EFFECT OF FLUORIDE VARNISHES CONTAINING DIFFERENT CALCIUM PHOSPHATE SOURCES ON MINERALIZATION OF INITIAL PRIMARY ENAMEL LESIONS

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Abstract. This study was conducted to evaluate the effect of fluoride varnishes containing different calcium phosphate sources on demineralization of initial primary enamel lesions. Forty-eight sound primary incisors were completely coated with nail varnish except for two 1x1 mm windows before being placed in demineralizing solution for 4 days. After demineralization, one of the windows in each tooth was coated with nail varnish. The teeth were randomly divided into four groups (A to D; *n* = 12), and then the other (exposed) window was treated with: Group A: deionized water, Group B: Duraphat® fluoride varnish, Group C: Clinpro™ White varnish and Group D: Enamel Pro® varnish. The pH-cycling regimen was carried out consisting of demineralization (6 hours) and remineralization (18 hours) for 7 days. Polarized light microscopy was used to evaluate the lesion depth initially and then after a seven-day pH cycle. Lesion depth was measured using a computerized method with the Image-Pro® Plus Program. The pair *t*-test was used to compare lesion depths before and after treatment. Differences in mean lesion depths among the groups were compared with the one-way ANOVA and Tukey's multiple comparison tests with 95% confidence intervals. The lesion depths had a significant difference between before and after treatment of the all groups. There was a significant increase in lesion depth in Group A compared to the other groups. No significant differences were seen among Groups B, C and D, containing fluoride and the different calcium phosphate sources in inhibiting progression of initial primary enamel lesions.

Keywords: calcium phosphate, demineralization, fluoride varnish, primary teeth

INTRODUCTION

Dental caries are a major public health problem among children (Chu *et al*, 2012). In recent decades, the most important concepts proposed for addressing dental caries occurs as a result of a continuum of cyclic demineralization and remineralization of

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enamel (Borges *et al*, 2011). Topical fluoride delivered via various vehicles is effective in reducing the risk of developing caries. (Marinho *et al*, 2004). Fluoride varnish was developed to prolong the contact time between fluoride and the tooth surface since it attaches to the tooth surface in a thin layer for a longer period of time (12 or more hours) and acts as a slow-release reservoir of fluoride (Ramaswami, 2009; Azarpazhooh and Main, 2009).

Fluoride has a dramatic effect on reducing the prevalence of caries but for remineralization, calcium and phosphate are also required. Several studies have found synergistic behavior among various minerals (*eg*, calcium, strontium, phosphate) along with fluoride leads to improved remineralization efficacy (Legeros, 1999; Schemehorn *et al*, 1999; Reynolds, 2008). It is important the calcium agent used should not interfere with the action of fluoride and should enhance fluoride's activity in remineralizing weakened enamel (Karlinsey *et al*, 2009).

Currently, the calcium phosphate sources added to fluoride varnish are amorphous calcium phosphate (ACP) and tri-calcium phosphate (TCP). These calcium sources have been evaluated clinically (Cochrane *et al*, 2010) but have never been compared with each other regarding their remineralization potential.

We conducted an *in vitro* study evaluating and comparing the effects of a TCP-fluoride varnish with an ACP-fluoride varnish and a plain fluoride varnish on the remineralization of caries-like lesions on primary enamel.

MATERIALS AND METHODS

Specimen preparation

This study was approved by The Ethics Committee of Mahidol University. Forty-eight sound extracted or naturally exfoliated human primary incisor teeth were obtained and polished with fine pumice to remove contaminants and then kept in normal saline until use.

All teeth were blot-dried with a piece of tissue paper and completely coated with two layers of acid resistant nail varnish except for two square windows on each tooth, each window was approximately 1x1 mm. The windows were made over sound intact surfaces on the labial side of the teeth. The root apices were then sealed with sticky wax. The teeth were then again immersed in deionized water until use.

