improve our understanding of FVs and find the best test conditions. We also tried to study how those variables relate to each other in an effort to establish relevant variables for *in vitro* FV research.

The aim of our study was to investigate the potential anticaries effect of 14 commercially available FVs on artificially created early caries lesions through the following outcome variables: 1) 24-hour fluoride release into artificial saliva (AS), b) surface microhardness, and c) enamel fluoride uptake (EFU), after pH cycling for five days with a second demineralization challenge.

METHODS AND MATERIALS

Specimen Preparation

Enamel specimens, 5 × 5 mm, were obtained from bovine teeth using a low-speed saw (IsoMet, Buehler, Lake Bluff, IL, USA). The teeth were stored in deionized water saturated with thymol during the sample preparation process. The specimens were ground and polished to create flat surfaces to facilitate surface microhardness testing using the Struers RotoPol 31/RotoForce 4 polishing unit (Struers Inc, Cleveland, PA, USA). The bottom side of the specimens was ground flat to a uniform-thickness using 500-grit silicon carbide grinding paper. The topside of the specimens was ground in a series of 1200-, 2400-, and 4000-grit paper until most of the tooth surface was flattened. The specimens were then polished using 1-μm diamond paste to eliminate any scratches that may have resulted from the grinding procedure. The specimens were sonicated in deionized water between each grinding/polishing step. As a final cleaning step, the polished specimens were sonicated in 2% microliquid. The specimens were assessed with a magnification of 10×.

To be acceptable for the study, a specimen was required to

1) have a minimum 5 × 5 mm polished facet across the surface;
2) not have any obvious cracks or other flaws in the enamel surface;
3) have an evenly polished, high-gloss enamel surface; and
4) have no contamination on the top surface from sticky wax or any other material.

Specimens were then secured, with the polished enamel side facing upward, on a one-inch square acrylic block using sticky wax to facilitate surface microhardness measurements. Then, all specimen surface areas apart from the polished top surface were covered with a colored nail varnish (Sally Hansen Advanced, Hard As Nails Nail Polish, Red, New York, NY, USA). Prepared specimens were stored at approximately 100% relative humidity and 4°C until further use. A total of 216 specimens were prepared for the present study (18 groups of 12 specimens each).

Sound Enamel Microhardness

Initial surface microhardness of the sound enamel specimens was determined using a Vickers microhardness indenter at a load of 200 g for 11 seconds (Instron T2100B Vickers Surface Microhardness Tester, Norwood, MA, USA). The average sound enamel microhardness (VHN_{sound}) was determined from five indentations on the surface of each specimen (Figure 1). Only specimens with $300 \leq VHN_{sound} \leq 400$ were accepted into the study.

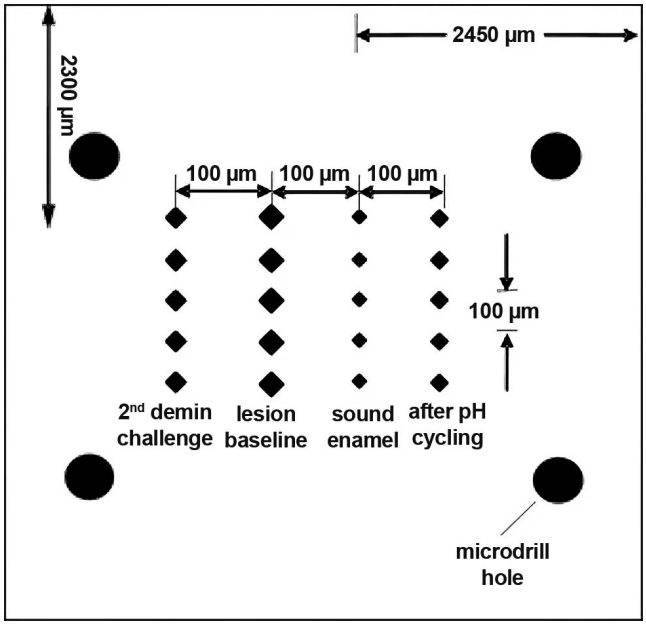

Figure 1. *Enamel specimen (5×5 mm) with microhardness indentations and microdrill holes.*

