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ORIGINAL ARTICLE

Fluoridated milk enhances the mineral density of artificial proximal carious lesions *in situ*

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ABSTRACT

Objective: To compare the mineral density (MD) of non-fluoridated-milk (non-F-milk), fluoridated-milk (F-milk), adjunctive to 1000-ppm-fluoride dentifrice (FD), and 1000-ppm-FD alone of proximal artificial enamel carious lesions (AECL) in high caries-risk patients.

Materials and methods: This double-blind, cross-over *in situ* study comprised seven high caries-risk volunteers. Orthodontic brackets with one slab of AECL were fixed randomly to each volunteer per phase. The study comprised three experimental periods with a 7-d wash-out period using FD between sessions; (1) A four-week tooth brushing with FD 2×/day by all subjects as a control. The participants were then randomly allocated to (2) drinking 2.5-ppm-F-milk 1×/day or (3) non-F-milk 1×/day, adjunctive to tooth-brushing with FD for 4-weeks. The subjects crossed over from each type of milk and continued the same protocol for another four weeks. After each phase, the MD of each specimen was analyzed using micro-computed tomography (Micro-CT).

Results: The baseline MD was not significantly differences ($p = .653$). When brushing with FD and drinking F-milk, the MD gain was significantly higher (11.68 ± 2.89%) compared with brushing with FD and drinking non-F-milk (4.59 ± 1.78%) ($p = .003$) or brushing with FD alone (5.30 ± 2.10%) ($p = .003$).

Conclusions: F-milk adjunctive to FD significantly increased MD gain compared with non-F-milk + FD or FD alone.

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KEYWORDS
Fluoridated milk; fluoride; proximal caries; remineralization; micro-CT

Introduction

Dental caries is caused by the interaction of plaque bacteria, sugar, and saliva, resulting in an acidic environment leading to tooth demineralization. When there is no more sugar for the bacteria to metabolize, the saliva plays a role in remineralization. When this balance shifts to demineralization, a white spot lesion with an intact, but porous, layer on the enamel surface occurs [1,2]. The current paradigm for managing caries is to manage early non-cavitated caries lesions using agents that remineralize tooth structure. A study found that remineralization was induced by super-saturating the white spot lesion microenvironment with fluoride. FD efficiently reduces caries [3,4], however, in high-risk patients, additional fluoride is recommended [5–7]. Fluoridated milk has been used to reduce caries in many countries [8–11]. Most of the studies reported caries increment in the cavitated lesions [8–11]. However, the topical effect of F-milk on white spot lesions and the adjunctive effect of F-milk to using FD has not been widely studied.

Many experimental techniques are available to detect changes after or during de- and remineralization. However, there are few techniques that are appropriate for intra-oral *in situ* studies, such as wet chemical analysis, polarized light microscopic analysis, microradiography [12], and Micro-CT [13]. Transverse microradiography (TMR) and polarized light microscopy methods have been used in studies on tooth demineralization or remineralization for decades [14]. However, Micro-CT offers several advantages compared with these methods. Micro-CT is a non-destructive technique, which allows high spatial resolution of the inner tooth structure to be examined [15].

The objective of this study was to use Micro-CT to compare the remineralizing effect of milk and F-milk in adjunct to using 1000-ppm-FD and 1000-ppm-FD alone (standard control) on the mineral density of proximal artificial enamel carious lesions in high caries risk volunteers, *in situ*. We hypothesized that F-milk adjunctive to FD would remineralize enamel early lesions better than FD alone, evaluated by MD and lesion depth changes.

Materials and methods

Ethical approval and subjects

The study protocols were approved by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University,

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No. 070/2017, and the Thai Clinical Trials Registry (TCTR), No. TCTR20180823006.

A sample size calculation based on our pilot study indicated that seven subjects were required to demonstrate an absolute difference of 5% in mean MD change between milk and F-milk with a 90% power of analysis ($\alpha = 0.05$, $\beta = 0.10$). Based on a 10% dropout rate, eight volunteers were recruited into our study.

The subjects comprised eight healthy post-orthodontic volunteers, aged 19–23 years old who were high caries risk as determined by the frequency of their between-meal sugar consumption of >3 times per day [16]. Intraoral examinations determined that each volunteer had at least 22 natural teeth with no current caries activity, periodontal disease, or other oral pathology. Each volunteer had an unstimulated salivary flow rate of ≥0.1 ml/min. The subjects were given verbal and written explanations of the experimental protocol and informed consent was obtained.

