Original Article

The efficiency of child formula dentifrices containing different calcium and phosphate compounds on artificial enamel caries

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Received: 22-08-16 Accepted: 27-10-16 Published: 12-12-16

Abstract

Objectives: Fluoride toothpaste has been extensively used to prevent dental caries. However, the risk of fluorosis is concerning, especially in young children. Calcium phosphate has been an effective remineralizing agent and is present in commercial dental products, with no risk of fluorosis to users. This *in vitro* study aimed to compare the effects of different calcium phosphate compounds and fluoride-containing dentifrices on artificial caries in primary teeth. **Materials and Methods:** Fifty sound primary incisors were coated with nail varnish, leaving two 1 mm^2 windows on the labial surface before immersion in demineralizing solution for 96 hours to produce artificial enamel lesions. Subsequently, one window from each tooth was coated with nail varnish, and all 50 teeth were divided into five groups ($n = 10$); group A – deionized water; group B – casein phosphopeptide–amorphous calcium phosphate (CPP–ACP) paste (Tooth Mousse); group C – 500 ppm F (Colgate Spiderman®); group D – nonfluoridated toothpaste with triple calcium phosphate (Pureen®); and group E – tricalcium phosphate (TCP). Polarized light microscopy and Image-Pro® Plus software were used to evaluate lesions. **Results:** After a 7-day pH-cycle, mean lesion depths in groups A, B, C, D, and E had increased by 57.52 ± 10.66%, 33.28 ± 10.16%, 17.04 ± 4.76%, 32.51 ± 8.99%, and 21.76 ± 8.15%, respectively. All data were processed by the Statistical Package for the Social Sciences (version 16.0) software package. Comparison of percentage changes using one-way analysis of variance and Fisher's least squares difference tests at a 95% level of confidence demonstrated that group A was significantly different from the other groups ($P < 0.001$). Lesions in groups B and D had a significant lesion progression when compared with groups C and E. **Conclusions:** All toothpastes in this study had the potential to delay the demineralization progression of artificial enamel caries in primary teeth. The fluoride 500 ppm and TCP toothpastes were equal in the deceleration of enamel caries progression and better than CPP–ACP paste and TCP toothpaste.

Key words: *Artificial caries, calcium lactate, casein phosphopeptide-amorphous calcium phosphate, dicalcium phosphate dihydrate, primary teeth, toothpaste, tricalcium phosphate*

Access this article online	
Quick Response Code:	Website: www.jispcd.org
	DOI: 10.4103/2231-0762.195517

How to cite this article: Rirattanapong P, Vongsavan K, Saengsirinavin C, Khumsub P. The efficiency of child formula dentifrices containing different calcium and phosphate compounds on artificial enamel caries. J Int Soc Prevent Communit Dent 2016;6:559-67.

INTRODUCTION

Dental caries is now recognized as a preventable disease. In young children, noninvasive intervention for initial caries should be considered because the routine operative treatment is hard to achieve and may need a special behavior management. The most common intervention is a daily tooth-brushing with fluoride dentifrice. The caries reduction of 1000–1500 ppm standard fluoride toothpaste was 24–29% in permanent teeth, although there were insufficient evidences for caries prevention in primary dentition,[1,2] which carried risk of developing dental fluorosis when used in very young children. Children who began brushing teeth at 18 months or less were seen to have lower caries prevalence but higher fluorosis especially for the 1000 ppm F than 400–550 ppm F toothpaste.[3] Hence, low-fluoride toothpastes were developed for small children who were at a risk of developing fluorosed teeth.[4,5] The low fluoride toothpaste (400–550 ppm F) was not statistically different with standard fluoride toothpaste (1055 ppm F) for caries prevention[6,7] and did not significantly decrease the risk of fluorosis in the permanent maxillary incisor.[8] Hence, there was an interest in other remineralizing agents including calcium phosphate-based remineralization system.

