The Remineralization Effect of Theobromine and CPP-ACP on Enamel of Primary Molars

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Abstract

Background: Studies have investigated remineralization process utilizing non-fluoridated agents, however the efficacy of theobromine and casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) remain relatively scarce, especially in primary teeth.

Objective: This study aimed to investigate the remineralization effects of theobromine and CPP-ACP on enamel of primary molars under carious simulation condition.

Methods: Extracted forty-five primary molars were collected. The crown was divided into 4 pieces. Three samples from each crown were randomly assigned to one of three treatment groups: deionized water (control), theobromine, and CPP-ACP for microhardness test and another sample was randomly used for ultrastructure and Energy-Dispersive X-ray Spectroscopy (EDS) evaluation. Microhardness testing was applied before and after pH cycling for all three groups (n = 15 each). Ultrastructure and EDS analyzed were performed on samples from four groups: untreated, deionized water, theobromine, and CPP-ACP (n = 10 each). The 7 days pH cycling process was employed to simulate a caries model. Data were described and compared statistically.

Result: After pH cycling, the microhardness of enamel decreased in all 3 groups. The theobromine group did not exhibit significant changes after pH cycling (220.72 ± 24.75 VHN from 275.84 ± 25.34) compared to deionized water (163.21 ± 28.08 from 275.18 ± 27.71 VHN) and CPP-ACP (184.74 ± 44.55 from 277.29 ± 28.48 VHN) groups. Theobromine and CPP-ACP groups showed mineral precipitation covered demineralized enamel surface, as well as deionized water group. The Wt.% of Ca and P elements in theobromine group (Ca 39.54 ± 4.11 and P 18.38 ± 1.61) were not significantly different from untreated enamel (43.33 ± 5.48 and 19.66 ± 1.70) while CPP-ACP (Ca 36.37 ± 3.77 and P 17.13 ± 1.71) and deionized water groups (Ca 36.88 ± 4.67 and P 17.24 ± 2.10) showed a significant reduction ($p < 0.05$).

Conclusion: Under the carious simulation conditions, theobromine might induce remineralization on demineralized enamel by increased Ca and P elements concentration and improved the microhardness while CPP-ACP did not show significant change from the deionized water group.

Keywords: Theobromine, CPP-ACP, enamel surface microhardness, SEM, EDS, primary molars

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Introduction

Dental caries is a multifactorial disease caused by an imbalance between demineralization and remineralization processes affecting enamel and dentin (1). The elements of crystal hydroxyapatite (HA), including calcium, phosphate play an important role in regulating demineralization and remineralization processes (2). When the pH of the oral environment drops below 5.5, demineralization occurs, resulting in a reduction in enamel hardness (3). The remineralization can effectively arrest the progression of active early-stage (non-cavitated) caries when the pH increased.

Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) has the capacity to promote the remineralization of subsurface enamel lesions and inhibit demineralization (4). The ability of CPP is to bind and stabilize calcium and phosphate in an amorphous form through a cluster of sequence phosphoryl residues in addition to its ability to adhere to dental biofilm and tooth enamel (4). CPP-ACP enhances enamel microhardness by creating a state of supersaturation with calcium and phosphate ions (5). However, CPP-ACP has a significant drawback, it has been reported to potentially cause anaphylactic reactions in some patients (6).

The natural substances might also be used as alternative approaches for remineralization due to their relative safety and affordability (3). Theobromine, an acrid alkaloid derived from the cacao plant, may contribute to future dental health, Theobromine is available in form of crystallized, white in appearance, odourless and hydrophilic powder (7). Previous research has established that theobromine solution showed an efficacy on strengthen enamel and prevent cavities (8). Application of theobromine enhances the microhardness of dental enamel that underwent demineralization comparable to those achieved with fluoride (9). Theobromine which also found in chocolate demonstrates superior remineralization and enamel hardening properties compared to fluoride when tested on human-extracted molar enamel (10) and as evidenced by the results of EDS (11). The application of 200 mg/L theobromine gel on either bovine incisor crown (3) or human premolar (12) has been shown to improve enamel hardness. Theobromine has been employed as a food ingredient, a pharmaceutical agent/product in various medical and dental applications. Examples of theobromine used in dentistry include its application in toothpaste formulations (Theodent^{TM}) Theobromine-containing dentifrice, Theodent^{TM}, also demonstrated a remineralization (13). Nassar et al (2021) (14) suggested that combining theobromine with fluoride did not enhance mineral uptake.

