Longer Retention Time of Fluoridated Varnishes Enhances Enamel Remineralisation In Vitro

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Purpose: Widely used in caries prevention, fluoridated varnish (FV) is also capable of remineralising carious lesions. However, the retention time of FV needed on enamel to ensure the maximum benefit is unclear. This study aimed to determine whether an increase in the retention time of 5% sodium fluoride (NaF) varnishes on enamel carious lesions enhances remineralisation.

Materials and Methods: Carious lesions were generated on bovine enamel slabs and treated with one of three 5% NaF commercial varnishes: Duraphat, Durashield or FlorOpal. After application, the varnishes were maintained on the slabs for 8, 12, 18, 24, 36 or 48 h while immersed in artificial saliva. Remineralisation efficacy was calculated and expressed as the percentage of surface microhardness recovery (%SHR). Untreated carious lesions served as negative controls. Data from each timepoint for each product were compared by ANOVA, followed by a post-hoc test ($p < 0.05$).

Results: There was a trend for increased remineralisation over time, reaching maximum %SHR values of about 30% after 18 to 24 h. No significant differences were detected after 18 h ($p > 0.05$). This remineralisation dynamic was similar among the three tested products.

Conclusion: Remineralisation of enamel lesions using 5% NaF varnish appears to be initially dependent on the retention time of the product. Higher remineralisation was observed upon 18 h of varnish retention on the lesions.

Key words: enamel, fluoridated varnish, fluoride, remineralisation, time

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Fluoridated varnish is one of the most widely used professionally applied fluorides (F^-).[3,6] Fluoridated varnish (FV) is mainly indicated to control caries, by both preventing demineralisation and enhancing remineralisation.[3] Although it has been clinically demonstrated that FVs have an effect on controlling caries,[23,24] their remineralising capabilities have been less explored. Instead of looking at remineralisation on enamel and thus controlling caries, the focus has been on in vitro studies assessing the release of F^- from FV to saliva.[9,10,20,28] Since professional applications of FVs are indicated once every three to six months,[23] their anticaries effect cannot be based on the F^- released to saliva a few hours after the application. Instead, the formation of F^- reservoirs (calcium fluoride-like products, or CaF_2) on dental surfaces is the mechanism of action of topical fluoride products.[31] These reservoirs are mostly responsible for the sustained provision of F^- over time, during the de- and remineralisation episodes.[31,33] The concentration of CaF_2 formed has been related to the retention time of FV on enamel.[5,14,27] Hence, mechanical removal of FV should be postponed to enhance CaF_2 formation. However, it is unclear whether FV retention on the dental surface affects remineralisation.

No consensus has been reached on the amount of time that FV should stay in contact with the hard dental tissues to achieve maximal effectiveness. Several clinical protocols and recommendations for the application of FV are available. Some of them recommend refraining from eating or keeping to a soft diet for a number of hours after the application. Likewise, toothbrushing after application is not advised, in order to maintain FV and dental surfaces in contact

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for longer times, which facilitates CaF_2 formation as an end-product from the sodium fluoride (NaF) contained in the varnishes. When the different commercial products are analysed, the time recommended before resuming toothbrushing ranges between 4 h to the next day, depending on the manufacturer (Table 1). Adding to the lack of clarity on the topic, published clinical protocols recommend refraining from toothbrushing for 12 to 24 h after FV application.[1,3,26,35]

Most FVs contain 5% NaF, equivalent to 22,600 ppm F^-, in a resin carrier. The resin carrier allows a longer contact time with dental surfaces,[3,8,30] increasing the absorption and distribution of fluoride.[18] Importantly, most of the NaF in the FV (~80% in Duraphat) remains insoluble in the varnish suspension.[5,14] Thus, longer retention time of FV on the tooth after the application (~24 h) will allow insoluble NaF in the varnish to be dissolved by saliva, promoting continuous CaF_2 formation and precipitation.[14]

Evidence is scare on how long FV should be in contact with the tooth before mechanical removal to actually achieve a remineralising effect. Likewise, there is disagreement between recommendations from manufacturers and researchers. The aim of this work, therefore, was to study the relation between retention time of different commercial FVs and their remineralising effect on enamel carious lesions. We hypothesised that longer retention time on the enamel increases the effectiveness of varnish remineralisation. Thus, an experimental remineralisation model was tested with different commercial FVs, using previously created enamel lesions.