Artificial Lesion Creation

Artificial lesions were formed in the enamel specimens by a 48-hour immersion at 37°C into a solution of 0.1 M lactic acid and 0.2% Carbopol C907, which was 50% saturated with hydroxyapatite and adjusted to pH 5.0 using KOH. After demineralization, the specimens were rinsed with deionized water for approximately one minute. Specimens were blotted dry with a tissue and stored at approximately 100% relative humidity and 4°C until further use.

Lesion Baseline Microhardness

Microhardness of the demineralized enamel specimens was determined as described above. The average specimen lesion baseline microhardness (VHN_{lesion}) was calculated. Only specimens with $25 \leq VHN_{lesion} \leq 60$ were accepted into the study. Specimens were assigned to treatment groups (n=12) based on a randomization procedure that resulted in treatment groups with mean VHN_{lesion} values that were not significantly different statistically.

Specimen Mounting

Once assigned to their treatment groups, specimens were removed from their acrylic blocks and mounted onto a 12-well microtiter plate lid. Acrylic blocks (12×12×9 mm) were mounted onto the inside of the lid using acrylic glue. Then, specimens were mounted with the enamel side facing upward onto the acrylic block using sticky wax.

FV Application

All products tested in this study contained 5% NaF (22,600 ppm fluoride ion) and were assigned to groups and labeled as per Table 1. In addition to the test FVs shown above, two additional experimental groups were included: one placebo group (O–; placebo varnish, no toothpaste treatment during pH cycling phase) and one positive control group (O+; no FV treatment, toothpaste treatment during pH cycling phase). The placebo varnish had the following composition (all w/w): 2% shellac, 10% ethyl cellulose, 40% ethyl acetate, 2% polyvinylpyrrolidone, 2% xylitol, 5% NaCl, and 39% ethanol and was manufactured in-house especially for this study.

The protective foil from the individual FV dose was removed, and the FV was mixed using the manufacturer's applicator for at least 10 seconds to homogenize the FV, as sedimentation of NaF and phase separation may have occurred during storage. Duraphat (group C) was supplied in a tube. For this FV, approximately 0.5 mL was squeezed into a small weighing cup and processed as described above. The placebo varnish was handled in a similar manner.

Subsequently, FV was applied to the surface of each specimen using a single brush stroke and using the manufacturer's applicator. Any unused FV was discarded.

Saliva Incubation

Immediately after FV application, the lid containing 12 specimens was placed onto the microtiter plate containing 4.0 mL of AS per well. AS had the same composition as in a previous experiment.[14] Two batches of this solution were prepared: one for the FV incubation phase in all specimens and one for the pH cycling phase for all specimens.

The microtiter plate was then placed in an incubator set at 37°C. Every hour for six hours, the lid was placed onto a new microtiter plate containing 4.0 mL fresh AS per well.

This procedure was repeated until a total AS exposure time of six hours had been reached. After six hours, the lid was placed onto a new microtiter plate containing 4.0 mL AS per well, which was placed in the incubator for 18 hours. All saliva samples were frozen immediately after each cycle and retained for analysis of ionic fluoride.