Studies by Ten cate et al (15) had shown that after demineralization the enamel surface hardness could be improved by remineralization during pH cycling. However, it is important to note that none of the studies compared theobromine with CPP-ACP in the primary teeth. Therefore, the aims of this study were to investigate the remineralization effects of theobromine and CPP-ACP on surface microhardness, ultrastructural changes, and EDS analysis of the enamel of primary molars subjected to 7 days of pH cycling.

Materials and Methods

The experimental protocol, including the use of human tissue, was approved by the Human Experimentation Committee of the Faculty of Dentistry, Chiang Mai University (No.15/2022). Forty-five extracted primary molar teeth were

collected from healthy children, aged 6 to 12 years at the Pedodontics Clinic, Faculty of Dentistry, Chiang Mai University, Thailand, with parental consent. The teeth were extracted due to prolonged retention or an inability to undergo pulpal treatment and had intact buccal and lingual surface. The samples were cleaned and stored in a 0.1% (w/v) thymol solution at 4°C until use.

The sample size calculation was calculated using the following formula:

$$n = \frac{\left(z_{\frac{\alpha}{2}} + z_{\beta}\right)^2 \left(\sigma_1^2 + \sigma_2^2\right)}{e^2}$$

Where 95% confidence interval, 80% power, 0.9 as a margin of error, standard deviation 1 = 0.4, and standard deviation 2 = 0.7. A sample size of 6 samples was indicated as the minimum to reveal statistical significance among groups. The sample size calculation was based on previous studies (3,16,17). A total of 45 primary molar teeth, 15 per group (for microhardness test), and 10 per group (for ultrastructure and EDS test) were utilized.

After the roots were cut off at the CEJ level, the crowns were sectioned into four pieces by cutting along the mesiodistal and buccolingual directions using a cylinder diamond bur (Intensive®, Swiss Dental Product, Switzerland) mounted on high-speed airotor handpiece under water cooling. One section from each tooth specimen (totally 45 samples) was used for the microhardness test. Forty sections were randomly selected from the 45 tooth samples and examined under scanning electron microscope (SEM : VEGA3, Tescan, Kohoutovice, Czech Republic) to assess ultrastructural changes, and EDS (Ultim Max 40, Oxford Instruments, High Wycombe, UK) analysis was performed (Fig.1).

For microhardness test, the 45 samples were randomly divided into 3 groups (n = 15 each) for testing 3 solutions: deionized water, Theobromine and CPP-ACP. The Vickers hardness tester (STARTECH SMV-1000, Guiyang Sunproc International Trade Co., Ltd., Guiyang, China) was used to measure the microhardness of the enamel surface before and after pH cycling. The red nail varnish (Maybelline New York, Thailand) was applied to the enamel surface to define a testing area and prevent excessive polishing. Monitoring the nail varnish allowed us to ensure that an area of 1 × 1 mm^2 of enamel was exposed. When the nail varnish was removed to achieve the desired area, polishing was promptly ceased.

Position the enamel surface, with the nail varnish coating facing downward in PVC circular block (20 mm in diameter, 4 mm height), and embedded in the clear liquid epoxy resin (Rungart, Thailand). After the epoxy resin cured, polished the nail varnish coated enamel surface with 800-grit silicon carbide abrasive paper until an approximately 1 x 1 mm^2 flat enamel was exposed. Further polishing was performed with 1000, 1500, and 2000 grit papers for an additional 5 seconds each under running water to achieve a smooth enamel surface. Additionally, before testing the Vicker surface microhardness, all specimens were examined under the light microscope at 40x magnification to confirm the condition of the enamel surface.