MATERIALS AND METHODS

Experimental Design

Caries-like lesions were formed on enamel slabs using a *Streptococcus mutans* UA159 biofilm model.[15,16] Surface hardness (SH) was measured before and after caries creation and slabs were randomly allocated to 18 experimental groups. Three commercial FVs were applied to the previously formed carious lesions and immersed in artificial saliva for 8, 12, 18, 24, 36 and 48 h. After each timepoint, varnish was carefully removed using a soft toothbrush and SH was reassessed. Percentage of SH recovery was calculated (%SHR). Three independent experiments were carried out, each one in triplicate (n = 9 per group).

Sample Preparation

Bovine teeth were used to obtain enamel slabs of 7 x 4 x 1 mm. Enamel surfaces were flattened and polished using Soflex disks (3M; St Paul, MN, USA), followed by autoclaving.[15] The SH of each sample was assessed using a Knoop microhardness tester (402 MVD, Wolpert Wilson Instruments; Norwood, MA, USA). Three indentations 100 µm apart were made with a pyramidal diamond on the central area using a 50-g load for 5 s. This initial measurement was called T_{-1}. Only slabs with a mean SH of 356.12 ± 18.07 Kg/mm^2 were used to form caries-like lesions with *S. mutans* biofilms.

Caries-like Lesion Formation

Enamel slabs were exposed to a demineralisation process using a cariogenic *S. mutans* biofilm model. *S. mutans* UA159 were grown on saliva-coated enamel slabs and maintained in a brain heart infusion (BHI) broth at 37°C, 10% CO_2 for 5 days, and exposed to 10% sucrose three times per day for 5 min.[15,16] This model demineralises enamel through intermittent episodes of 'feast and famine'. These cycles create pH-cycling conditions, which favor mineral loss. Demineralisation was determined once lesions were formed by removing the biofilms from the slabs and measuring SH again (T_0). Only slabs with a SH of 210.73 ± 22.03 Kg/mm^2 were selected and randomly assigned to the experimental groups to assess remineralisation.

Remineralisation Experimental Model

Enamel samples were treated with one of three commercial 5% NaF FVs: 1. Duraphat (Colgate-Palmolive; Waltrop, Germany); 2. DuraShield (Sultan; Englewood, NJ, USA); or 3. Flor Opal (Ultradent; South Jordan, UT, USA) (Table 1). Enamel samples were carefully dried using absorbent paper, and FV was applied using the brush supplied by the manufacturer. FV was carefully applied by one previously trained operator placing a layer of approximately 0.3–0.5 mm on the enamel surface[3] for all treatments. One group remained untreated and was used as the negative control for remineralisation. Each slab was immersed in 2.5 ml of prescription formula of artificial saliva for clinical use made in a drugstore (pH = 6.8, containing 0.55 mM Ca and 0.55 mM Pi [inorganic phosphate]) in a well of a 24-well sterile culture plate. Samples were maintained at 37°C for 8 h, 12 h, 18 h, 24 h, 36 h or 48 h. To replenish the medium for remineralisation, two-thirds of the saliva contained in each well was removed and replaced for fresh artificial saliva, 3 times per day.

