Clin Oral Invest (2016) 20:1655–1662
DOI 10.1007/s00784-015-1648-4

ORIGINAL ARTICLE

The effect of fluoride varnishes on caries lesions: an in vitro investigation

Laila Al Dehailan[1,2] · **Esperanza Angeles Martinez-Mier**[1] · **Frank Lippert**[1]

Received: 28 October 2014 / Accepted: 2 November 2015 / Published online: 10 November 2015

Abstract
Objectives The purpose of this study was to investigate the effect of five commercially available fluoride varnishes (FV) on caries lesions.
Materials and methods Ninety bovine enamel specimens were assigned to five varnish groups (n=18). Early caries lesions were created in the specimens and characterized using Vickers surface microhardness number (VHN). FV was applied to each group of specimens. Immediately afterwards, 7.5 ml of artificial saliva (AS) were pipetted over each group of specimens, collected and renewed every 15 min for 6 h. AS samples were analyzed for fluoride using an ion-specific electrode. Enamel fluoride uptake (EFU) was determined using the acid etch technique. Each group was then subjected to a pH cycling regimen for 5 days after which VHN was determined again. One-way analysis of variance (ANOVA) was used for data analysis.
Results FVs differed in their rehardening capability (highest mean value was for Enamel Pro=32.3±5.8 and lowest mean value was for Vanish=18.9±11.3). No significant difference in EFU was found among groups. Total fluoride release over 6 h was in the order of MI Varnish (303 μg/ml)>Enamel Pro (217 μg/ml)>Flor-Opal (153 μg/ml)>PreviDent(84 μg/ml)>Vanish(28 μg/ml).
Conclusions ΔVHN and fluoride release characteristics differ among FV products. These differences may be attributed to the different compositions and physical properties of the tested FV.
Clinical relevance Fundamental, comparable research on FV and how different formulations affect early caries lesion rehardening, fluoride release into saliva, and uptake by teeth is scarce.

Keywords Fluoride varnish · Fluoride release · Enamel fluoride uptake · Microhardness

Introduction

Dental caries remains the most common global chronic disease, affecting 60–90 % of school-aged children and a significant number of adults [1]. Topically applied fluoride has contributed to major reductions in both the incidence and prevalence of dental caries. It has also been shown to be safe and effective [2]. Fluoride has the ability to inhibit the demineralization process, enhance remineralization, and inhibit bacterial enzymes found in dental plaque [3, 4]. Nowadays, a vast range of professionally applied topical fluoride products exists, including rinses, gels, foams, drops, and varnishes. Fluoride varnishes (FVs) are relatively simplistic delivery vehicles for cariostatic amounts of fluoride and typically contain 5 %

✉ Laila Al Dehailan
laldehai@iu.edu

Esperanza Angeles Martinez-Mier
esmartin@iu.edu

Frank Lippert
flippert@iu.edu

[1] Department of Cariology, Operative Dentistry and Dental Public Health, Indiana University School of Dentistry, 415 Lansing Street, Indianapolis, IN 46202, USA

[2] Department of Restorative Dental Sciences, College of Dentistry, University of Dammam, P. O. Box 1982, Dammam 31441, Saudi Arabia

sodium fluoride. FVs offer several advantages over other modalities of topical fluoride treatment such as effectiveness, relative safety, and ease of application [5–8]. The prolonged contact time with the dentition and extended release of fluoride over a long period of time give FV an advantage over other forms of fluoride delivery [9–11].

In 1994, the US Food and Drug Administration approved fluoride varnishes' usage as cavity liners and dentin hypersensitivity treatments. However, most dental professionals in the USA use fluoride varnishes off-label for the prevention of dental caries [9].

Despite the American Dental Association Council on Scientific Affairs' evidence-based clinical recommendation for people at risk of developing dental caries that "FV containing 2.26 % fluoride applied at least twice per year is effective in preventing caries for patients 6 years or older," the current regulatory situation has created a "grey area" for manufacturers (a recent search brought more than 30 different manufacturers to daylight) [12]. Thus, unlike for fluoride dentifrices and rinses, no efficacy testing is required for FV, or in other words—the majority of currently marketed FV have not been evaluated for their effectiveness in preventing caries or their toxicity. Considering the importance of a professional caries intervention and the fact that FV are typically only applied every 3–6 months, every effort should be taken to ensure a maximum benefit to risk ratio.