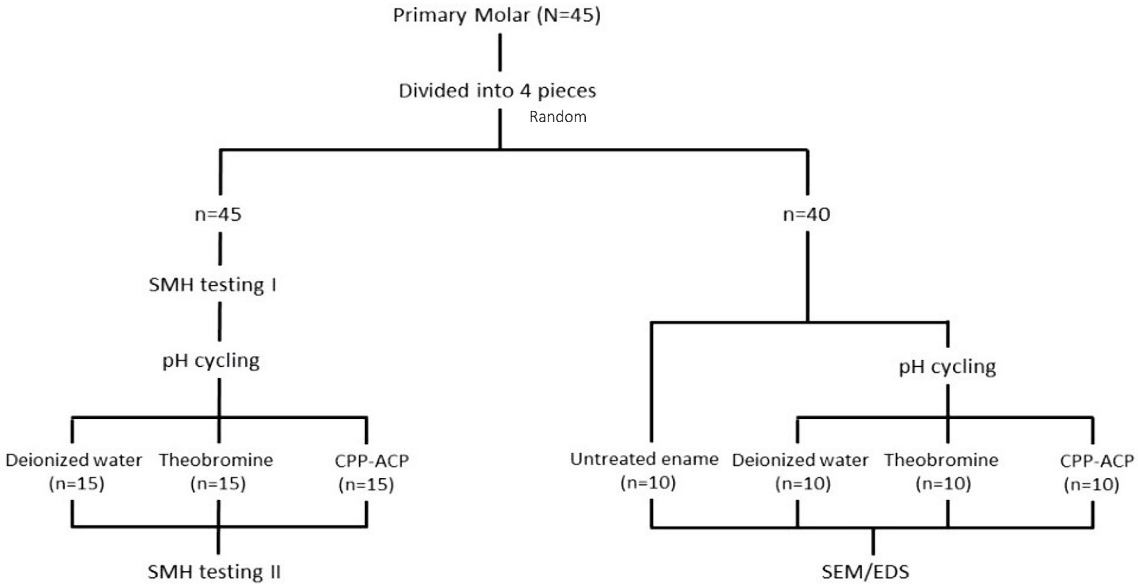

Fig.1 Forty-five primary molars were divided into 4 pieces. One piece from each tooth was randomly allocated into 3 groups for surface microhardness test (SMH). Another piece from each tooth was divided into 4 groups for SEM and EDS analysis.

A Vickers diamond indenter was positioned on the polished flat enamel surface (1 x 1 mm^2) under a x40 objective lens and applied a 100 grams load for 15 seconds. Five indentations were placed randomly along occluso-gingival direction with approximately 100 microns apart. The locations of the indentations varied between pre and post-treatment measurements, and consistently within the designated 1 × 1 mm^2 flat surface. Any samples showing cracks after indentation were excluded from the analysis. The Vickers hardness number (VHN) was calculated from each indentation by using formular:

$$VHN = 1.8544 \frac{F}{D^2}$$

Whereas F in the formular stands for the load in kilogram force (kgf), while D represents the mean of 2 diagonals lengths measured in millimeters (mm) using built-in scaled in microscope.

After microhardness test, the specimens were processed through an artificial caries model utilizing a slightly modified pH cycling method from Featherstone et al (1986) (18). The demineralized and remineralized conditions were cycling for 7 days. For one cycle which involved 24 hour, the specimen was immersed in 0.5 ml of demineralization solution composed of 2.0 mmol/L (0.437 g/L) calcium nitrate ($Ca(NO_3)_2.4H_2O$), 2.0 mmol/L (0.2722 g/L) potassium phosphate (KH_2PO_4), and 0.075 mol/L (4.3 ml) acetic acid (CH_3COOH) at pH 4.8 (pH adjusted with NaOH) slightly modified from Ten Cate J and Duijsters P (19) for 4 hours. The specimen was then immersed in 5 ml of either distilled water (Group A), 5 ml of 200mg/L of theobromine (3,7-Dimethylxanthine: (Sigma Aldrich, Hamburg, Germany) (Group B) or applied onto the test surface with a single fold (approximately 0.01 g) of CPP-ACP paste (GC Tooth Mousse Plus® contains CPP-ACP RECALDENTTM, GC America,