Table 1: *Study Test Products*

Group	Product	Manufacturer	Noteworthy Ingredient
A	CavityShield 5% Varnish	3M ESPE	—
B	Vanish 5% NaF Varnish w/TCP	3M ESPE	Functionalized tricalcium phosphate (fTCP), Xylitol
C	Colgate Duraphat Varnish	Colgate Oral Pharmaceuticals	—
D	Colgate Prevident Varnish	Colgate Oral Pharmaceuticals	Xylitol
E	Sparkle V Varnish	Crosstex International	Xylitol
F	Nupro 5% Fluoride Varnish	Dentsply Professional Division	—
G	Kolorz Clearshield Varnish	DMG America	Xylitol
H	MI Varnish	GC America	Casein phosphopeptide-ACP (CPP-ACP, Recaldent)
I	Duraflor Halo 5% Sodium Fluoride Varnish	Medicom	—
J	Enamel Pro Varnish Clear	Premier Dental	Amorphous calcium phosphate (ACP), Xylitol
K	Vella Fluoride Varnish	Preventive Technologies	Xylitol
L	Butler White Fluoride Varnish	Sunstar Americas, Inc	Xylitol
M	Flor-Opal Varnish White Fluoride Varnish	Ultradent	Xylitol
N	Waterpik UltraThin Varnish	Waterpik Technologies Inc	Xylitol
O	Placebo Varnish	(manufactured in-house)	—

Then, a soft toothbrush (Oral B P40, Procter & Gamble, Mason, OH, USA) was used to brush each specimen by hand. A slurry of Crest Cavity Protection (0.243% sodium fluoride; Procter & Gamble) at a ratio of 1:2 w/w (dentifrice: AS) was prepared in a beaker with a magnetic stirrer. The toothbrush was dipped into the slurry briefly (approximately two seconds). Each specimen was then brushed under a stream of deionized water for 20 seconds, with the specimen being rinsed another 10 seconds after brushing. This procedure was repeated until all specimens on the lid were brushed. A new toothbrush and slurry was used for each lid. After this procedure, the pH cycling phase commenced on the same day.

pH Cycling Phase: Remineralization

The present study was separated into three phases, and each phase contained a placebo group (ie, O-a, O-b, O-c) to allow for comparisons between phases. The daily cyclic treatment regimen for each lid containing the specimens consisted of one, one-minute treatment period with a slurry of aforementioned toothpaste (prepared as described above), followed by a two-hour exposure to AS, four hours per day acid challenge in the lesion-forming solution, and exposure to AS for the remainder of the day and overnight. One slurry per day was prepared and pipetted into each well of the used microtiter plates.

The pH cycling was performed by placing the lid containing the specimens onto different microtiter plates containing 4.0 mL each per well of toothpaste slurry, AS, or lesion-forming solution. After each treatment, the specimens were rinsed under running deionized water briefly (approximately two seconds per specimen). The regimen was repeated for five days. The study was conducted at room temperature.

After completion of the pH cycling phase, all specimens were carefully removed from the lids and remounted onto an acrylic block to facilitate microhardness and EFU measurements.

Posttreatment Microhardness

The average specimen microhardness (VHN_{post}) was determined again in the same manner as when obtaining lesion and baseline microhardness.

Enamel Fluoride Uptake

The fluoride content of the enamel specimens was determined using the microdrill technique. The enamel specimens were mounted perpendicular to the long axis of a micro end mill attached to a specially designed microdrill and drilled to a depth of 100 μm through the entire lesion. The drilling and sample collection were performed in a static-controlled atmosphere to prevent loss of enamel powder due to charging effects. The enamel powder sample was transferred to a diffusion dish and then analyzed for fluoride. The diameter of the drill hole was determined using a calibrated microscope interfaced with an image analysis system. Indentations for microhardness testing and microdrill holes were placed on enamel specimens according to Figure 1.

Acid Resistance Test

To test whether the various FVs impart acid resistance to the enamel specimens after pH cycling, a second *in vitro* demineralization treatment was performed and followed the same protocol as described above but using a demineralization time of only eight hours. The average specimen microhardness (VHN_{art}) was determined again as described above.

AS Fluoride Analysis

The collected, frozen AS samples were thawed. An aliquot was removed and analyzed for fluoride by comparison to a similarly prepared standard curve using an ion-selective electrode (Model 9609BNWP, Orion Research, Boston, MA, USA) and meter. Individual as well as cumulative F release data were calculated to determine $[F]_{max}$ (highest F concentration found in any collected AS sample over the 24-hour period) as well as F_{total} (the total amount of F released from FV).