Several recent studies highlighted considerable differences in fluoride release characteristics between FV from different manufacturers, not only in terms of cumulative amount of fluoride released over time but also in the kinetics of fluoride release [9, 13]. The clinical relevance of these findings is unknown due to the lack of comparable FV clinical trials on caries progression and reversal. Fundamental research on FV and how different formulations affect adherence to teeth, fluoride release into saliva, and uptake by teeth—to name their most important aspects, is virtually non-existent. Thus, further research is required first to establish a baseline before experimental work can commence.

pH cycling models were designed to simulate the dynamic variations in mineral saturation and pH associated with the natural caries process. They mimic specific events of the caries process under controlled conditions and allow the investigation of individual mechanistic variables which would be extremely difficult to do under in vivo conditions [14]. At the same time, it is important to recognize the limitations of in vitro experiments in their ability to reproduce the whole complexity of caries dynamics. In vitro experiments provide only limited information on the effects of different variables on the caries process. This must be taken into consideration when in vitro data are extrapolated to in vivo conditions.

Many response variables can be used to investigate the efficacy of fluoride treatments. One of which is hardness measurement that can quantitatively describe the depth of artificial lesions [15]. Hardness measurement has been proven to have adequate sensitivity to detect early changes in the outer 15–20-μm layer of enamel [16, 17]. However, this technique has its limitations. The size of the indentation is highly influenced by water and organic content of tissue. This has a bigger impact when analyzing dentin and may affect the analysis of results [18]. Also, hardness measurement is unable to give a clear explanation on changes that occur deep within a carious lesion [19].

Fluoride uptake is a widely used assessment tool to determine the amount of fluoride that has been incorporated in enamel lesions following fluoride treatment [19]. It is considered as an important research method for testing new formulations for their anticaries activity. Reduction in dental caries, increased levels of remineralization, and elevated resistance to acid challenge have been linked to increased incorporation of fluoride into enamel; however, it is still unclear how enamel fluoride uptake (EFU) correlates with aniticaries activity [20–24]. One way to assess the enamel fluoride uptake is by using the acid etch technique which has demonstrated excellent precision and accuracy [25, 26].

The mode of action of FV is not fully understood; however, the bioavailability of fluoride in the oral cavity has been proven to be essential in caries prevention. Low levels of fluoride over prolonged periods of time have been shown to be effective in preventing demineralization and enhancing remineralization [27, 28]. Measuring the levels of fluoride over time is one way to demonstrate its bioavailability and consequently its effect on caries activity. This method has been used as a research tool to investigate the anticaries efficacy of several fluoride treatments [9, 29, 30].

The purpose of this in vitro study was to determine the effect of five different commercially available FVs on caries lesions by investigating their fluoride release into artificial saliva (AS) and their ability to fluoridate and remineralize early carious lesions.

Materials and methods

Specimen preparation

Enamel specimens obtained from bovine teeth were used as the hard tissue test substrate. The teeth were cut into 4×4-mm specimens using a Buehler Isomet low-speed saw. The teeth were stored in thymol during the sample preparation process. The 4×4 mm specimens were ground and polished to create flat surfaces to facilitate surface microhardness testing using Struers Rotopol 31/Rotoforce 4 polishing unit (Struers Inc., Cleveland, PA, USA). The dentin and enamel sides of the specimens were ground flat to a uniform thickness with 500-grit silicon carbide grinding paper. As a final cleaning step, the

specimens were sonicated in a detergent solution (Micro-90 concentrated cleaning solution with 2 % dilution) for 3 min. The specimens were finally assessed under Nikon SMZ 1500 stereomicroscope at ×10 magnification. Accepted specimens had no obvious cracks, areas of hypomineralization, or other flaws in the enamel surface. Specimens were then embedded in acrylic resin (ClaroCit Kit, Struers) using a 1.5-inch mounting mold (Struers FlexiForm). Specimens were arranged to ensure that they were not in contact with each other and with the enamel surface facing downward. The resin was poured carefully over the specimens to a height of approximately 1 to 2 cm. Once the resin had cured, the specimens embedded in the disk (18 specimens per disk) were polished to mirror flatness as described above with a final polishing step using 4000-grit paper followed by 1-μm diamond polishing suspension. Eighteen specimens per FV treatment group were used for this study with a total of 90 specimens.