USA) (Group C) for 5 minutes (according to the manufacturer instruction). Finally, all specimen immersed in the 5 ml artificial saliva contained 1.5 mmol/L (0.3542 g/L) Calcium nitrate $Ca(NO_3)_2.4H_2O$, 0.9 mmol/L (0.1224 g/L) Potassium phosphate (KH_2PO_4), 150 mmol/L (9.692 g/L) potassium chloride (KCl) and 20 mmol/L (4.28 g/L) cacodylate buffer (NaCacodylate) at a pH of 7.2 (pH adjusted with citric acid) for 20 hours at 37°C. Before and after immersing the specimen in each solution, it was rinsed with deionized water for 10 seconds each time. All solutions used in the pH-cycling process were freshly prepared and replaced daily to ensure consistency and accuracy throughout the experiment. After finished 7 days pH cycling, the specimens underwent microhardness testing once again.

For ultrastructure and EDS analysis, the 40 samples were randomly divided into 4 groups (n = 10 each): a negative control (untreated group) and three test groups (deionized water as a positive control for pH cycling, Theobromine and CPP-ACP).

The specimens in the deionized water, theobromine and CPP-ACP groups were undergoing 7 days pH cycling of demineralization and remineralization similar to the microhardness test specimens described above. These specimens, along with the untreated enamel group serving as the negative control, were prepared for examination SEM EDS. At the end of the pH cycling, each specimen was rinsed in deionized water for 10 seconds. Then, each specimen which processed for SEM was placed in a screw cap containing deionized water and cleaned in an ultrasonic cleaner for 10 minutes to eliminate any debris from the tooth surface. Each specimen was dried by storing it in a desiccated jar with silica gel at room temperature for 3 days and then gold-coated using a gold sputtering machine (SPI-Module^{TM} Sputter Coater, West Chester, Pennsylvania, USA). The specimens were examined at x10,000 and x30,000 magnification. Nine major elements: Calcium (Ca), Phosphorus (P), Carbon (C), Sodium (Na), Manganese (Mg), Fluorine (F), Chlorine (Cl), Oxygen (O) and Nitrogen (N) as well as the proportion of Ca and P, were measured using EDS as weight percent (Wt. %).

The mean microhardness of the three groups at baseline and after treatment was compared by two-way repeated ANOVA and multiple comparison tests. The ultrastructure morphologies of enamel surfaces for all four groups were descriptively compared at x10,000 and x30,000 magnification, while the weight percentage (Wt. %) of major elements was quantitatively compared using one-way ANOVA. The p value less than 0.05 was considered statistically significant.

Results

After undergoing pH cycling for 7 days, the microhardness of all samples declined significantly. The deionized water groups showed the greatest reduction in microhardness, while the theobromine group showed the least reduction. When evaluating the ability to protect the enamel surfaces from demineralization from pH cycling, theobromine demonstrated a greater capacity to restore microhardness than CPP-ACP ($p < 0.05$). The data of microhardness was summarized in Table 1.

Table 1. Mean microhardness of the test groups at baseline (before treatment) and after 7 days pH cycling with remineralizing agents, analyzed by two-way repeated ANOVA using multiple comparison with Bonferroni test.

Group	Vickers hardness number (Mean ± SD)		
	Baseline	At day 7 post pH cycling	Mean difference
Deionized water	275.18 ± 27.71	163.21 ± 28.08	111.98 ± 39.07
Theobromine	275.84 ± 25.34	220.72 ± 24.75	55.12 ± 39.20
CPP-ACP	277.29 ± 28.48	184.74 ± 44.55	92.55 ± 64.68

In SEM images, untreated enamel showed a homogenous surface (Fig. 2A), while the enamel subjected to pH cycling and stored in deionized water showed enamel porosity, forming a fish scale like characteristic on the surface (Fig. 2B). Both the theobromine and CPP-ACP groups (Fig. 2C and 2D, respectively) showed evidence of remineralization, with deposition of amorphous calcium phosphate on demineralized enamel. At higher magnification of x30,000, there were fine granules were found on the surface treated with theobromine while larger granules of amorphous calcium phosphate coating on the surface treated with CPP-ACP. (Fig. 2E and 2F, respectively)