Statistical Analysis

A one-way analysis of variance statistical method was used for data analysis. An overall significance of (α=0.05) was used. Pairwise comparisons between the groups were conducted using Tukey's method. Pearson correlation coefficients were calculated to investigate associations between the study variables.

RESULTS

Specimens treated with Nupro had the highest $\Delta VHN_{(post\ -\ lesion)}$, ranging from 24.3 for Nupro to 11.7 for Vella for the specimens receiving active treatment (Figure 2). No significant differences were found among FV; however, Nupro was the only FV significantly different from all placebo varnishes.

Specimens treated with Sparkle had the highest rehardening values following the second acid challenge, $\Delta VHN_{(art\ -\ lesion)}$, but were significantly different only from those treated with Vella. All other FVs did not statistically affect lesion rehardening differently, but all FVs were significantly different from the placebo varnishes (Figure 3).

Fluoride release trends were somewhat similar for all tested FVs (Figure 4). The highest release for most FVs was within the first hour of testing, and then varnishes exhibited a steady decrease in fluoride release over time. MI released significantly more fluoride in the first hour than all the other test products (9.71 μg/mL). Enamel Pro came second (5.44 μg/mL), followed by Flor Opal (4.37 μg/mL); however, the latter two were not statistically different from each other. Flor Opal released in the first hour of the experiment approximately half that of MI. Butler White released significantly less fluoride (0.12 μg/mL) in the first hour than all other test products. Vella and Kolorz demonstrated atypical release behavior where peak release occurred at the five-hour collection point (2.55 μg/mL).

For fluoride release total (F_{total}), MI released significantly more fluoride over the 24-hour incubation period of the experiment (F_{total}=14.97 μg/mL) than all other FVs (Table 2). In addition, Enamel Pro and Flor Opal released more fluoride than the remaining FVs, except Nupro. Butler White released significantly less fluoride (F_{total}=0.50 μg/mL) than all other FVs.

The highest concentration of fluoride at any given time point was for MI ($[F]_{max}$=9.71), which was significantly higher than all FVs except for Enamel Pro ($[F]_{max}$=5.44), with Enamel Pro and Flor Opal having higher peak concentrations than all remaining FVs. Butler White exhibited the lowest peak concentration ($[F]_{max}$=0.17) and was significantly lower than all other FVs under study (Table 2).

Differences between FVs were less prominent for EFU than for F_{total}. PreviDent, Duraphat, and Vanish had significantly higher EFU than Cavity Shield, MI, Flor Opal, and Butler White but were not significantly different from the other FVs (Figure 5).

There were statistically significant but weak linear associations between $\Delta VHN_{(post\ -\ lesion)}$ and EFU (r=0.36; p<0.0001), $\Delta VHN_{(post\ -\ lesion)}$ and F_{total} (r=0.22; p=0.0011), and F_{total} and EFU (r=0.21; p=0.0022).

DISCUSSION

In this *in vitro* study, we aimed to investigate the efficacy of 14 commercially available FVs using the same outcome variables from one of our previous *in vitro* investigations.[14] While the two studies investigated the effect of FVs on caries lesions, the study models were fundamentally different. The wide variation in performance of FV in our first experiment prompted the design of this *in vitro* study. We aspired to have a better understating of how different formulations affect the efficacy of FVs. Therefore, in this experiment, we aimed to investigate a larger variety of FVs in an attempt to have a range of products that represent different formulations. Also, in this study, FVs were left on the