Dicalcium phosphate dihydrate (DCPD) has been used in the fluoride dentifrice to enhance the remineralizing effect of fluoride. Calcium ions in DCPD not only dissolved intraorally and increased the level of calcium ions in plaque fluid but also acted as a fluoride carrier and enhanced fluoride uptake in artificial enamel caries in primary bovine tooth to improve remineralization.[9,10] DCPD-containing dentifrice alone exhibited anti-caries property in a *in vivo* rat caries study against the silica-based dentifrice.[11]

Calcium lactate is a soluble calcium compound and is commonly used in food products. The rats that consumed diets containing calcium lactate had lower caries incidence than those that consumed normal diet.[12] Casein phosphopeptide–amorphous calcium phosphate (CPP–ACP) is one of the commercial calcium phosphate-based remineralizing agent. It was claimed that calcium and phosphate ions were freely bioavailable and diffused down into enamel subsurface lesions, thereby effectively promoting remineralization.[13] The primary artificial early enamel lesions with CPP–ACP treatment had significantly increased surface microhardness than the 500 ppm F solution.[14]

Recently, Karlinsey introduced a functionalized beta-tricalcium phosphate (fTCP), which was modified from a mechanochemical reaction between beta-tricalcium phosphate (β-TCP) and sodium lauryl sulfate (SLS) or fumaric acid.[15] It was designed to work in synergy with fluoride to create a more acid-resistant mineral relative to that achievable with fluoride alone. The addition of 50 ppm fTCP in 500 ppm F produced greater remineralization than 500 and 1100 ppm F.[16] The combination of 1000 ppm NaF plus fTCP in incipient bovine enamel lesions had greater surface microhardness relative to the 1000 ppm F dentifrice, which was statistically equivalent to 1450 ppm F dentifrice.[17]

According to ten Cate, the *in vivo* patterns and effects of caries-preventive materials regarding demineralization and remineralization could best be examined with an *in vitro* pH-cycling system.[18] Thaveesangpanich *et al*. suggested that a 7-day pH-cycling model was appropriate for primary enamel.[19] Polarized light microscopy is one of the standard methods for evaluation of carious lesion. This technique allows to assist the outer enamel surface and measure the lesion depths.

Because there are no experiments which compared the different calcium phosphate compounds in children dentifrices, the aim of the present *in vitro* study was to evaluate the effect of these dentifrices on artificial enamel caries in primary teeth using the polarized light microscopy technique. Two null hypotheses were tested: (1) dentifrice containing calcium does not increase the enamel's resistance to caries and (2) there are no significant differences in prevention of enamel caries among different types of calcium phosphate compounds.

MATERIALS AND METHODS

Specimen preparation

Fifty sound extracted or naturally exfoliated human primary anterior teeth were cleaned to remove soft tissue debris and inspected for cracks; fluorosis; enamel hypoplasia; white, yellow, or brown spots; and tetracycline lesions. The teeth were painted with two layers of acid resistant nail varnish and two windows were created of size 1 mm^2 1 mm apart on the labial surface.

Artificial lesion formation

Specimens were immersed for 96 hours in the demineralizing solution (D1; 2.2 mM, $CaCl_2$ 2.2 mM, NaH_2PO_4 0.5 M acetic acid, pH was adjusted to 4.4

with 1M KOH) to produce artificial carious lesions approximately 60–100 μm deep.[19] Subsequently, the teeth were humidly collected by immersion in artificial saliva containing 0.65 g/l KCl (British Pharmacopoeia, UK), 0.058 g/l $MgCl_2$ (British Pharmacopoeia, UK), 0.165g/l $CaCl_2$ (British Pharmacopoeia, UK), $K_2(HPO_4)_2$ (Pharmacopoeia, USA), $KH_2(PO_4)_3$ (British Pharmacopoeia, UK), 2g/l $NaCO_2CH_3$ cellulose (Pharmacopoeia, USA), and deionized water to prepare 1 liter, as adapted from Amaechi *et al.*[20] until use.