Remineralisation Assessment

At the end of each timepoint (8 h, 12 h, 18 h, 24 h, 36 h or 48 h), slabs were removed from the saliva and the varnish was mechanically removed using a soft toothbrush, ensuring complete removal of the FV as determined by visual examination. A new row of triplicate indentations was made 100 µm from the previous indentations (T_1), as described above. The %SHR was calculated using SH values from the different steps of the experiment: T_{-1}: sound substrate; T_0: after demineralisation by the cariogenic biofilm; T_1: after remineralisation. Calculation of %SHR was performed by the formula $(T_1\text{-}T_0)/(T_{-1}\text{-}T_0) \times 100$.[21]

Statistical Analysis

Assumptions of homogeneity of variances and normal distribution of errors were checked using the Shapiro-Wilk test. The analysis showed normal distribution of the data. Comparisons among the treatment groups and the timepoints were carried out by ANOVA followed by the Bonferroni post-hoc test, using SPSS 14.0 software, at a 5% significance level.

Table 1 Manufacturer's recommendations for post-treatment patient

Product	Total F concentration	Brushing and flossing	Eating	Other sources of fluoride
Duraphat	5% NaF, or 22,600 ppm F, or 2.26% F	Refrain for 12 h	Refrain for at least 2 h	Interrupt for 2–3 days
Durashield		Wait until next morning	–	–
Flor Opal		Refrain for 4–6 h	Refrain for 4–6 h	Interrupt for 4 days

RESULTS

In general, %SHR increased over time when FV remained in contact with dental enamel without removal (Figs 1 to 3). Untreated control slabs that were maintained in saliva without FV showed almost imperceptible %SHR variations (0.1, -2.4, -2.7, 0.2, 0.8, 0.4 at 8 h, 12 h, 18 h, 24 h, 36 h and 48 h, respectively). Remineralisation was enhanced by Duraphat varnish over time (Fig 1). Increases seemed to stabilise after 18 h of FV retention, without statistical differences between the following timepoints ($p > 0.05$), but with a non-significant peak at 36 h. Durashield (Fig 2) and FlorOpal (Fig 3) FV showed behaviour similar to that of Duraphat. When the three commercial FVs were compared, no significant differences were detected at any of the timepoints tested ($p > 0.05$).

DISCUSSION

Despite the vast worldwide use of FV, recommendations on when to resume toothbrushing and what and when to eat after professional application remain unclear and can be confusing for clinicians. Discrepancies exist not only between manufacturers, but also between clinical protocols developed by researchers. This study aimed to shed some light on this issue, contributing objective data on the efficacy of remineralisation when the products are allowed to act for different times on carious enamel lesions. Previous data showed that CaF_2 formation, with the maximum formation is time dependent, occurring after 24 h of FV retention.[14] The fact that CaF_2 is formed does not necessarily mean that remineralisation will take place. Little evidence is available that longer retention time of FV on initial carious enamel lesions will lead to enhanced remineralisation. In that context, therefore, this study provides novel information on the effect of FV on remineralisation and its relation with the time of FV retention. Our results showed that increasing the contact time of 5% NaF as a FV on a carious enamel surface increases remineralisation (%SHR) over time. Although it seems that the retention time of the FV and the resulting remineralisation have a linear relation, the three commercial products tested here showed that this effect reaches a peak at about 18 h to 24 h, and does not rise significantly after this period.

The manufacturers of Duraphat and FlorOpal recommend refraining from toothbrushing for 4 to 12 h, whereas Durashield recommends brushing the next morning (Table 1). The present experimental remineralisation model indicates that a longer time of about 18 h might be preferable to optimally remineralise initial carious lesions. Clinical recommendations to refrain from toothbrushing until the next morning[1,35] or for 24 h[3] have been previously mentioned. These recommendations differ from those given by manufacturers, who recommend suspending flossing and toothbrushing for times as diverse < 12 h to the day after the clinical application. It is important to highlight that clinical recommendations should also consider mechanical removal due to oral tissue rubbing and food friction that will inevitably detach some of the varnish from the smooth tooth surfaces. Nevertheless, residual amounts of FV retained in the pits, fissures and proximal areas of the teeth will release F^- to induce CaF_2 precipitation.[14]