Demineralizing and remineralizing solution preparation

Two demineralizing solutions and one remineralizing solution were prepared. Demineralizing solution 1 (D1) was comprised of 2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4, 0.05 M acetic acid at a pH of 4.4 adjusted using 1M KOH. Demineralizing solution 2 (D2) contained the same components as D1, but the pH was adjusted to 4.7 using 1M KOH. The remineralizing solution (R) was comprised of 1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4, and 0.15 M KCl at a pH of 7.0 adjusted using 1 M KOH (ten Cate and DuijSters, 1982). The demineralizing and remineralizing solutions were freshly prepared for each pH cycle and kept in separate plastic containers.

Caries like lesion formation

Each tooth was immersed in 3 ml of D1 and incubated at 37°C (Sheldon Manufacturing, model 1545, Cornelius, OR) for 4 days to produce carious like lesions 100-150 μm deep (initial enamel lesion) (Rirattanapong *et al*, 2014). Each tooth was then rinsed in 15 ml deionized water and wiped off carefully with a piece of tissue paper. All the teeth were processed in the same manner and immersed in artificial saliva containing 0.65 grams per liter of potassium chloride (British Pharmacopoeia, BP, Norwrich, UK), 0.058 g/l magnesium chloride BP, 0.165 g/l calcium chloride BP, 0.804 g/l dipotassium hydrogen phosphate (US Pharmacopeia, Rockville, MA), 0.365 g/l potassium dihydrogen phosphate, 2 g/l sodium carboxymethyl cellulose BP and deionized water was added to make 1 liter as modified from Amaechi *et al* (1999) until use.

Grouping

Following formation of the carious like lesions, one of the two windows in each tooth was randomly assigned to be used as a "baseline lesion" and was coated with 2 layers of acid resistant nail varnish (Revlon, New York, NY), while the other window was used as the "experimental lesion" window and exposed to the test products and pH-cycling process. Forty-eight teeth were used in total and randomly divided into four groups of 12 teeth each. The fluoride varnish used in this study were purchased from manufacturers as shown in Table 1. The 4 groups were treated as follows: Group A (control group): no treatment; Group B: Duraphat® varnish; Group C: Enamel Pro® varnish; Group D: Clinpro™ White varnish.

The varnishes were applied according to the manufacturer's instructions and the treated teeth were stored for 24 hours in a moist environment. This storage period was followed by brushing to remove visible fluoride varnish and rinsing with deionized water, after which they were subjected to pH-cycling for 7 days.

The pH-cycling process

The pH-cycling process was intended to imitate the changes in pH in the oral environment for 7 days. Each daily cycle involved three hours of demineralization followed by two hours of remineralization and then another three hours of demineralization (ten Cate and Duijsters, 1982). All the specimens were kept in remineralizing solution overnight at 37°C in an incubator shaker (Series 25 Incubator Shaker®, Ramsey, MN).

Thin section preparation

After completion of 7 days of pH-cycling, the specimens were removed from the solution and the acid-resistant nail varnish was removed with acetone solvent. The lesions were transected longitudinally along inciso-gingival axis using a slow speed diamond saw under water spray (Accutom-50, Struers, Ballerup, Denmark) to create a 400 μm thick section of the tooth. The thin sections were then ground with wet 800 and 1,000 grit silicon carbide papers. The thin sections were ground to 100-150 μm thickness and measured using an electronic digital caliper (Mitutoyo® model CD-6C, Kawasaki, Japan).

Polarized light microscopic measurement

All the sections were placed in deionized water, mounted on glass-slides and the caries like lesion depths were analyzed using a polarized light microscope (Nikon® model eclipse E400 pol, Tokyo, Japan) at 10x magnification. An average lesion depth was calculated from the maximum depth of the lesion at three points. Photomicrographs were taken and analyzed using a computerized calculation with Image-Pro® Plus (Media Cybernetics, Bethesda, MD). The lesion depths were recorded using a single-blind technique.

Intra-examination reliability

Polarized light microscopy measurements were calibrated. Ten sections (20%

Table 1
Three types of fluoride varnish used in this study.

Active ingredients	Trade mark	Manufacturing company
5% sodium fluoride	Duraphat® Varnish	Colgate Oral Pharmaceuticals, New York, NY
5% sodium fluoride with ACP	Enamel Pro® Varnish	Premier, MDSS GmbH Schiffgraben, Hannover, Germany
5% sodium fluoride with TCP	Clinpro™ White Varnish	OMNI Preventive Care, A 3M ESPE Company, West Palm Beach, FL

Table 2
Means and standard deviations of lesion depths and the percentage changes after pH-cycling.