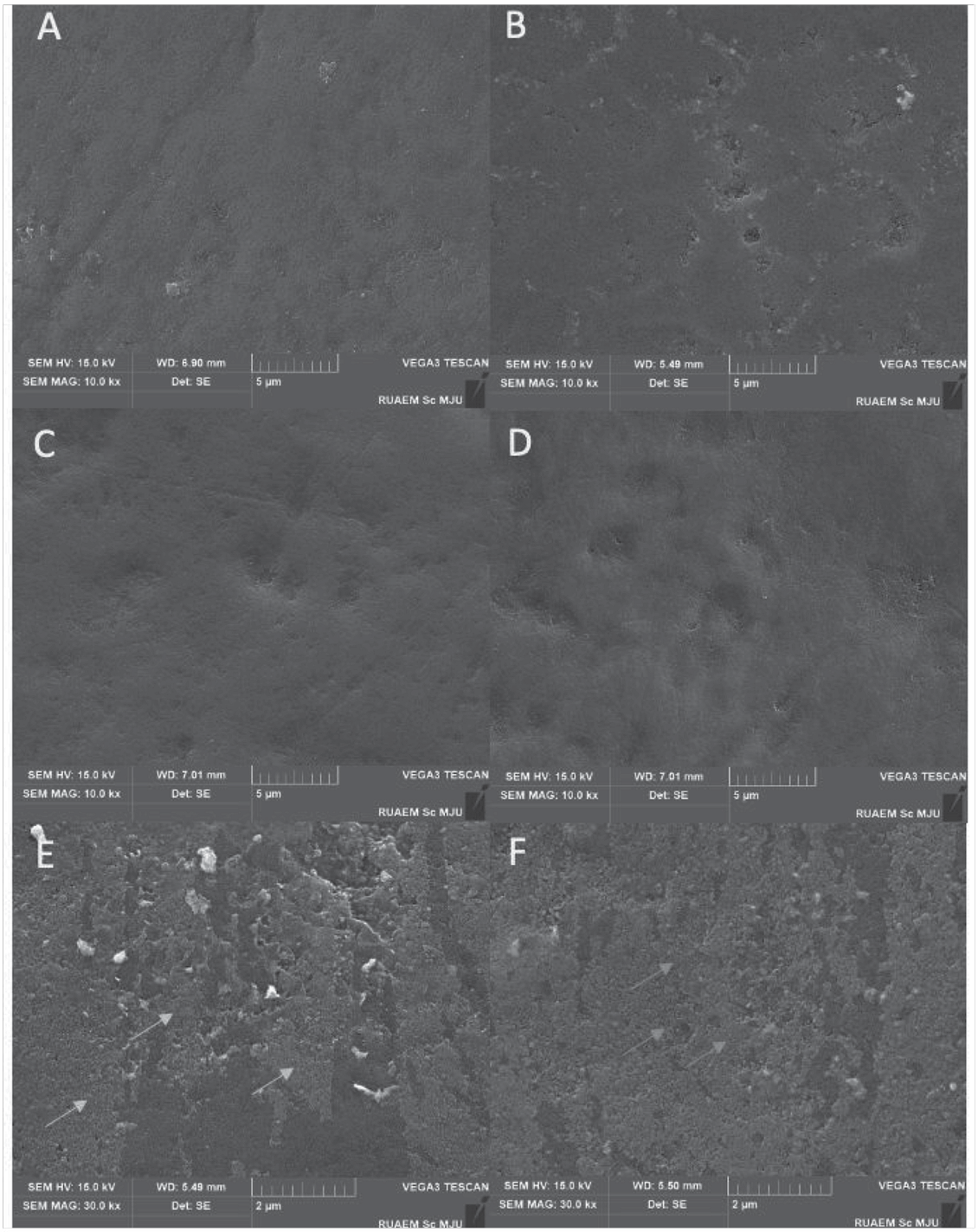

Fig.2 The SEM images of enamel surface taken at x10,000 magnification; untreated enamel (A),deionized water (B), Theobromine (C) and CPP-ACP (D). The SEM images of enamel surface taken at x30,000 magnification; Theobromine (E) with the yellow arrows to show finer granules, and CPP-ACP (F) with the orange arrows to show larger granules.