Early carious lesion creation

The demineralization protocol is based on that by White (1987) and has been extensively studied using a variety of techniques over the years [31, 32]. Artificial lesions were formed in the enamel specimens of each disk by a 48-h immersion 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). Demineralization was performed at 37 °C at a ratio of 10 ml of solution per specimen. The resulting lesions are early, shallow, subsurface lesions with an average depth of approximately 50 μm.

Demineralization (baseline) microhardness

Initial hardness of the demineralized specimens was determined using a Vickers microhardness indenter (M247AT Leco Corporation, St. Joseph, MI, USA) at a load of 200 g for 15 s. The average specimen surface microhardness (VHN_{lesion}) was determined from four indentations on the surface of each specimen.

Fluoride varnish application

A list of the tested products and their active ingredients can be found in Table 1. Each disk with the polished, embedded specimens was placed back into the mounting mold.

The protective foil from the individual FV dose was removed, and the FV was mixed using the manufacturer's application (typically a microbrush) for at least 10 s to homogenize the FV, as sedimentation of NaF and phase separation may have occurred during storage. Subsequently, FVs were evenly applied to the surface of each of the disks using the manufacturer's applicator. The amount of FV applied was recorded. The average amount applied to each treatment group consisting of 18 specimens was 0.13 g and ranged between 0.10 and 0.18 g.

Saliva incubation

Immediately after FV application, 7.5 ml of AS that had been pre-heated to 37 °C was pipetted carefully over the disk in the mounting mold. The mold was then placed in an incubator set at 37 °C. AS formulation was based on that by Hara et al. (2008) and had the following composition: 2.20 g/l gastric mucin, 1.45 mM $CaCl_2 \times 2H_2O$, 5.4 mM KH_2PO_4, 28.4 mM NaCl, and 14.9 mM KCl and was adjusted to pH 7.0 with KOH [33].

Every 15 min for a total of 6 h, the 7.5 ml AS was renewed by pouring the AS in the mold into a separate pre-weighed container to determine the weight of AS, then carefully pipetting fresh AS into the mold and placing the mold back into the incubator for another 15 min.

These collected AS samples were then processed for fluoride analysis. An aliquot was removed and analyzed for fluoride using an ion-selective electrode (Model 9609BNWP, Orion Research, Boston, MA, USA) and meter by comparison to a similarly prepared standard curve. Fluoride data were calculated as μg F/mg FV.

Fluoride varnish removal

After the last AS sample collection, 10 ml of chloroform was poured over the disk to dissolve any remaining FV. The mold was placed into a suitable container to prevent evaporation of the chloroform. The mold/container was gently shaken for 5 min to accelerate the dissolution process. This process is repeated at least once and until there are no visible signs of FV left on the specimens.

Enamel fluoride uptake

The fluoride content of the enamel in each of the disks was determined using a modification of the acid etch technique by Sakkab et al. [34]. Approximately half of the enamel surface of each specimen was covered with nail varnish to protect an area of the specimen for the subsequent pH cycling phase. Each disk was placed back into its mold. Specimens in each disk were acid etched by pouring 5 ml of 1 M $HClO_4$ over each disk for 1 min. The acid etch solution was then collected. Immediately after the etching, the specimens were rinsed thoroughly with deionized water. The acid etch procedure was repeated four more times, with each acid etch solution being collected separately. A sample of each acid etch solution was buffered with total ionic strength adjustment buffer (TISAB) II (0.25 ml sample, 0.5 ml TISAB II, and 0.25 ml 1 N NaOH) and the fluoride content determined by comparison to a similarly prepared standard curve (1 ml standard + 1 ml TISAB

Table 1 Study test products

Fluoride varnish	Manufacturer	Fluoride source and concentration	Carrier	Other active ingredient
Enamel Pro	Premier Dental	5 % NaF	Rosin	Amorphous calcium phosphate (ACP) and xylitol
Flor-Opal	Ultradent	5 % NaF	Hydrogentated rosin	Xylitol
MI Varnish	GC America	5 % NaF	Hydrogenated rosin and polyvinyl acetate	Casein phosphopeptide-ACP (CPP-ACP, Recaldent)
PreviDent	Colgate-Palmolive	5 % NaF	Synthetic resin	Xylitol
Vanish	3M ESPE	5 % NaF	Pentaerythritol glycerol ester of colophony resin	Functionalized tri-calcium phosphate (fTCP) and xylitol