Group	Treatment	Mean lesion depth ± SD (microns)		Percent change
		Baseline lesion	Experimental lesion	
A	Deionized water (Control group)	128.07 ± 16.62[a]	429.97 ± 37.84[b]	242.33 ± 62.32[d]
B	Duraphat®	130.54 ± 16.41[a]	229.43 ± 14.50[c]	77.57 ± 17.74[e]
C	Enamel Pro®	129.71 ± 8.94[a]	220.52 ± 22.96[c]	69.90 ± 11.54[e]
D	Clinpro™	133.70 ± 21.14[a]	222.42 ± 21.17[c]	70.33 ± 31.12[e]

Different superscript letters indicate significant differences ($p<0.05$, ANOVA, Tukey's test).
The same superscript letter indicates no significant difference.

of all the sections) were randomly selected and re-examined by the same examiner under the same conditions using the same equipment. Intra-examination reliability was tested using Pearson's correlation coefficients.

Statistical analysis

One way-analysis of variance (ANOVA) and Tukey's multiple comparison test were used to test for differences in the mean lesion depths and the percentage change in the lesion depth by group (SPSS version 20.0 for Windows; Armonk, College Station, TX). The pair-*t* test was used to compare the lesion depths before and after treatment within each group. Significance was set at $p<0.05$.

RESULTS

Intra-examiner reliability for lesion depth, tested by the Pearson's correlation coefficient was good (0.958).

The means and standard deviations (SD) for lesion depths of each of the groups at baseline and after pH-cycling are shown in Table 2; these ranged from 125.98 ± 5.09 µm to 130.54 ± 16.41 µm. No significant differences were seen in lesion

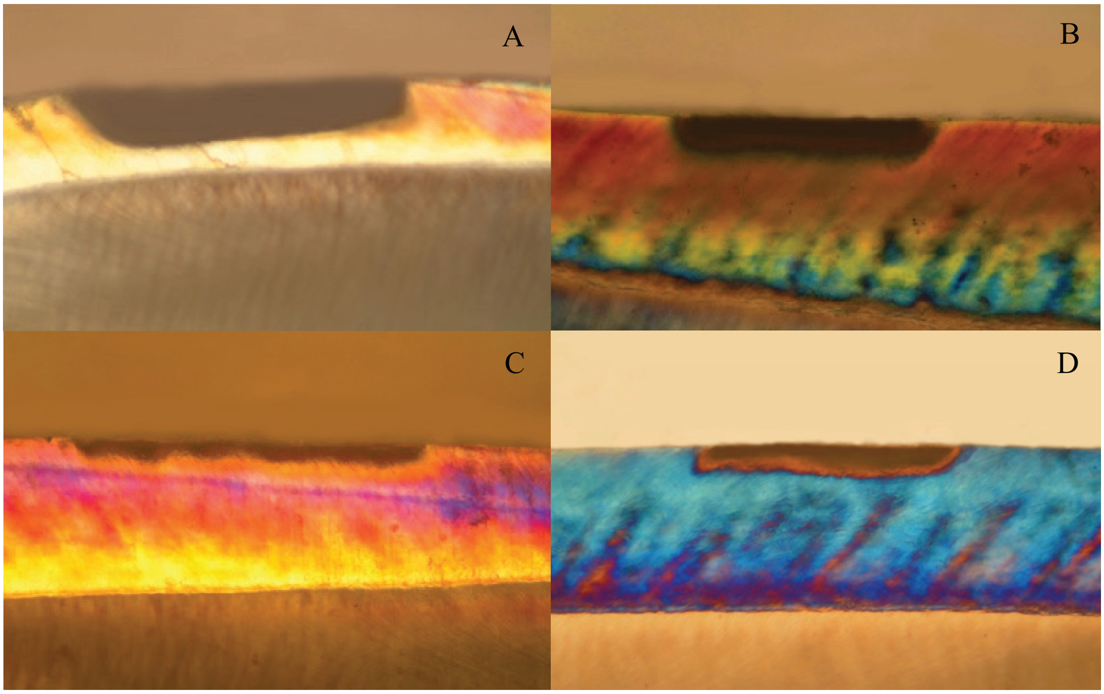

Fig 1–Polarized light photomicrograph at 10x magnification of lesions from deionized water group (A), Duraphat® group (B), Enamel Pro® group (C) and Clinpro™ White Varnish group (D).

depth by group at baseline ($p = 0.232$).