The results from the EDS analysis suggested that the weight percentages of calcium (Ca) and phosphorus (P) in the deionized water group which represented the enamel that underwent pH cycling was significantly reduced ($p < 0.05$) compared to the untreated enamel. Two remineralization groups showed different results: the theobromine group showed no significant difference from untreated enamel, but CPP-ACP group still showed a significant reduction in both elements compared to the untreated enamel ($p < 0.05$). Other elements were not significant different among groups. However, the Ca/P ratio did not show significant differences among the four groups (Table 2).

Table 2. EDS analysis of the two major elements (Ca and P) and Ca and P ratio of four groups presented in weight percent (Wt. %).

Elements	EDS analysis (Wt. %)			
	Untreated	Deionized water	Theobromine	CPP-ACP
Ca	43.33 ± 5.48	36.88 ± 4.67*	39.54 ± 4.11	36.37 ± 3.77*
P	19.66 ± 1.70	17.24 ± 2.10*	18.38 ± 1.61	17.13 ± 1.71*
Ca/P ratio	2.21 ± 0.11	2.14 ± 0.05	2.15 ± 0.05	2.12 ± 0.06
O	27.95 ± 5.75	32.65 ± 6.07	33.12 ± 6.21	34.69 ± 4.79
C	7.05 ± 1.73	10.84 ± 9.23	7.21 ± 2.10	9.37 ± 5.12
Na	0.39 ± 0.08	0.39 ± 0.08	0.39 ± 0.09	0.37 ± 0.07
Mg	0.17 ± 0.17	0.11 ± 0.12	0.17 ± 0.12	0.12 ± 0.15
F	0.39 ± 0.16	0.49 ± 0.24	0.48 ± 0.21	0.56 ± 0.23
Cl	0.55 ± 0.17	0.45 ± 0.10	0.44 ± 0.09	0.44 ± 0.05
N	0.13 ± 0.4	0.61 ± 0.15	0.00	0.3 ± 0.79

*Indicated significant different $p < 0.05$ from other groups.

Discussion

The pH cycling process involves repeatedly alternating demineralization and remineralization to simulate the progression of dental caries (17). The research by L.J. Wang et al. (2006) (20) demonstrated that the rate of demineralization in developing lesions varies with the direction and location of acid attacks, with primary enamel exhibiting a higher vulnerability to dissolution compared to permanent enamel. In this study, the demineralization and remineralization solutions were modified from Featherstone et al. (1986) (18) for use with primary teeth in order to reduce the rapid decalcification of dental hard tissue. Featherstone's pH-cycling model, commonly employed to induce artificial caries in permanent enamel, may have limitations when applied to primary teeth, which are more vulnerable to acid dissolution (20). The higher vulnerability of primary enamel to demineralization leads to increased lesion progression compared to permanent enamel (20,21). To address these challenges, a pilot

study was conducted to modify the pH conditions, adjusting the demineralized pH from 4.3 to 4.8 and the remineralized pH from 7.0 to 7.2. Additionally, the duration of acid challenges was reduced from 6 hours to 4 hours, and the cycle length shortened from 14 days to 7 days. Consistent with findings by Kargul et al (2012) (16) after primary tooth specimens were processed through 7 days of pH cycling, the microhardness of the enamel surface decreased, as shown in the deionized water group.

The use of both CPP-ACP and theobromine agents resulted in a lesser reduction in enamel hardness, indicating remineralization of artificial caries caused by pH cycling. Corresponding with Syafira et al (2013) and Amaechi et al (2013) (8, 10) studies, theobromine showed a smaller reduction in enamel microhardness compared to CPP-ACP, suggesting a higher remineralization potential. The application of theobromine gel significantly increased microhardness more than CPP-ACP in bovine teeth (3). Furthermore, Amaechi et al (2015) (22) suggested that theobromine enlarges hydroxyapatite crystal size, preventing dissolution and aiding in caries prevention. In contrast, Kargul et al (2012) (23) suggested that theobromine and CPP-ACP had equivalent remineralization effects and increased enamel surface microhardness in human third molar stored in a demineralized solution.