Sample size calculation[21]

$$N = 2 \times Z_\alpha(\sigma_1^2 + \sigma_2^2)/d^2$$
$$= 2 \times 1.96\,(32^2 + 57^2)/42^2$$
$$= 9.48 >> 10$$

Treatment groups

Fifty anterior teeth were randomly divided into five groups of treatment ($n = 10$) as follows:

- Group A: Deionized water (Control)
  Group B: CPP–ACP paste (Tooth Mousse, GC Corp, Tokyo, Japan)
- Group C: 0.11% Sodium fluoride (500 ppm F) toothpaste (Colgate® Ultimate Spiderman, Colgate-Palmolive Ltd., Bangkok, Thailand)
- Group D: Nonfluoridated children toothpaste containing DCPD, calcium lactate, and calcium pyrophosphate (Pureen®, AmLion Toothpaste Mfg., Malaysia)
- Group E: TCP toothpaste (Faculty of Dentistry, Mahidol University, Thailand).

Toothpaste slurry preparation

The slurry solution of each experimental toothpaste was freshly prepared for each cycle by mixing a pea-sized (0.32g)[19] portion in 30 ml deionized water and stirring at 250 rounds per minute for 3 minutes with a magnetic stirrer (Heidolph®, MR Hei-standard model, German).[22]

Demineralizing and remineralizing solution preparation

Demineralizing and remineralizing solutions for the pH-cycle process were freshly prepared in each cycle with deionized water. According to Rirattanapong *et al.*,[22] the demineralizing solution (D2) contained 2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4, and 0.5 M acetic acid, and pH adjusted to 4.7 with 1 M KOH. The remineralizing solution (R) contained 1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4, and 0.15 M KCl, and 1 M KOH was used to adjust the pH to 7.0.[22]

The pH-cycling process

The five groups of specimens were subjected to the remineralization–demineralization process with a pH-cycling model for 7 days.[19] The model was composed of 3 hours of demineralization in the demineralization solution (D2) twice daily and 2 hours of remineralization between the periods of demineralization. Before the first demineralizing cycle and both before and after of the second demineralizing cycle, the specimens were immersed in the experimental treatment slurries for 3 minutes.[22] The specimens were stored in the remineralizing solution (R) overnight at 37°C in the control temperature chamber.

After the 7-day pH cycle, specimens were removed from the solution. The acid resistant nail varnish was carefully erased with acetone solvent and immersed in deionized water before measurement.

Thin specimen preparation

Each specimen was removed from the acrylic block and sectioned longitudinally in the labiolingual direction through the enamel lesion in each window, using a microcutting instrument (Accutom-50, Struers, Denmark) to produce approximately 400 µm thick section. Then, the thin sections were grinded with wet 1000 grit silicon carbide paper to produce the 150–250 µm thin section measuring by electric digital caliper (Mitutoyo model CD-6C, Japan).[22]

Polarized light microscopy measurement

All sections were imbibed in deionized water, and mounted on glass-slides. The photomicrographs of the artificial caries lesion, both before and after treatment, were taken under a polarizing light microscope (Nikon model eclipse E400 pol, Japan) at 5× magnification with a digital camera (Nikon® Coolpix 990, Japan) and analyzed with Image-Pro® Plus Program (Media Cybernatics, MD, USA) software. The average lesion depths were calculated from the maximum depth at three points of lesions and recorded in the single-blinded technique.[22]

Data analysis

Statistical analysis of the data was conducted with significance set at 0.05. Mean lesion depths were examined for normality and homogeneity of variance. Comparison of pre and posttreatment lesion depth in each group was conducted with the paired-sample *t*-test at 95% confidence level. Percentage changes of mean lesion depth among groups were compared using the one-way analysis of variance (ANOVA) and least squares difference (LSD) tests ($P = 0.05$). Intraexamination reliability was performed using the Pearson's correlation coefficients. The result showed good reliability with correlation coefficients of 0.979.