Specimen preparation and artificial caries lesion induction

Human premolars extracted for orthodontic reasons were collected from private dental clinics in Bangkok with consent and kept in 0.9% normal saline until used. The inclusion criteria were no white spot lesions, restorations, cracks, or any defects, including hypoplasia, and the proximal surface areas must be at least $4 \times 3\,mm^2$ on each side of the tooth.

The proximal enamel surface of each tooth was polished using a polishing machine (DPS3200, IMPETECH, South Africa) at 100 rpm for 45 s to remove the fluoride-rich zone. Each tooth was painted with acid-resistant nail varnish (Enchanting, Revlon Nail Lacquer, USA), except at an area of enamel $\sim 1 \times 1\,mm^2$ at the proximal contact zone on the mesial and distal surfaces (Figure 1). Artificial caries lesion formation was performed by immersing each specimen in a demineralizing solution ([0.2% (g/100 ml)] polyacrylic acid (Carbopol C907, BF Goodrich, Cleveland, OG, USA) 0.1 M lactic acid (Sigma Aldrich, Saint Louis, MO, USA), and 50% saturated with hydroxyapatite (Sigma Aldrich, Saint Louis, MO, USA) at pH 4.8 and 37 °C) [17] for 7 days and the specimens were then rinsed with deionized water and dried. We prepared longitudinal sections of artificial caries specimens in our pilot study which revealed that the artificial caries lesion formed was ~250 μm deep measured using a polarized light microscope.

Three mesial and/or three distal specimens with artificial carious lesions were obtained from each tooth by sectioning the proximal surfaces into enamel slabs ($1 \times 3 \times 2\,mm^3$) containing a $1 \times 1\,mm^2$ artificial caries lesion window in the middle 1/3 of the slab with a cutting machine (ISOMET 1000™, Buehler, USA). Each of the three slabs was randomly allocated to a baseline phase and two experiment phases and then randomly assigned to a participant. Initial tomographic sample images were obtained using Micro-CT (μCT 35, Scanco, Switzerland) and the baseline mineral density was determined. The specimens were sterilized with ethylene oxide for 12 h and stored in a humidified environment until used.

Intraoral appliance preparation

For the *in situ* study, one specimen per volunteer in each phase was inserted in an orthodontic bracket between the

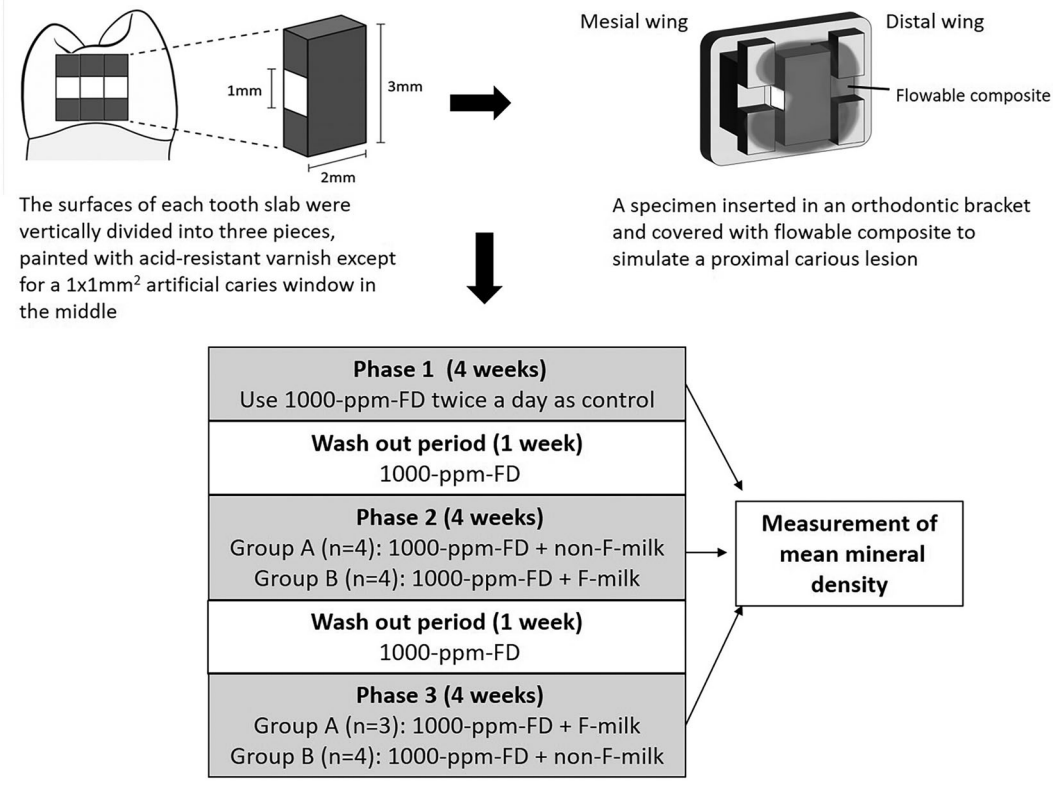

Figure 1. Specimen preparation and diagram of the experimental procedures.