The mean ± SD of the lesion depths after pH-cycling ranged from 215.21 ± 18.81 μm to 429.97 ± 37.84 μm. The mean lesion depths after pH-cycling in the treated groups varied significantly from the control group ($p = 0.000$). There were no significantly differences in lesion depth after pH-cycling among the treatment groups (Fig 1).

DISCUSSION

Several studies have demonstrated the remineralization effects of calcium phosphate containing products (such as toothpaste, cream, mouth rinse) on artificial caries lesions similar to this study (Hicks and Flaitz, 2000). None of these studies compared the de/remineralization effects of fluoride varnish containing different calcium phosphate sources. Our study had the advantage of a baseline control for each experimental group allowing a more accurate comparison of treatment results. These baseline levels did not differ significantly from each other; therefore the treatment results are comparable, even though they used different teeth.

Fluoride varnish is professionally applied, and highly concentrated (22,600 ppm). Multiple studies have shown fluoride varnish can promote enamel remineralization (Arends and Schuthof, 1975; Marinho *et al*, 2002; Castellano and Donly, 2004). Fluoride varnish can also reduce the incidence of caries in permanent dentition. However, previous studies have not evaluated their effectiveness in primary dentition. In this study fluoride varnish did reduce lesion depth progression in primary teeth. This study confirms the protective effect of fluoride varnish in primary teeth similar to a study by Santos *et al* (2009).

However, fluoride is not the only agent involved in remineralization. Calcium and phosphate are also involved in remineralization.

Enamel Pro® varnish contains the same amount of 5% sodium fluoride as Duraphat® (fluoride only) (22,600 ppm) and has ACP. A synergistic cariostatic effect was seen with Enamel Pro® containing 5% sodium fluoride and ACP. The superior efficacy of ACP and fluoride combined in toothpaste over fluoride alone has been seen in a number of *in vitro* studies (Legeros, 1999; Schemehorn *et al*, 1999; Hicks and Flaitz, 2000; Ramaswami, 2008). However, de/remineralization studies of varnish forms need to be conducted.

Our findings showed the mean experimental lesion depths after pH-cycling in the Enamel Pro® varnish group and the Duraphat® varnish group were not significantly different (p>0.05). No studies have been conducted comparing the remineralizing effect of Enamel Pro® varnish with Duraphat® varnish.

TCP has also been found to have remineralization potential (Karlinsey *et al*, 2010). Our study found no significant difference between Clinpro™ varnish group and Duraphat® varnish similar to a study by Rirattanapong *et al* (2014). Clinpro™ has poorly soluble TCP and together with the large particle size and low amount of calcium phosphate could explain the poor release of calcium and phosphate ions from the product and its inability to significantly increase calcium phosphate levels (Shen *et al*, 2011). Hence, neither Clinpro™ varnish nor Enamel Pro® varnish had a greater remineralization effect than Duraphat® varnish in this study.

Comparisons between the two fluoride varnish products containing calcium and phosphate have been studied. Schemehorn *et al* (2011) found ACP varnish delivered significantly more fluoride to the enamel than TCP varnish. However, they did not include a calcium and phosphate-free varnish formulation in their study (Schemehorn *et al*, 2011). No remineralization or demineralization comparison studies for these products has been published.

The varnishes in our study were all applied for 24 hours, similar to previous studies (Dijkman *et al*, 1983; Santos *et al*, 2009). This is because patients who receive the varnish are instructed not to brush their teeth for 24 hours (Vaikuntam, 2000), even though the manufacturer recommends to varnish, it needs to only be applied to the teeth for a few seconds.