RESULT

Mean and standard deviation of lesion depth before a pH-cycle ranged from 93.35 ± 12.41 to 100.04 ± 10.45 μm. No statistically significant difference of mean lesion depth was obtained among the pretreatment groups ($P = 0.656$, ANOVA) [Table 1 and Figure 1].

The results from the lesion depth measurements after the 7-day pH-cycle were significantly different from pretreatment in each group ($P = 0.000$) [Table 1 and Figure 1].

Group A (deionized water), Group B CPP–ACP paste (Tooth Mousse); Group C 500 ppm F toothpaste (Colgate Spiderman®), Group D nonfluoride toothpaste with triple calcium phosphate(Pureen®), and Group E TCP toothpaste had increased in lesion depth by 57.52 ± 10.66%, 33.28 ± 10.16%, 17.04 ± 4.76%, 32.51 ± 8.99%, and 21.76 ± 8.15%, respectively [Figures 2-7]. The comparison of percentage changes using one-way ANOVA and LSD tests at a 95% level of confidence demonstrated that Group A deionized water was significantly different from the other groups ($P < 0.001$). Progression of lesions in Group B CPP–ACP paste was not statistically significant different from group D nonfluoride toothpaste ($P = 0.848$), whereas group C 500 ppm F kids toothpaste and group E TCP were also not significant different from each other ($P = 0.243$). Lesions in groups B and D had a significant progression compared with groups C and E ($P < 0.05$).

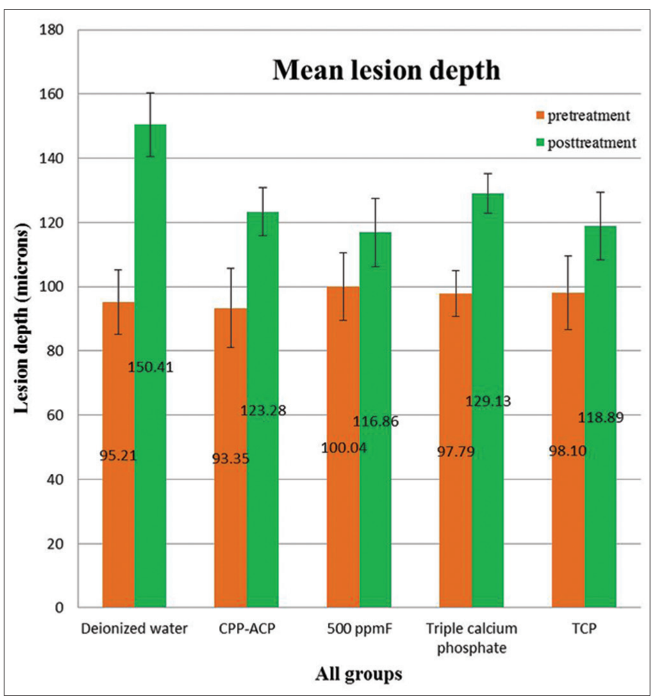

Figure 1: Graph showed the mean lesion depth in all treatment groups before and after a 7-day pH-cycling process

DISCUSSION

The *in vitro* experiment with a pH-cycle model was chosen in this study because of many advantages in providing preliminary assessment of anti-caries activity of tested products, mainly due to inhibition of demineralization and enhancement of remineralization, controllable factors and conditions, and being inexpensive. The pH-cycle model could mimic an *in vivo*

Table 1: Mean and percentage change of lesion depth

Group	Treatment	Mean lesion depth±SD (microns)		Percent change
		Before	After	
A	Deionized water	95.21±10.07	150.41±9.82*	57.52±10.66[a]
B	500 ppmF	100.04±10.45	116.86±10.59*	17.04±4.76[b]
C	CPP-ACP	93.35±12.41	123.29±7.42*	33.28±10.16[c]
D	Triple calcium phosphate	97.78±7.14	129.13±6.16*	32.51±8.99[c]
E	Tricalcium phosphate	98.10±11.46	118.89±10.55*	21.76±8.15[b]