mesial and distal wings of an orthodontic bracket by aligning the specimen window against the mesial wing of the bracket and flowable composite resin was applied between the upper and lower mesial wings to simulate proximal contact (Figure 1). The artificial caries window was fixed at the centre and flowable composite resin was applied (Filtek Flow®, 3MESPE, St. Paul, MN, USA) to cover the whole surface, except for the $1 \times 1\,mm^2$ artificial caries window between the upper and lower mesial wings (Figure 1). The bracket was bonded (Transbond XT®, 3 M Unitek, Monrovia, CA, USA) to the buccal surface of the volunteer's upper permanent molar.

In situ study experimental protocol

This was a randomized, cross-over design, and double-blinded *in situ* study (Figure 1). The study was performed in three four-week phases with a one-week washout period between each phase as follows:

1. 1000-ppm-FD alone (control): Brushing with 1000-ppm-FD (Colgate regular flavour, Colgate-Palmolive Company, Chonburi, Thailand) 2×/day. All volunteers were instructed to use 1 gram of FD in each trained modified Bass technique twice daily (after breakfast and before bedtime). The analytical data of the dentifrice used demonstrated that the total fluoride and total soluble fluoride in 1 gr FD was 1020 ± 20 and 1000 ± 20 ppm, respectively.
2. 1000-ppm-FD + F-milk: The subjects drank 2.5 ppm ultra-heat treated (UHT) F-milk (200 ml) 1×/day (Wangnamyen Dairy Cooperative limited, Sakaeo, Thailand) and brushed with 1000-ppm-FD 2×/day
3. 1000-ppm-FD + non-F-milk: The subjects drank UHT milk (200 ml) 1×/day (Wangnamyen Dairy Cooperative limited, Sakaeo, Thailand) and brushed with 1000-ppm-FD 2×/day

Each volunteer participated in all sessions. After the control phase, the volunteers were then randomly allocated to the (2) 1000-ppm-FD + F-milk or (3) 1000-ppm-FD + non-F-milk intervention for the next four weeks. After the wash-out period, the interventions were switched between the two groups and the experiment continued for another four weeks. The volunteers were blinded to the type of milk. Masking tape was wrapped around the cartons. The volunteers drank the milk after lunch every day. The volunteers were instructed to swish the milk around the oral cavity before swallowing and not to eat, drink, or rinse their mouth after drinking the milk for 30 min.

After each experimental phase, the specimens were removed and their mineral density was measured using Micro-CT. After a one-week wash-out period, a new specimen from the same tooth was attached as before in the next experimental phase until each volunteer had gone through both experimental phases. During the wash-out periods, the volunteers brushed their teeth with 1000-ppm-FD. They were instructed to maintain their normal dietary habits without any other fluoride supplements or products used and were given a diary to record their diet and oral hygiene procedures so they could maintain similar diet habits and oral hygiene practice during each phase. After each experimental phase, the brackets were detached and the specimens were removed from the brackets.

Micro-computed tomography

The MD of each specimen was analyzed using Micro-CT (μCT 35, Scanco Medical AG., Switzerland) before and after each phase, the specimens were scanned and the mineral density was measured every 12 μm through the lesion depth. The same scanning parameters were used for each specimen: 70 kVp, 114 μA, standard resolution (1024×1024 pixels), 180° rotation, 1000 projections, 40 slides, and 400 ms. A 0.5-mm-thick aluminium filter was placed in the beam path to reduce the beam hardening effects. To calibrate the Micro-CT, a series of hydroxyapatite phantom standards with a range of densities were scanned [18].