Various methods have been used to study remineralization of carious lesions, such as microradiography (Featherstone *et al*, 1983), polarized light microscopy (Hick and Flaitz, 2000), microhardness (Magalhães *et al*, 2010), mineral analysis of calcium phosphate and fluoride phases (Buzalaf *et al*, 2010), transmission and scanning electron microscopy (Whittaker, 1982). The most commonly used qualitative method in depth-related properties of artificial lesions are polarized light microscopy (Hick and Flaitz, 2000). We used polarized light microscopy and the Image-Pro® Plus program to analyze demineralization depth. This method is accurate but time consuming (Lo *et al*, 2010). The different methods used to determine lesion depth may have contributed to the different results. Polarized light microscopy provides an accurate measurement of demineralization (Rana *et al*, 2007). Although the same model is used in multiple laboratories, some variations have occurred in testing due to differences in available equipment, specimen sources, elements and other details.

In this study, pH-cycling was conducted for 7 days, similar to Yimcharoen *et al* (2011) who evaluated remineralization on primary enamel lesions. A previous study found artificial carious lesions in primary teeth became too extensive to be evaluated after 7 days (Thaveesangpanich *et al*, 2005). The solution concentrations and pH were maintained in the range reported to exist in oral fluids (ten Cate and Duijsters,1982). To avoid the risk of the solution becoming saturated, fresh demineralizing and remineralizing solutions were made each cycle and the pH was checked regularly in our study.

The pH-cycling model was used for our study because of the low cost and the lack of ethical limitations. The role of the pH-cycling model is to facilitate the generation of sufficient quantitative data to give investigators the confidence to appropriately design clinical trials (Buzalaf *et al*, 2010). Further *in vitro* and clinical trials are needed.

In conclusion, our study findings showed all the fluoride varnishes: Duraphat®, Clinpro™, Enamel Pro® and TCP fluoride varnish exhibited the same ability to inhibit progression of initial primary enamel lesions. Even though previous studies have demonstrated a synergistic effect of calcium phosphate and fluoride, we did not see this effect in our study.

REFERENCES

Amaechi BT, Higham SM, Edgar WM. Factors influencing the development of dental erosion *in vitro*: enamel type, temperature and exposure time. *J Oral Rehabil* 1999; 26: 624-30.

Arends J, Schuthof J. Fluoride content in human enamel after fluoride application and washing - an *in vitro* study. *Caries Res* 1975; 9: 363-72.

Azarpazhooh A, Main PA. Fluoride varnish in the prevention of dental caries in children and adolescents: a systematic review. *Hawaii Dent J* 2009; 40: 6-7, 10-3.

Borges BC, de Souza Borges J, de Araujo LS, Machado CT, Dos Santos AJ, de Assunçao Pinheiro IV. Update on nonsurgical, ultraconservative approaches to treat effectively non-cavitated caries lesions in permanent teeth. *Eur J Dent* 2011; 5: 229-36.

Buzalaf MA, Hannas AR, Magalhães AC, Rios D, Honório HM, Delbem AC. pH-cycling models for in vitro evaluation of the efficacy of fluoridated dentifrices for caries control: strengths and limitations. *J Appl Oral Sci* 2010; 18: 316-34.

Castellano JB, Donly KJ. Potential remineralization of demineralized enamel after application of fluoride varnish. *Am J Dent* 2004; 17: 462-4.

Chu CH, Ho PL, Lo EC. Oral health status and behaviors of preschool children in Hong Kong. *BMC Public Health* 2012; 12 : 767.

Cochrane NJ, Cai F, Huq NL, Burrow MF, Reynolds EC. New approaches to enhanced remineralization of tooth enamel. *J Dent Res* 2010; 89: 1187-97.

Dijkman AG, de Boer P, Arends J. *In vivo* investigation on the fluoride content in and on human enamel after topical applications. *Caries Res* 1983; 17: 392-402.

Featherstone JD, ten Cate JM, Shariati M, Arends J. Comparison of artificial caries-like lesions by quantitative microradiography and microhardness profiles. *Caries Res* 1983; 17: 385-91.

Hicks MJ, Flaitz CM. Enamel caries formation and lesion progression with a fluoride dentifrice and a calcium-phosphate containing fluoride dentifrice: a polarized light microscopic study. *ASDC J Dent Child* 2000; 67: 21-8.