**Showed significant different before and after treatment (P = 0.000). [a,b,c]different letters showed significant difference among groups (P < 0.05)*

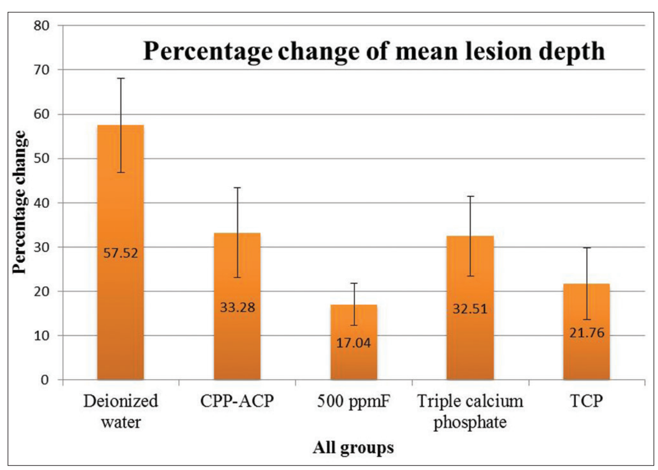

Figure 2: Graph showed the percentage change of mean lesion depths in all treatment groups before and after a 7-day pH-cycling process

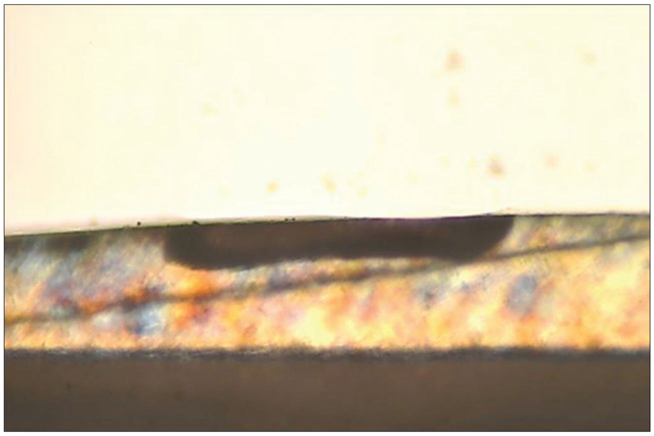

Figure 3: Polarized light photomicrograph of enamel lesion after treatment in Group A (deionized water)

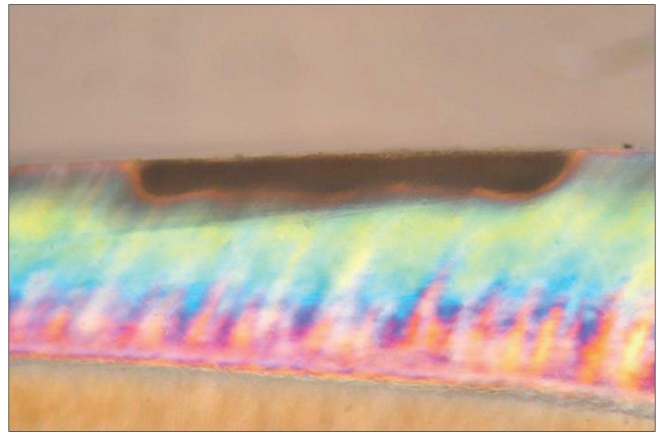

Figure 4: Polarized light photomicrograph of enamel lesion after treatment in Group B (CPP-ACP paste)

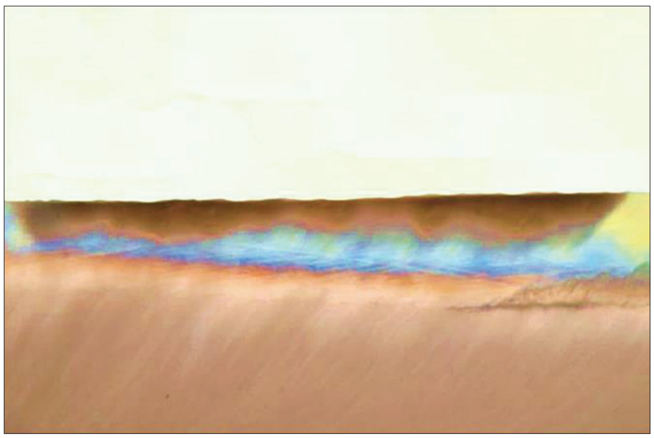

Figure 5: Polarized light photomicrograph of enamel lesion after treatment in Group C (500 ppm F dentifrice)