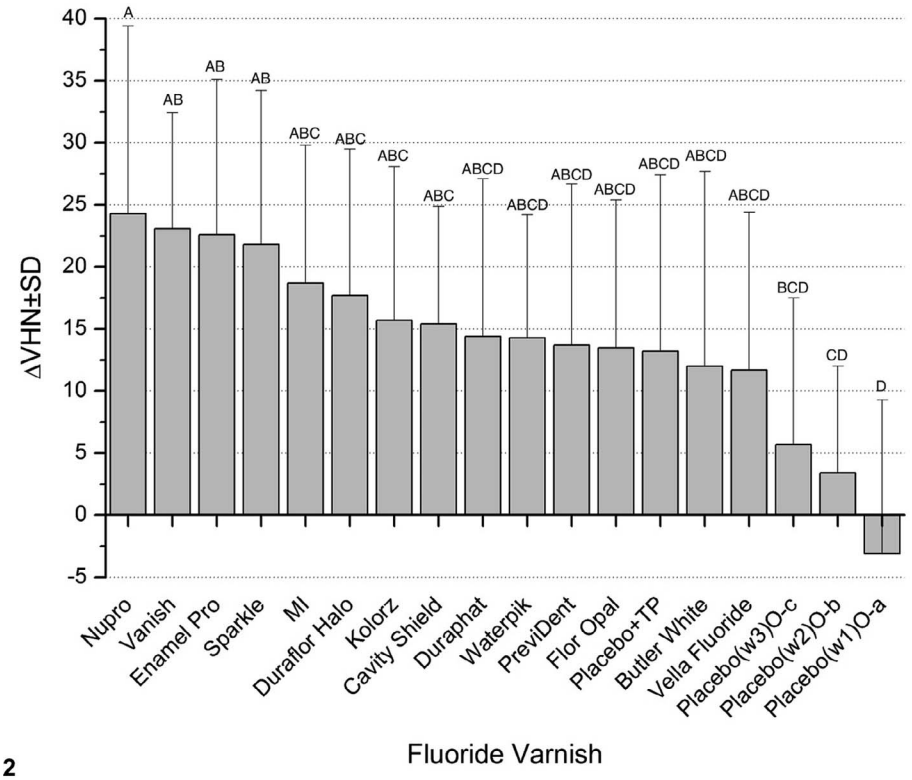

Figure 2. *Mean change in surface microhardness ($\Delta VHN_{(post - lesion)}$) as a function of fluoride varnish treatment. Significant differences between varnishes are highlighted by different letters. Error bars denote standard deviations.*