The MD of each specimen (mg HA/cm^3) was determined using volumetric measurements, and the greyscale value was calculated from the Micro-CT scan images. The MD change was calculated for each specimen, every 12 μm through the lesion depth. To determine the mineral gain at each lesion depth, the mean MD was plotted against the lesion depth. The lesion depth was marked at the depth where the MD was equivalent to 95% of sound enamel. The mean MD gain (MD gain) was calculated by subtracting the pre-treatment area under the curve (AUC) from the post-treatment AUC of each group [18–20]. The MD of the lesion was calculated from the AUC, which is the sum of the trapezium areas (grey colour area). The trapezium area was calculated from the sum of the length of two scanning levels (D1 + D2) divided by 2 and multiplied by the scanning level depth (=12 μm) (Figure 2).

The proportion of the MD gain and the original lesion's mineral density obtained from the equation % Remineralization$=(AUC_{post}-AUC_{pre}/AUC_{pre})\times 100$ was shown as the percent remineralization [19].

Statistical analysis

The difference in baseline MD in each group was analyzed by one-way Analysis of variance (ANOVA). The MD changes and lesion depth after each phase were analyzed by Friedman and Wilcoxon analysis with Bonferroni correction at a 0.05 significance level. The carryover effect was determined with generalized linear models (GLMs analysis) using the MD values from the two experimental phases. All analyses were performed using the SPSS program version 16 (SPSS Inc., Chicago, IL, USA) with a significance level of 0.05.

Results

At the end of the phases, one subject was excluded due to the loss of one subject's orthodontic bracket. Thus, seven

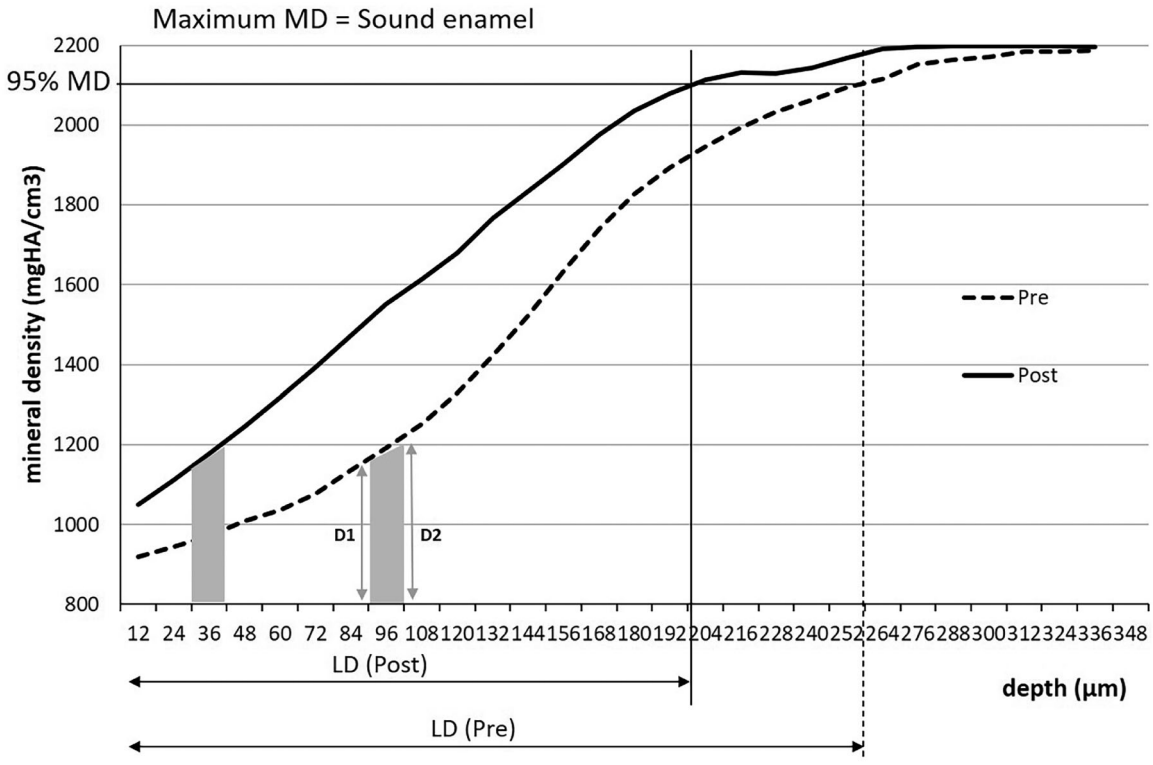

Figure 2. The graph shows the calculation of the mineral density and lesion depth. MD: mineral density; LD: lesion depth.

Table 1. Mineral density/1 µm, mineral gain, % Remineralization, and % increase compared with control.