Karlinsey RL, Mackey AC, Stookey GK. *In vitro* remineralization efficacy of NaF systems containing unique forms of calcium. *Am J Dent* 2009; 22: 185-8.

Karlinsey RL, Mackey AC, Walker ER, Frederick KE. Preparation, characterization and *in vitro* efficacy of an acid-modified beta-TCP material for dental hard-tissue remineralization. *Acta Biomater* 2010; 6: 969-78.

Legeros ZR. Calcium phosphate in demineralization/remineralization processes. *J Clin Dent* 1999; 10: 65-73.

Lo EC, Zhi QH, Itthagarun A. Comparing two quantitative methods for studying remineralization of artificial caries. *J Dent* 2010; 38: 352-9.

Magalhães AC, Moron BM, Comar LP, Wiegand A, Buchalla W, Buzalaf MA. Comparison of cross-sectional hardness and transverse microradiography of artificial carious enamel lesions induced by different demineralising solutions/gels. *Caries Res* 2010; 43: 474-83.

Marinho VC, Higgins JP, Logan S, Sheiham A. Fluoride varnishes for preventing dental caries in children and adolescents. *Cochrane Database Syst Rev* 2002: CD002279.

Marinho VC, Higgins JP, Sheiham A, Logan S. One topical fluoride (toothpastes, or mouthrinses, or gels, or varnishes) versus another for preventing dental caries in children and adolescents. *Cochrane Database Syst Rev* 2004: CD002780.

Ramaswami N. Fluoride varnish: a primary prevention tool for dental caries. *J Mich Dent Assoc* 2008; 90: 44-7.

Rana R, Itthagarun A, King NM. Effects of dentifrices on artificial caries like lesions: an *in vitro* pH cycling study. *Int Dent J* 2007 Aug; 57: 243-8.

Reynolds EC. Calcium phosphate-based remineralization systems: scientific evidence? *Aust Dent J* 2008; 53: 268-73.

Rirattanapong P, Vongsavan K, Saengsirinavin C, Pornmahala T. Effect of fluoride varnishes containing tri-calcium phosphate sources on remineralization of initial primary enamel lesions. *Southeast Asian J Trop Med Public Health* 2014; 45: 499-504.

Santos L de M, Reis JI, Medeiros MP, Ramos SM, Araujo JM. *In vitro* evaluation of fluoride products in the development of carious lesions in deciduous teeth. *Braz Oral Res* 2009; 23: 296-301.

Schemehorn BR, Orban JC, Wood GD, Fischer GM, Winston AE. Remineralization by fluoride enhanced with calcium and phosphate ingredients. *J Clin Dent* 1999; 10: 13-6.

Schemehorn BR, Wood GD, McHale W, Winston AE. Comparison of fluoride uptake into tooth enamel from two fluoride varnishes containing different calcium phosphate sources. *J Clin Dent* 2011; 22: 51-4.

Shen P, Manton DJ, Cochrane NJ, *et al*. Effect of added calcium phosphate on enamel remineralization by fluoride in a randomized controlled in situ trial. *J Dent* 2011; 39: 518-25.

ten Cate JM, Duijsters PP. Alternating demineralization and remineralization of artificial enamel lesions. *Caries Res* 1982; 16: 201-10.

Thaveesangpanich P, Itthagarun A, King NM, Wefel JS, Tay FR. *In vitro* model for evaluating the effect of child formula toothpastes on artificial caries in primary dentition enamel. *Am J Dent* 2005; 18: 212-6.

Vaikuntam J. Fluoride varnishes: should we be using them? *Pediatr Dent* 2000; 22: 513-6.

Whittaker DK. Structural variations in the surface zone of human tooth enamel observed by scanning electron microscopy. *Arch Oral Biol* 1982; 27: 383-92.

Yimcharoen V, Rirattanapong P, Kiatchallermwong W. The effect of casein phosphopeptide toothpaste versus fluoride toothpaste on remineralization of primary teeth enamel. *Southeast Asian J Trop Med Public Health* 2011; 42: 1032-40.