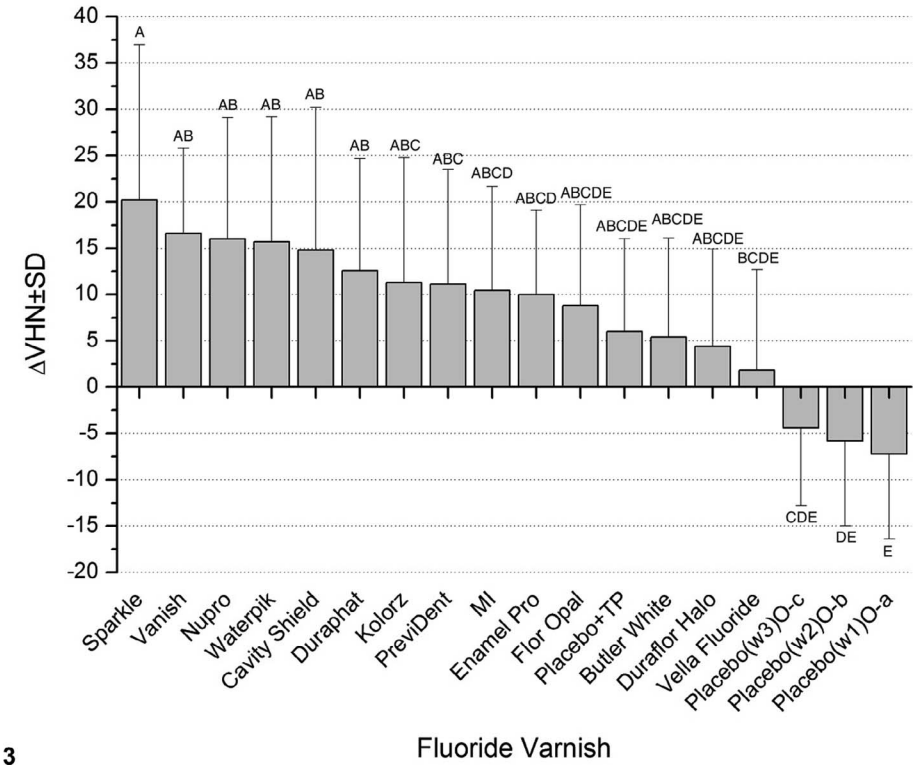

Figure 3. *Mean change in surface microhardness ($\Delta VHN_{(art - lesion)}$) as a function of fluoride varnish treatment. Significant differences between varnishes are highlighted by different letters. Error bars denote standard deviations.*

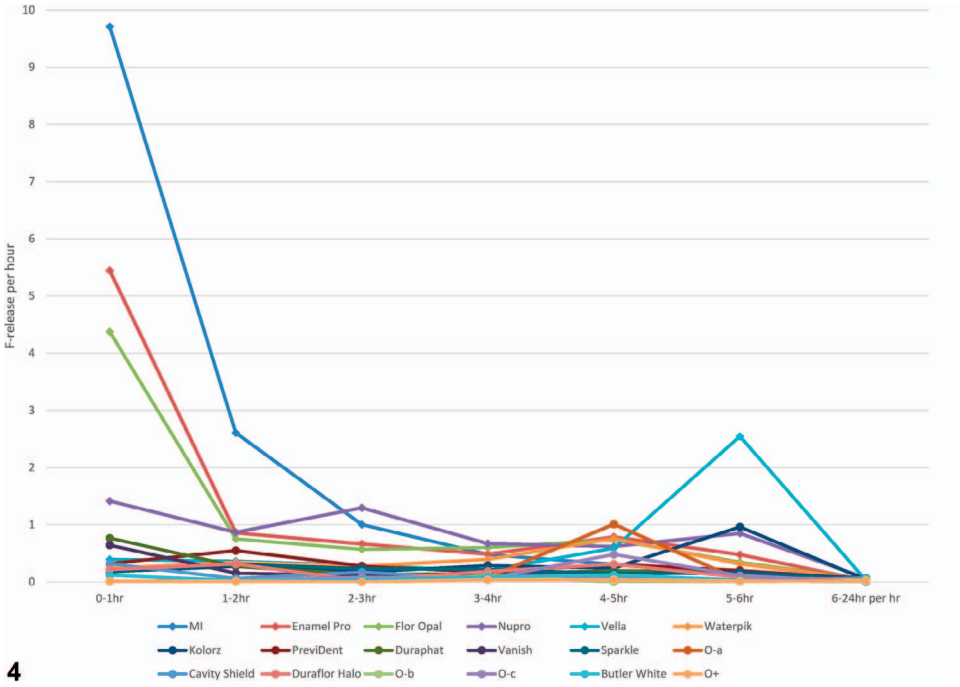

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Figure 4. *Mean hourly fluoride release from fluoride varnishes into saliva.*