	Mineral density/1 µm (mg_{HA})				% R	
Treatment	Pre	Post	MD gain (mg_{HA})	*p*-Value		*p*-Value
1000-ppm-FD	1554.75 ± 78.49	1636.86 ± 84.15*	82.11 ± 31.43^{A}	.003	5.30 ± 2.10^{A}	.003
1000-ppm-FD + non-F-milk	1529.07 ± 112.45	1600.67 ± 141.35*	71.60 ± 32.24^{A}		4.59 ± 1.78^{A}	
1000-ppm-FD + F-milk	1495.11 ± 155.65	1669.21 ± 172.81*	174.10 ± 42.82^{B}	.003	11.68 ± 2.89^{B}	.002

MD gain: mineral gain; % R: % remineralization.
Different superscript letters indicate a significant difference ($p < .05$) between the groups in the same column.
*Indicate a significant difference ($p < .05$) between the pre- and post-op mineral density in the same row.

subjects completed all treatment phases and 21 specimens from the seven subjects were analyzed using Micro-CT.

The Micro-CT results demonstrated no significant differences in the baseline MD in the three groups ($p = .653$) (Table 1). Specimen remineralization was represented by the MD gain, which was the difference in MD between the pre- and post-treatment specimens (Table 1). The GLMs analysis indicated that there were no significant carryover effects on the mineral density gain in the two groups ($p = .565$).

The MD gain in the three groups was different ($p = .002$). The FD + F-milk group demonstrated a significantly higher MD gain compared with the FD + non-F-milk group and FD alone ($p = .003$ and $p = .003$, respectively) (Table 1). The % Remineralization (%R) in The FD + F-milk group was significantly higher than those of the FD + non-F-milk ($p = .002$) and FD groups ($p = .003$) (Table 1).

The lesion depth at baseline in the three groups was not different ($p = .723$). The FD + F-milk group had the significantly lowest post-op depth ($p = .010$). The depth changes in the FD + F-milk group presented a significant reduction compared with the FD + non-F-milk and the FD groups ($p = .006$ and $p = .009$, respectively) (Table 2). The % depth change (decrease) in the FD + F-milk group also showed a similar pattern compared with the FD + non-F-milk and the FD alone ($p = .006$ and $p = .009$, respectively). In the FD alone group, the mean MD was higher than its baseline at 12–96 µm (Figure 3), however, those of the FD + non-F-milk and the FD + F-milk groups were higher than their baseline at 72–96 µm (Figure 4) and 36–108 µm (Figure 5), respectively ($p = .018$).

Discussion

The present study demonstrated that the use of F-milk adjunctive to 1000-ppm-FD promoted greater remineralization of proximal artificial caries lesions *in situ* compared with non-F-milk adjunctive to 1000-ppm-FD and 1000-ppm-FD alone. Because our study evaluated interproximal enamel caries, the specimens were obtained from the proximal surfaces of human premolars and used simulated proximal contact to mimic the normal oral condition.

Our study focussed on the high caries risk group. If the volunteers did not use fluoride dentifrice, a greater MD loss or increased lesion depth might have occurred. The fluoride

Table 2. Depth, depth change, and % depth change.

	Depth (microns)						
Treatment	Pre	Post	Depth change (microns)	*p*-Value	% depth change	*p*-Value
1000-ppm-FD	201.61 ± 30.38	201.73 ± 40.97	−0.12 ± 20.47^{A}	.009	0.31 ± 9.74^{A}	.009
1000-ppm-FD + non-F-milk	214.81 ± 34.94	217.17 ± 42.12	−2.37 ± 19.58^{A}		−0.91 ± 9.53^{A}	
1000-ppm-FD + F-milk	223.92 ± 60.20	178.00 ± 37.33*	45.92 ± 32.96^{B}	.006	19.02 ± 9.70^{B}	.006

Different superscript letters indicate a significant difference ($p < .05$) between the groups in the same column.
*Indicate a significant difference ($p < .05$) between the pre- and post-op mineral density in the same row.