II). Data from multiple etches for each group were combined to calculate EFU.

pH cycling phase

Before pH cycling, the nail varnish that protected half of the specimen during etching for EFU was removed using acetone, and the etched half was painted with nail varnish. The cyclic treatment regimen for each of the five disks containing the demineralized specimens is provided in Table 2. Fluoride treatments were performed using slurries of Crest Cavity Protection (0.243 % sodium fluoride; Procter and Gamble, Mason, OH, USA). The slurry was prepared by adding toothpaste to AS at a ratio of 1:2 *w*/*w* (dentifrice:AS) in a beaker with a magnetic stirrer. A fresh treatment for each subgroup was prepared just prior to each treatment. After the treatments, the specimen disks were rinsed with running deionized water and placed back into AS. At the remaining time (~20 h), the disks were in AS. The regimen was repeated for 5 days.

Post-treatment microhardness

The average specimen microhardness was determined, as previously described, from four indentations on the surface of each specimen, next to the baseline indentations (VHN_{post}).

Table 2 Daily pH cycling treatment schedule

Time	Treatment
8:00–8:01 a.m.	Toothpaste treatment
8:01–10:00 a.m.	Artificial saliva
10:00 a.m.–2:00 p.m.	Acid challenge
2:00–4:00 p.m.	Artificial saliva
4:00–4:01 p.m.	Toothpaste treatment[a]
4:01 p.m.–8:00 a.m.	Artificial saliva

[a] On the last day, this treatment was not given; the test ended with the AS treatment at 4 p.m.

The difference between the hardness after lesion creation and the pH cycling phase was calculated as follows: $\Delta VHN = VHN_{post} - VHN_{lesion}$.

Statistical analysis

Data analysis was performed using one-way analysis of variance (ANOVA). An overall significance of (α=0.05) was used. Pairwise comparisons between the groups was conducted using Student-Newman-Keuls test. Pearson's correlation coefficients were calculated to investigate associations between the study variables.

Results

The results for all study variables can be found in Figs. 1 (ΔVHN), 2 (fluoride release profiles), and 3

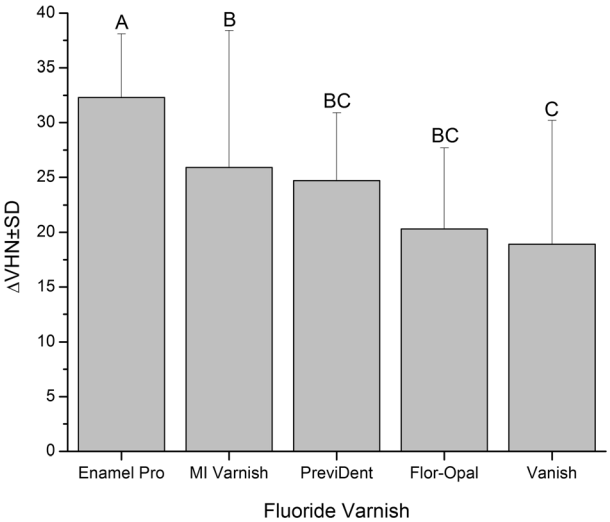

Fig. 1 Mean change in surface microhardness (ΔVHN) as a function of fluoride varnish treatment. Significant differences between varnishes are highlighted by *different letters*. *Error bars* denote standard deviations

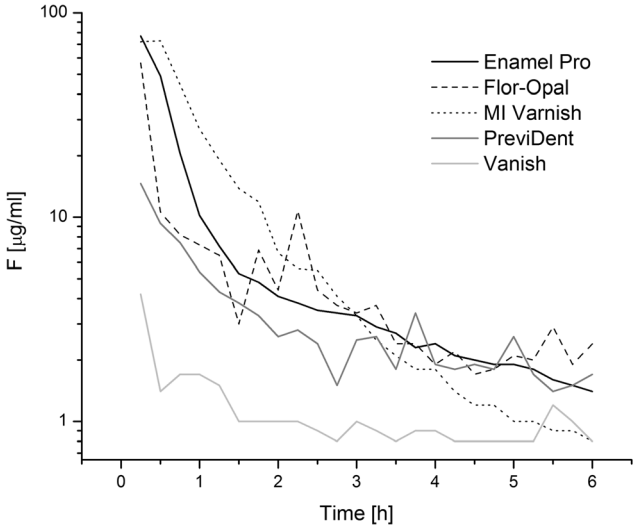

Fig. 2 Fluoride release (log10 scale for better clarity) from fluoride varnishes into saliva as a function of time (n=1)

(EFU) and Tables 3 (ΔVHN) and 4 (cumulative fluoride release and peak fluoride concentration).