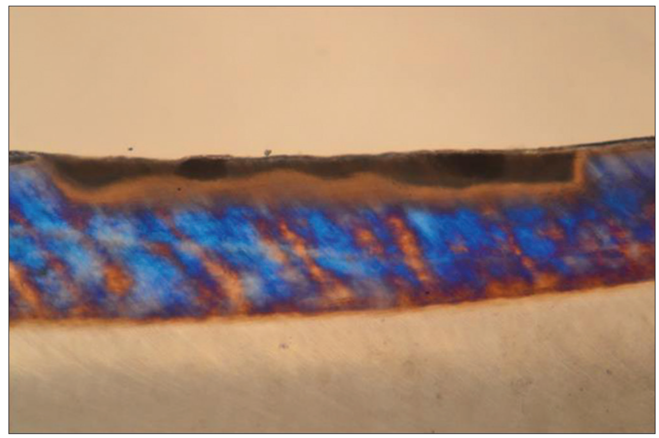

Figure 6: Polarized light photomicrograph of enamel lesion after treatment in Group D (triple calcium phosphate dentifrice)

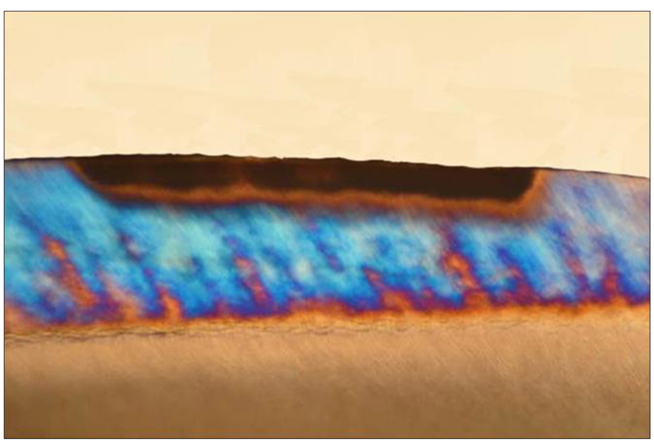

Figure 7: Polarized light photomicrograph of enamel lesion after treatment in Group E (TCP dentifrice)

caries development,[23,24] however, *in vivo* experiments are still necessary due to a significant limitation of an *in vitro* study to simulate complex biological processes in the mouth. The pH-cycle process was conducted for 7 days based on the previous study which found that a 10-day pH-cycle model for permanent teeth was not suitable for primary teeth because enamel lesions became eroded and progressed into dentin after 7 days.[19]

The human primary enamels were chosen in this study according to Lippert *et al*. who suggested that bovine enamel caries had progressed more rapidly and became more remineralized than human enamel.[25] Hence, it might not well represent a de- and remineralization challenge in primary human enamels.

The primary incisors were selected in this study because of wide flat surface area of enamel of labial surface and limited variations in the morphology of individual teeth that were similar to previous studies.[22,26,27]

We used a polarized light microscope to identify the effect of toothpastes. However, it was much more laborious and time-consuming to prepare thin specimen sections and could not provide the quality of mineral changes in lesions. It was a less expensive method and gave an accurate measurement when the main outcome was lesion depth.[28]

The baseline mean lesion depth of artificial enamel caries was not significantly different among all five groups. It can be implied that there were no variations of lesions even though the specimens were from different primary teeth, and thus there was no effect on demineralization. The artificial caries-like lesions of enamel was more constantly reproducible than natural lesions, and hence it was suitable for an *in vitro* experiment. In the present study, artificial caries formation followed the previous studies by Thaveesangpanich *et al*.[19,26] and Rirattanapong *et al.*[22] The mean lesion depths were slightly deeper than the previous reports.