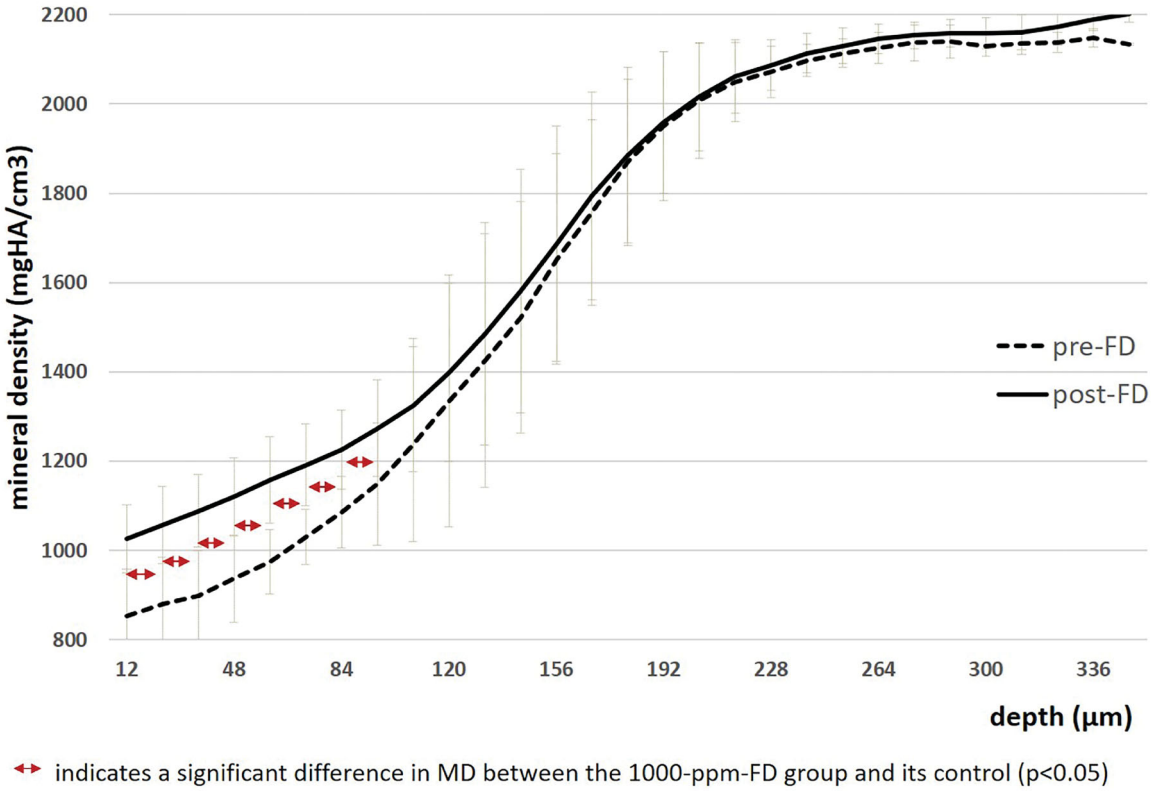

Figure 3. The mineral density profile of the 1000-ppm-FD group. The data is expressed as mean ± *SD*.

in the dentifrice helped remineralize the lesion [21]. We found the MD in the post-treatment groups was significantly higher than baseline (Table 1).

Studies have found that the 2.5 ppm fluoride concentration in milk is the lowest concentration that effectively remineralizes carious lesions [22,23]. Therefore, we decided to use F-milk with a concentration of 2.5 ppm F in our study.

A study on the mode of drinking F-milk found that there were no significant differences in plaque F concentration or salivary F concentration between the children drinking milk directly from the bottle, through a straw with the tip between the lips, or through a straw with the tip deep into their oral cavity with the same F-milk concentration [24]. In our study, volunteers were instructed to use a straw with the tip between their lips and swish the milk around their mouth before swallowing and not to eat drink or rinse with water for 30 min to maximize the contact of the F-milk with the oral hard and soft tissues, which presumably release retained fluoride into the saliva over time [25].

Drinking F-milk resulted in significantly elevated F levels in unstimulated saliva [26]. One study found no significant influence in F concentration in unstimulated whole saliva after rinsing with F-milk, while drinking F-milk resulted in higher fluoride concentration in unstimulated whole saliva [27]. These results indicate that drinking F-milk raised the salivary fluoride level compared with rinsing alone, implying that the salivary F concentration after drinking F-milk is due to topical and systemic sources.