Treatment with Enamel Pro resulted in significantly greater lesion surface rehardening compared to all other tested FV. MI Varnish exhibited greater rehardening than Vanish but was similar to PreviDent and Flor-Opal. There were no differences between PreviDent, Flor-Opal, and Vanish.

The fluoride release from FV showed commonalities and dissimilarities. Overall, fluoride release profiles were somewhat similar between FV as all showed a gradual decrease in released fluoride over time. However, differences were apparent in the shape and slope of the release curves as well as the cumulative amount of released fluoride and the highest

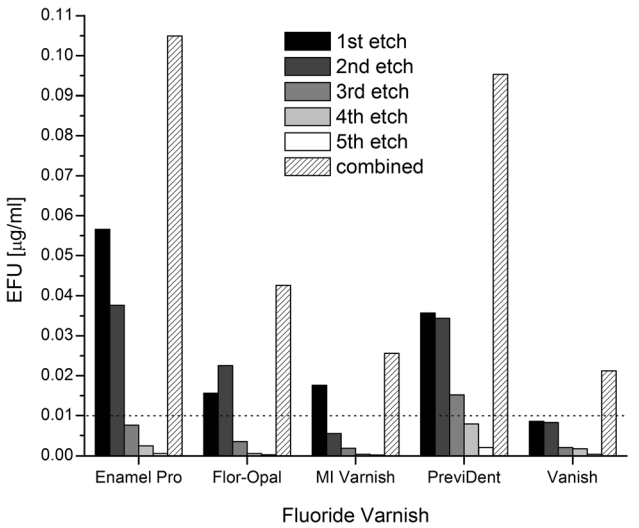

Fig. 3 Enamel fluoride uptake (consecutive etches and combined data) as a function of fluoride varnish treatment. The *dashed, horizontal line* represents the lowest fluoride concentration of the calibration curve. EFU was normalized per milliliter of acid etch solution. Values lower than 0.01 µg/ml were calculated based on extrapolation of the calibration curve

Table 3 Mean ΔVHN and SD

Fluoride varnish	Number	Mean ΔVHN (SD)
Enamel Pro	15	32.3 (5.8)[a]
Flor-Opal	18	20.4 (7.4)[bc]
MI Varnish	18	25.9 (12.5)b
PreviDent	16	24.7 (6.2)[bc]
Vanish	16	18.9 (11.3)[c]

Superscript letters represent significant differences of ΔVHN means

released fluoride concentration. For example, while both MI Varnish and Enamel Pro exhibited similar initial fluoride releases, MI Varnish released more fluoride than Enamel Pro during the first 3 h, whereas Enamel Pro showed a more gradual decrease and released more fluoride during the latter 3 h of the chosen experimental period. Vanish released less than 1/10 of fluoride in comparison to MI Varnish, and Vanish' peak fluoride concentration was approximately 1/20 of that of Enamel Pro.

The EFU data were not significantly different for all FVs tested. Enamel Pro had the highest EFU followed by PreviDent with both delivering more than twice as much fluoride compared to the other FV.

There was no linear correlation between the following main variables: ΔVHN versus cumulative fluoride release (r=0.61; p=0.28), ΔVHN versus EFU (r=0.72; p=0.17), and cumulative fluoride release versus EFU (r=0.01; p=0.99).

Discussion

The efficacy of fluoride varnishes in preventing dental caries has been well documented in the literature [13, 35]. Since the introduction of the first fluoride varnishes, researchers have been striving to improve FV by testing new formulations that aim to better deliver fluoride in varnishes [36, 37]. Fluoride varnishes last for a limited time in the oral cavity and are quickly removed by the action of mastication and oral hygiene practices. Therefore, varnishes are designed to release their

Table 4 Cumulative fluoride release and peak fluoride concentration (n=1)

Fluoride varnish	Cumulative fluoride release (µg/ml)	Peak fluoride concentration (µg/ml)
Enamel Pro	216.7	76.9
Flor-Opal	153.0	56.7
MI Varnish	303.0	72.9
PreviDent	84.3	14.6
Vanish	27.6	4.2

active ingredients in a relatively short time that has been estimated to be up to 24 h [29, 38].