After the 7-day pH-cycle, mean lesion depths in the control group were significantly different from the other experimental groups ($P < 0.001$). Therefore, the first null hypothesis of the present study was rejected. The polarized light photomicrographs showed progression of lesions, and no actual remineralization was observed. It can be concluded that the experimental toothpastes could only slow down the progression of demineralization. The result was similar to the previous studies in primary teeth that they also noticed the capability to decelerate progression of demineralization and no remineralization effect of a pea-size children toothpaste on artificial enamel caries.[19,26,27] The possible reasons may range from the unique primary enamel characteristics which are thinner, imperfect hydroxyapatite crystal, to lower mineral composition than permanent tooth.

This study found lesions in groups B and D had a significant lesion progression when compared with groups C and E. Therefore, the second null hypothesis was also rejected.

Low fluoride toothpaste was formulated for young children who might be at a risk of excessive ingestion of fluoride. In this study, the slurry solution of a pea-sized 500 ppm F children toothpaste had a retarding effect on lesion progression, which was similar to the *in vitro* studies by Thaveesangpanich *et al*.[19,26] and Rirattanapong *et al*.[22] Moreover, the posttreatment enamel lesion depths by Rirattanapong *et al*. showed that no significant difference existed between 500 and 1000 ppm F dentifrice, however, there was significant difference when compared with nonfluoride toothpaste.[22] Ekambaram *et al*.[29] and Itthagarun *et al.*[30] had observed the actual remineralization effect of 500 ppm F toothpaste if the amount increased to 3:1 by weight of deionized water and toothpaste.

CPP–ACP paste is designed to apply and leave on tooth surfaces. Many parents of young children were advised from dentists to brush their children's teeth with this paste rather than a fluoride dentifrice without any scientific support. This study proved that toothbrushing with CPP–ACP paste had enamel lesions progressing deeper than a 500 ppm F and TCP toothpaste. The unlikely results were observed after following the manufacture direction. Zhou *et al.*[31] revealed that a thin layer application of CPP–ACP paste and 500 ppm NaF solution decreased nanohardness of lesions on primary incisors with no significant differences between the materials. The 30-day remineralization process by Zhang *et al.*[14] found the enamel lesions treated with 5-minute application of CPP–ACP paste had significantly increased percentage of surface microhardness than the lesions treated with NaF solution. The result of CPP–ACP in the present study was against to prior studies, which might be due to a very small amount of CPP–ACP paste and immersion in the slurry solution technique, resulting in an insufficient amount of calcium and phosphate ions to create remineralization.

The available commercial nonfluoride toothpaste for children in this study proposed the benefit of three types of calcium phosphate compounds (DCPD, calcium pyrophosphate, and calcium lactate) against caries activity. However, none of the scientific evidences were reported about this product. DCPD and calcium pyrophosphate are common abrasive agents in toothpaste. DCPD was proved to enhance the remineralization effect of fluoride. An *in vitro* study on human third molars enamel caries had significantly increased the remineralization area after treatment with the saturated DCPD and inorganic 1 ppm F solution.[32] DCPD and calcium pyrophosphate are sparsely soluble in a neutral solution, such as deionized water, so they were not expected to be the remineralizing agents. The capability to delay the lesion progression might from calcium lactate, which is the only soluble calcium in this toothpaste.