Although TMR is considered the optimum technique for the determination of MD *in vitro*, the samples are destroyed during processing, which is incompatible with evaluating changes that occur in a sample over time and is time-consuming [28,29]. Hamba et al. has validated Micro-CT measurement of enamel lesions compared with TMR under various X-ray conditions. Lesion depth (µm) and mineral loss (vol% µm) were used to compare these two methods. A Micro-CT analysis similar to our study showed Pearson's correlation of $r > 0.86$ between the measurement of Micro-CT

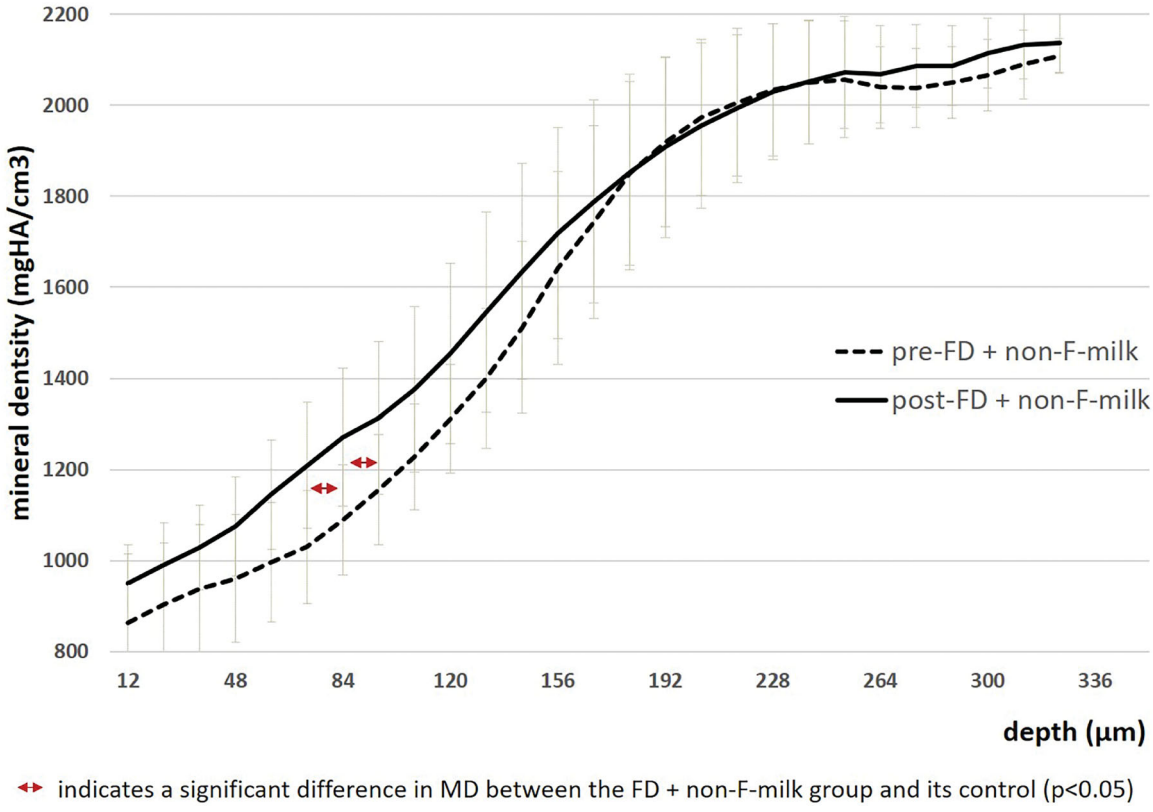

Figure 4. The mineral density profile of the FD + non-F-milk group. The data is expressed as mean ± *SD*.