The SEM images of the deionized water group, serving as the control for pH cycling, showed fish scale characteristic as the mineral on the surface and enamel was dissolved, similar to the results from other studies (17,24). This is one of the three basic etching patterns for human enamel after exposure to acid (24). Both theobromine and CPP-ACP showed evidence of remineralization, characterized by the deposition of calcium phosphate on demineralized enamel and the disappearance of fish scale pattern. At higher magnification (x30,000), fine granules of amorphous calcium phosphate were presented in the theobromine group, whereas larger granules were found in CPP-ACP group. Similarly, SEM images from enamel treated with 200 mg/L theobromine solution for 5 minutes showed the highest quantity of globular precipitates on the surface (16).

The result from EDS analysis suggested that Ca and P elements levels in the theobromine-treated enamel were not significantly different from untreated enamel. In contrast, the Ca and P levels in the CPP-ACP treated enamel were significantly lower than in untreated enamel, suggesting that theobromine has higher remineralization potential than CPP-ACP. It possible that the amorphous calcium phosphate precipitation caused by CPP-ACP was dissolved during the demineralization process of pH cycling (25). Similar to the EDS study by Farhad et al (2021) (26), theobromine showed higher calcium deposition than artificial saliva and 0.05% sodium fluoride, while hydroxyapatite crystals produced by CPP-ACP are amorphous and do not generate HA crystals in various sizes and forms (3).

The small theobromine ($C_7H_8N_4O_2$) molecule can penetrate into the microchannels of hydroxyapatite crystals and bind to the crystals by replacing of crystalline ions (26). Its higher electronegativity attracts calcium and phosphate ions, forming of a novel hydroxyapatite crystal known as theobromine apatite [$Ca_{10}(PO_4)_6(OHC_7H_8N_4O_2)$] (26). This may clarify why calcium levels in the theobromine group are higher than those in the other treatment groups. This new apatite increased both crystallite size and crystallinity by the growing hydroxyapatite

in an apatite-forming system with a sufficient quantity of partially alkylated xanthine (10). Additionally, the new crystal apatite formed from theobromine is four times larger (2 μm) than normal hydroxyapatite crystals (0.5 μm) (22). Larger crystal sizes may result in a slower dissolution rate in acid compared to smaller crystal sizes. Smaller crystals require a much greater surface area for reactivity (28).

The application of CPP-ACP induces precipitation of various sizes and forms of amorphous calcium phosphate crystals ($Ca_9(PO_4)_6$) (29) on the enamel surface instead of HA crystal formation (3), which easily breaks down. After exposure to acid, H+ ions break down the amorphous calcium phosphate into calcium and phosphate ions ultimately forming CaHPO4 (30). This corresponds with the EDS analysis results, which showed that the Ca and P levels in the CPP-ACP group were significantly lower than those in the untreated enamel and theobromine groups, and the result from microhardness test of CPP-ACP group showed significant reduction compared to the untreated enamel group though less reduction than theobromine group.

According to a study from Karlinsey et al (2012) (31), an increase in enamel surface porosity is related to a reduction in enamel surface microhardness. Calcium and phosphate ions play an important role in stopping demineralization and promoting remineralization, thereby strengthening the crystal and restoring the microhardness of the structures (32). A naturally extracted substance such as theobromine may be used for remineralization by promoting the formation of larger hydroxyapatite crystals, especially in enamel, and retarding the progression of dental caries.

Theobromine is considered safe for use in young children due to its negligible negative effects and high safety dose response (27). Therefore, it is a suitable option for children who may be more prone to swallow toothpaste or other dental products. A limitation of our study is that the in vitro pH-cycling model does not fully replicate the oral conditions; thus, a clinical trial would be advisable for the future research.

Conclusion

This *in vitro* pH cycling model suggests that theobromine might induce some degree of remineralization by increasing microhardness and the concentration of calcium and phosphate on the surface of demineralized primary enamel. In contrast, CPP-ACP did not show significant changes compared to the deionized water group, which is consistent with the ultrastructural observations from SEM.

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Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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