Although differences in the amount of F^- released into saliva from varnishes containing 5% NaF have been reported,[4,20,28] this outcome is only a proxy for the remineralising effect of F^-. The actual anticaries effect of professionally applied fluoridated products is based on the formation of calcium-fluoride-like products (CaF_2).[31] Hence, F^- release to artificial saliva is no measure of the efficacy of a FV.[4] Indeed, F^- released from the FV into saliva was not maintained longer than 4 h in a continuous flow model[14] or longer than 8 h in a static model.[28] In consequence, FVs that are applied 2-4 times per year[23] to form CaF_2 reservoirs must not be understood as other fluoridated products intended for daily use, such as toothpastes. In the case of FV, CaF_2 acts as a F^- reservoir to slowly release F^- to saliva or to the biofilm fluid.[31,33] These minerals can persist for weeks or months after application,[7,11] and are dissolved when the pH falls.[31,33]

CaF_2 formation is key to promote remineralisation of carious lesions, as F^- released from these deposits will induce remineralisation in the body of the lesion and around the direct application site by gradually releasing F^- ions into the medium.[2] Considering the latter, our results, which showed enhanced remineralisation over time, support the recommendation of extended retention times of at least 18 h to 24 h. Although we did not measure the amount of CaF_2 formed by each varnish at each time, our findings are supported by the evidence showing that the reactivity be-

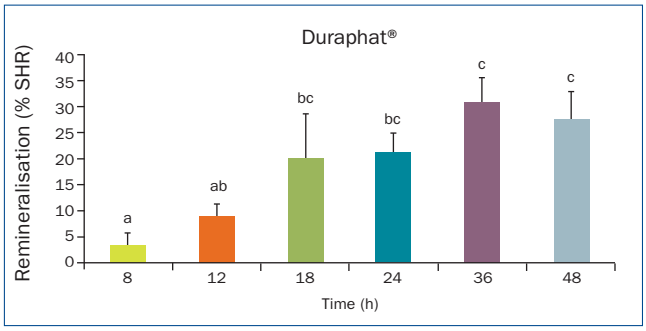

Fig 1 Percentage of surface hardness recovery (% SHR) on enamel treated with Duraphat varnish, at each of the indicated timepoints of varnish retention. Bars represent mean values of three independent experiments, each in triplicate. Error bars depict standard deviations of the mean.

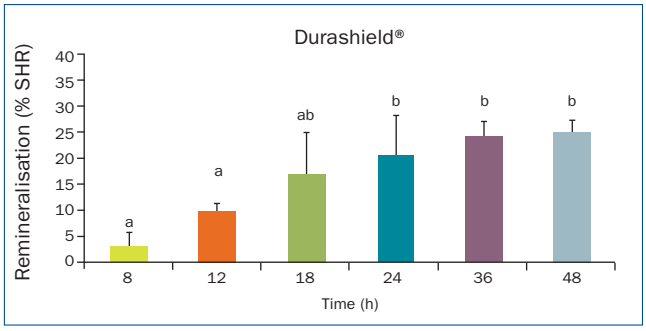

Fig 2 Percentage of surface hardness recovery (% SHR) on enamel treated with Durashield varnish, at each of the indicated timepoints of varnish retention. Bars represent mean values of three independent experiments, each in triplicate. Error bars depict standard deviations of the mean.