This study aimed to evaluate the effect of five commercially available FV products on caries lesions (Table 1) by investigating the amount of fluoride released from each varnish into AS, the amount of fluoride delivered to early enamel carious lesions, and the extent of surface rehardening of these lesions as a result of a FV treatment. To the authors' knowledge, this is one of the first in vitro studies to employ three response variables (F release, EFU, and VHN) to investigate the effect of FV on caries and to study the correlation between them. The chosen experimental design was based on previous studies [9, 13, 29, 39] while taking into account findings from preliminary in-house investigations (unpublished data).

The present study has shown that FV differ in their ability to reharden (Fig. 1) and fluoridate early carious lesions (Fig. 3) and release fluoride into the surrounding medium (Fig. 2) with no linear correlation being observed between any of these variables. The present findings indicate that varnishes containing amorphous calcium phosphate (ACP)-forming salts and casein phosphopeptide-ACP (CCP-ACP) demonstrated significantly higher ability to reharden early carious lesions than the other tested FVs. This may be explained by the higher amounts of available calcium and phosphate ions from varnishes containing ACP-forming salts. Recently, it was shown that ACP forming varnish formulations delivered more fluoride than formulations containing tri-calcium phosphate (TCP) to both sound and demineralized enamel. This was likely due to the non-crystalline structure of ACP that makes it more soluble and reactive compared to TCP that is an insoluble crystalline form of calcium phosphate [38].

The results of our study demonstrate a wide variation in total fluoride release over 6 h from the five varnishes under investigation. This wide variation in fluoride release amount and characteristics is difficult to explain since manufacturers are not required to provide exact formulation details. However, this variation may be due to the differences in additives or type of resin carriers (natural vs. synthetic) used. It has been postulated that fluoride ion diffusion is slower in varnishes with a natural resin base; however, this was not observed in this study [39, 40]. For example, Flor-Opal has a natural resin base (rosin) and released more fluoride than Vanish that has a synthetic resin base.

The highest release from all varnishes was within the first 15 min to 1 h of application and is similar to another study [39]. In our study, it was found that the highest total fluoride release over the period of 6 h was from a varnish containing CPP-ACP as an additional active ingredient, while the least amount of release was from a varnish with functionalized tri-calcium phosphate. These findings are in agreement with another study and are consistent with the high water solubility and bioavailable nature of CPP-ACP contained within these varnishes [41].

The present findings for EFU results are in contrast to our expectations for some of the evaluated FVs. For example, MI exhibited the greatest level of total fluoride release and a high level of rehardening value but a low level of EFU. This FV contains CPP-ACP and was found to release relatively high amounts of inorganic phosphate [29]. High levels of inorganic phosphate have been found to negatively impact the formation of CaF_2 thereby reducing the amount of bioavailable fluoride ion that is required for remineralization, and this may be an explanation for the lower level of EFU for MI varnish.

In the present study, we were unable to observe correlations between the outcome variables. This is in agreement with a prior study in our laboratory which employed a similar range of FVs [42]. For example, a FV that demonstrated a high fluoride release into saliva did not necessarily result in a high EFU value or enhanced remineralization. It is important to note that while there are similarities in the experimental models between studies, they were inherently different. Most importantly, the present study was concerned with FV effects on lesions after a pH cycling phase to mimic the short-term effect of FV on lesions, whereas our previous study was solely concerned with the immediate effect of FV on lesions. The observed differences in FV performance but consistencies in lack of correlation between variables highlight some of the shortcomings of laboratory research on FV. In the absence of a clinically validated in vitro model to determine the efficacy of FV, results from the present and previous laboratory studies need to be seen with caution.

One or all of the investigated variables may predict the efficacy of FVs. However, it is impossible to foresee at this point the best predictive variable for clinical performance. There is a need to develop and validate clinical and laboratory models that will help us better understand the mode of action of FVs and predict clinical efficacy.

Conclusions

(1) The present study has shown that the effect of the five tested FVs on early caries lesions varies greatly.
(2) The observed differences may be attributed to different compositions and the presence of other active ingredients besides fluoride.

Acknowledgments The authors thank George Eckert for his statistical analysis of presented data.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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