This is the first study of toothpaste which used TCP as both an active ingredient and abrasive agent. The experimental TCP toothpaste was dissimilar with fTCP regarding both structure and concentration. The fTCP was produced by altering 98% w/w β-TCP with 2% w/w sodium lauryl sulfate (SLS) using a ball milling technique,[33] whereas the TCP toothpaste was composed of 35% w/w native β-TCP and 1% w/w SLS without any special technique. fTCP worked with fluoride to produce more acid-resistant crystal relative to fluoride, β-TCP, or fTCP alone. The bovine and human enamels treated with 500 ppmF/fTCP dentifrices produced significantly greater surface microhardness relative to 500 ppmF and were comparable to a 1150 ppmF dentifrice alone.[34] Furthermore, the lesions treated with 1000 ppmF/fTCP had surface microhardness statistically equivalent to 1450 ppmF toothpaste and superior than CPP–ACP plus fluoride.[35] However, the statistical analysis in this study demonstrated that TCP toothpaste was not different from 500 ppmF toothpaste to retard the progression of artificial caries in primary enamel. It might be a suitable toothpaste for very young children who have initial caries lesions to avoid unintentional fluoride ingestion from fluoride toothpaste.

The ability of TCP test toothpaste to impede demineralization in this study may be explained with the aforementioned studies by Karlinsey *et al*.[33,36] First, based on the infrared (IR) spectroscopy test, the native β-TCP spectral feature was slightly distinct to the lesioned enamel, in which alteration with small molecules might help facilitate the remineralization of demineralized enamel. Second, although β-TCP had poor bioavailability when compared with fTCP and milled-TCP (mTCP), it had actually also been promoted into the lesioned enamel. Third, the dissolution profile showed that calcium ions were released from β-TCP in half the amount of fTCP. This confirmed the solubility of TCP in the present study. Finally, SLS is known to strongly adhere to enamel surface and uptake mineralizing ions to enamel surface; hence, SLS might play an important role in the experimental toothpaste.

Evidence-based review and research studies on the calcium phosphate agent for prevention of tooth caries in children indicated that calcium and phosphate ion promote remineralization of early enamel caries,[37-49] which is in line with the results of this study; however, which type of calcium phosphate compound is the best remineralizing agent is still controversial.[42-49]

Evidence suggests the initial enamel caries can be remineralized using appropriate remineralizing agents, both fluoride and calcium based.[39-41] With these alternative remineralizing strategies, we would be able to re-establish the health of oral tissues without a risk of fluoride toxicity if ingested in high levels, in particular in young children.[22,27] For young children who are likely to inadvertently ingest a dentifrice, a dentifrice-containing calcium phosphate compound could be a safer option.

However, because most studies were conducted *in vitro*, randomized clinical trials evaluating the effectiveness of these remineralizing agents in the prevention of enamel caries are scarce. Thus, the performance of the long-term *in vivo* studies is of extreme importance to evaluate the effective of products and the development of clinical protocols for their application against dental caries.

Strength and limitation

In our study, we have used pH cycling model. This combination of remineralization and demineralization experiments are designed to stimulate the dynamic variations in mineral saturation and pH associated with the natural caries process. To avoid the risk of solutions becoming saturated, fresh demineralizing and remineralizing solutions were made daily and the pH was checked routinely prior to use.

Polarized light microscope is a standard tool for demineralization/remineralization studies as well as for the assessment of the depth of lesions and their extension. In our study, we were concerned with lesion depth measurement, the polarized light microscope was considered to be the best method of choice. The depth of lesions was measured at three different points in order to errors and were readings using a single-blinded technique.

The primary incisors were used in this study for limited variation of the tooth morphology. The same tooth surface was fully evaluated prior to the experiment and then again after the exposure period.

This *in vitro* study had certain limitations such as difficulty in simulating the oral environment, lower level of salivary proteins, lack of bacteria in the artificial saliva solution used, control over the salivary flow rate, and a harsher acidogenic challenges used in a shorter period of time.

CONCLUSIONS

Based on the data obtained from this study, all examined children toothpastes had the potential to retard the demineralization progression of artificial enamel caries in primary teeth. The experimental TCP toothpaste had the efficacy to retard the lesion progression similar to a 500 ppm fluoride-containing toothpaste, and was significantly superior than a calcium phosphate commercial children toothpaste and CPP–ACP paste.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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