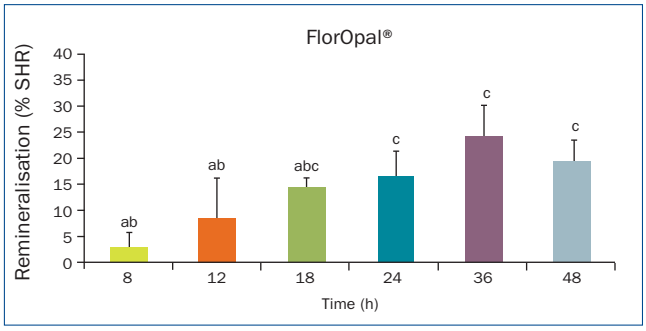

Fig 3 Percentage of surface hardness recovery (% SHR) on enamel treated with FlorOpal varnish, at each of the indicated timepoints of varnish retention. Bars represent mean values of three independent experiments, each in triplicate. Error bars depict standard deviations of the mean.

tween varnish and enamel is time dependent.[5,14] Thus, FV retained for a longer time will allow the formation of more CaF_2, which can be indirectly formed by salivary dissolution of insoluble F^- in the varnish.[14]

Like any in vitro model, the present experimental model of remineralisation has limitations derived from its nature.[34] Of course, the clinical situation includes multiple factors that are difficult to control, but a model like the one used here offers the unique possibility to control all the variables and to test only those of interest. The amount of FV used was standardised for all the treatment groups by the application of a single layer of FV, which was applied as carefully and homogeneously as possible by one single, trained investigator. To develop caries-like lesions, a microbiological model was chosen which simulates de- and remineralisation periods that allow the formation of sub-superficial lesions without surface loss.[13] Thus, caries-like noncavitated lesions were obtained with a mean depth of 75 µm. Although some microscopic changes can be observed in noncavitated incipient lesions,[17] the decision was made here to use SH to assess de- and remineralisation, as no erosion or any surface lost occurred after 5 days using the cariogenic model. Although SH is an indirect method to assess loss or gain of mineral content, it has been widely used in cariology for many years[38] and validated to study remineralisation on carious enamel.[36] In fact, SH has been studied and found to correlate well with remineralisation measured via conventional radiographic methods.[36] The technique has also been used in very recent studies similar to the present work.[22] Furthermore, remineralisation is superficial, so that SH is a valid technique to study superficial lesions, as in the present model. Nevertheless, further analysis of the surface structure using advances microscopy techniques will provide a better understanding of how varnish application or other fluoridated products may enhance remineralisation.

Despite the fact that our model was static, saliva was periodically renewed to avoid F^- saturation. It is expected that if a flow model were used, the behaviour of lesion remineralisation would be similar. CaF_2 formation was time dependent in a flow model, which exhibited similar kinetics to those found in the present study.[14]

The use of bovine enamel as a substitute of human enamel is widely accepted in caries research,[38] and was employed in the current study. The validity of this approach is based on a more uniform chemical composition of the bovine tissue, which decreases variability in the experimental response. In additon, it is easier to collect and manipulate.[25]

The present results showed a maximum %SHR of 35% at 36 h and a mean of 19.5% at 24 h. Those values are in agreement with other remineralisation studies,[19,36] showing that SH cannot be completely restored to the initial values of sound enamel. Lesion remineralisation from FV occurs preferentially on the surface and not in the body lesion.[12,37] Thus, mineral deposition on the dental surface will prevent F^- from penetrating inside the carious lesion. Although it has been argued that the layer of CaF_2 globules formed on the tooth surface[29] may restrict F^- diffusion into the body of the lesion, CaF_2 can be formed even inside the lesions, remineralising from within the tissue.[32] It is very important to understand that an optimal effect of F^- in caries treatment comprises the rather sporadic application of

high F^- doses (FV) as well as the exposure to constant, low F^- concentrations to remineralise deeply within the lesions (e.g. with dentifrices). As an additional mechanism, low F^- concentrations will be released from the CaF_2 reservoirs to become available during future demineralisation episodes.[31,33]

CONCLUSION

Within the limitations of an in vitro study, the present data show that the remineralisation of enamel lesions using 5% NaF varnish appears to be dependent on the retention time of the product. Remineralisation improved over time, suggesting that mechanical removal by eating hard foods and toothbrushing should be postponed for at least 18 h to 24 h after FV application.

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