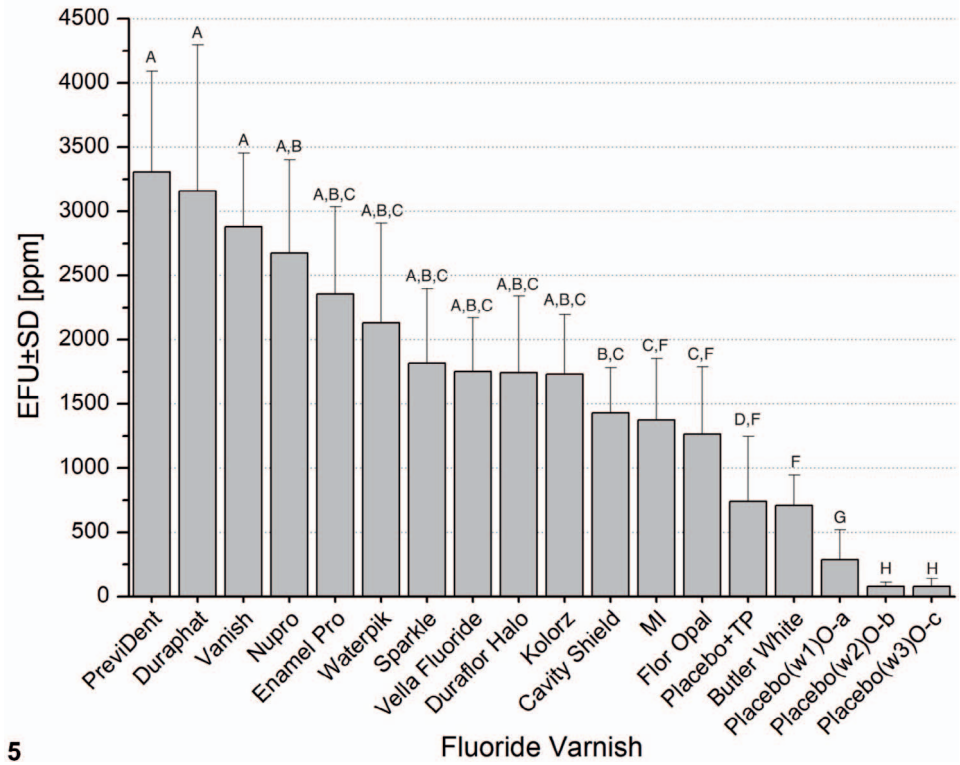

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Figure 5. *Enamel fluoride uptake (EFU) as a function of fluoride varnish treatment. Different letters highlight significant differences between varnishes. Error bars denote standard deviations.*

specimens for a prolonged period of 24 hours versus the six-hour period of the first study to better simulate the clinical situation.

This *in vitro* experiment's results for $\Delta VHN_{(post\ -\ lesion)}$ and for $\Delta VHN_{(art\ -\ lesion)}$ were unanticipated. As there are numerical differences in lesion rehardening values from different FVs, most of the FVs did not statistically differ from each other. Unfortunately, it is hard to thoroughly interpret the results because of the lack of detailed information on different FV formulations. However, there seems to be a superior effect on lesion rehardening when

Table 2: *Cumulative Fluoride Release and Peak Fluoride Concentration (n=12)*[a]

Fluoride Varnish	Cumulative Fluoride Release [μg/mL], Mean (SD)	Peak Fluoride Concentration [μg/mL], Mean (SD)
MI	14.97 (2.38) A	9.71 (1.40) A
Enamel Pro	9.20 (1.71) B	5.44 (1.05) A,B
Flor Opal	8.20 (1.91) B	4.37 (0.85) B
Nupro	6.96 (1.26) B,C	1.69 (0.43) C,D
Vella	4.91 (2.02) C,D	2.57 (2.17) C
Waterpik	3.07 (0.81) D,E	0.93 (0.30) D,E
Kolorz	2.91 (0.46) D,E	1.00 (0.11) C,D,E
PreviDent	2.82 (0.59) E,F	0.98 (0.09) C,D,E
Duraphat	2.64 (0.53) E,F	0.97 (0.12) C,D,E
Vanish	2.63 (0.53) E,F	1.28 (0.09) C,D,E
Sparkle	2.19 (0.47) E,F	0.95 (0.15) C,D,E
O-a	2.15 (0.56) E,F	1.20 (0.30) C,D,E
CavityShield	1.97 (0.14) E,F	1.28 (0.11) C,D,E
Duraflor Halo	1.82 (0.54) F	0.70 (0.22) E
O-b	0.85 (0.17) G	0.71 (0.16) E
O-c	0.81 (0.55) G,H,I	0.55 (0.51) F
Butler White	0.50 (0.15) H,I	0.17 (0.09) H
O+	0.49 (0.16) I	0.37 (0.16) G

[a] *Significant differences between varnishes are highlighted by different letters.*

calcium- and phosphate-containing ingredients are added to the formulation. This is in agreement with our previous *in vitro* study.[14] This may be attributed to the abundant amount of calcium and phosphate from these formulations that is readily available to interact with the teeth. It is important to note that the enhanced rehardening effect from calcium- and phosphate-containing ingredients did not withstand the second acid challenge. This is in contrast to a recent systematic review that suggested a possible long-term effect (>three months) of casein phosphopeptide–ACP (CPP-ACP) complexes on early caries lesions; however, the results cannot be extrapolated as the review investigated CPP-ACP alone because of insufficient evidence on the complex's synergistic effect with fluoride.[15] Another noteworthy finding is that lesions treated with Vanish, an FV-containing functionalized tricalcium phosphate (fTCP), had a higher $\Delta VHN_{(post\ -\ lesion)}$ than those treated with FVs containing ACP and CPP-ACP. This may be due to the protective effect of functionalization of the TCP molecule that prevents premature interaction between calcium and fluoride and aids in remineralization in a manner similar to that of fluoride.[16]

The fluoride release data in our experiment are in agreement with our previously mentioned in-house experiment and the 2014 study by Cochrane and others.[12,13] Calcium-containing FVs, with the exception of fTCP-containing varnishes, were able to release significantly higher levels of fluoride into saliva. This suggests a synergistic effect of adding casein complexes to FVs on fluoride release and may be explained by the bioavailable nature of ACP and CPP-ACP compared with the less-soluble fTCP.[16-18]

EFU data from this study extend our results from our aforementioned *in vitro* investigation. Once again, a CPP-ACP–containing varnish delivered less fluoride into caries lesions. This may be a result of the negative impact that inorganic phosphate released from CPP-ACP varnishes has on the formation of CaF_2, thus reducing bioavailable fluoride. Vanish, an FV-containing fTCP, delivered more fluoride into lesions compared with CPP-ACP and ACP-containing FV despite its low fluoride release into saliva. This is in agreement with a study that compared EFU from two varnishes, one containing fTCP and the other containing CPP-ACP.[17] Also, another study compared EFU with and without fTCP. It was found that lesions exposed to fluoride in conjunction with fTCP had significantly higher fluoride uptake than those exposed to fluoride alone, and the effect of fTCP was dose dependent. The mechanism of action of fTCP on enhancing lesion uptake of fluoride is not fully understood but may be attributed to the ability of fTCP to promote fluoride-based nucleation.[16]

Contrary to our findings in our preceding *in vitro* study, we were able to demonstrate a significant but weak linear association between $\Delta VHN_{(post\ -\ lesion)}$ and EFU. To the best of our knowledge, this is the first time an association was established between ΔVHN and EFU in FV research. We are also in agreement with another study in the lack of meaningful correlation between fluoride release and EFU.[19] Based on our findings, we believe that fluoride release cannot be used as a predictive measure for EFU, and therefore, one should be careful in relating fluoride release to FV efficacy.

While our findings are in agreement with some studies and in disagreement with others, it is important to remember that all FV *in vitro* models have their limitations to the extent that the *in vivo* situation cannot be mimicked because of the complexity of *in vivo* caries. With FV in particular, there is the issue of infrequent applications, which makes modeling much harder. In our model, we tried to mimic the *in vivo* application more closely. Most other models measure only the direct effect of an FV treatment while ignoring the effect of topical fluorides applied between FV applications. This makes our study more clinically relevant but also more challenging, as there are competing effects of the topical fluorides.

CONCLUSION

The results of this study demonstrated variation in FV efficacy in *in vitro* conditions, most likely because of differences in composition. Further investigations are needed to develop models that will help us understand FV behavior.

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Conflict of Interest

The authors of this article certify that they have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

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