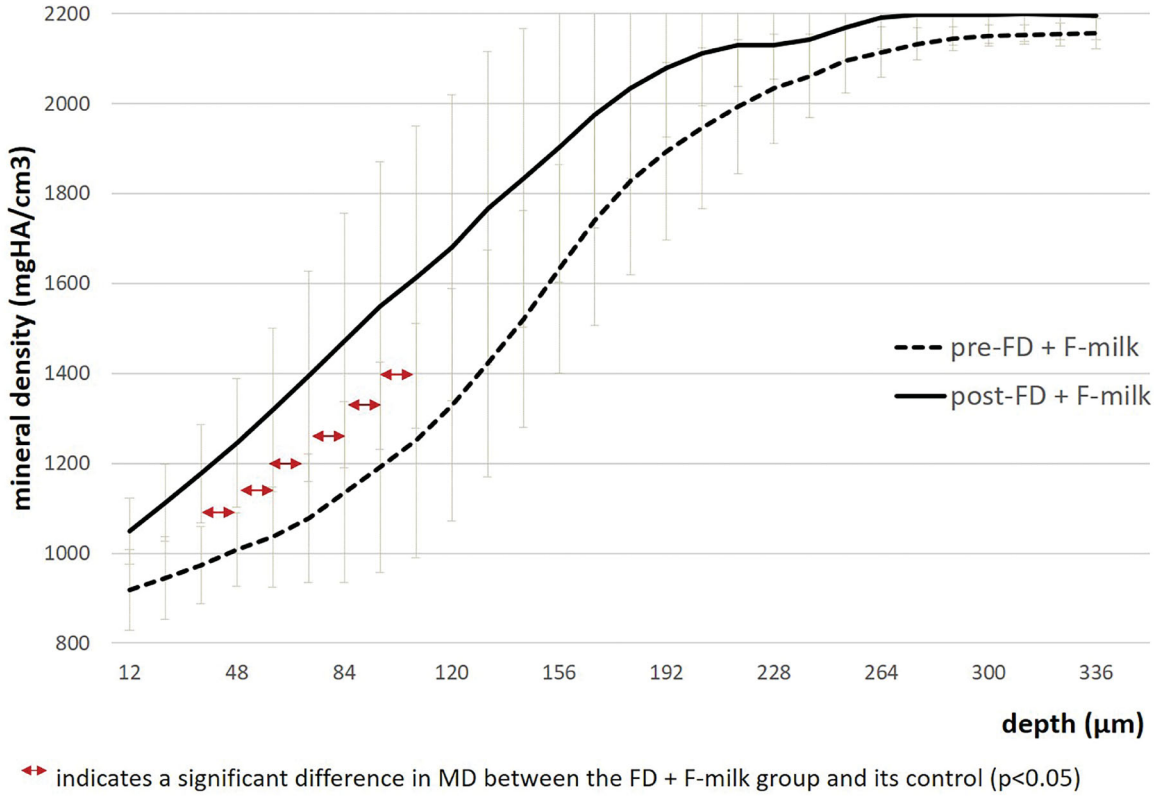

Figure 5. The mineral density profile of the FD + F-milk group. The data is expressed as mean ± *SD*.

and TMR ($p < .001$) [30]. Therefore, Micro-CT can be an alternative technique to TMR for the study of enamel caries lesions.

Notably, the pattern of the significant MD increase in the three groups was different. In the FD group, the MD increase was in the superficial lesion (12–96 μm), similar to a previous

study [31]. This was described as preferential surface layer precipitation [32]. In the FD + F-milk group, the significantly higher MD was at the deeper levels (36–108 μm), presumably due to the additional exposure of fluoride, in combination with the calcium and phosphate in milk that promotes remineralization at a deeper lesion level. In contrast, a significant MD increase in the FD + non-F-milk group was found in a narrow range (72–96 μm). Several studies have reported the role of milk in preventing caries [22,23], suggesting that calcium and phosphate ions increase remineralization. In contrast, demineralization can be caused by increased bacterial metabolism due to milk lactose [33]. This could be the reason for the lowest MD increase found in the milk group.

An *in situ* study showed that F-milk was not effective in significantly reducing enamel and dentine caries, which is different from the present study. The main reason might be that the effect of fluoride was evaluated for only a short time. This study was conducted for only 5 days, and remineralization was not observed [34]. Another study reported no additional effect of F-milk on enamel caries lesion remineralization [35]. However, this *in situ* study was performed using elderly subjects who wore full dentures with small enamel blocks embedded. After experiments were finished, enamel blocks were removed from the denture. The mineral loss and the lesion depth were evaluated by TMR. The difference in our study was from the method that the milk was swished around the mouth before swallowing and the instructions not to eat or drink or rinse for 30 min. Due to the large area of soft tissue covered by the denture, the area left for fluoride reservoir was less in their study, thus, the action of fluoride release over time might not be distinctly observed. Reduced resting and stimulated saliva flow has been reported in healthy elderly people (independent of drug intake or dental status) compared with young adults [36,37]. This might be the reason why the FD + F-milk group had the highest MD gain in our study. The authors stated that the main mechanism of their results was from FD, while our study demonstrated an additional effect of F-milk on FD.

The present study found that the MD gain from FD adjunctive to drinking F-milk was ~2-fold of those found when using FD alone or with additional drinking plain milk. Our F-milk was drunk for 4 weeks, therefore, the change in MD revealed by Micro-CT might not be observed clinically. If the exposure time of F-milk is longer, the activity of the lesion might be halted and the lesion could be converted to an inactive condition in which restoration may not be needed. This would reduce the cost to the parents, third-party payers or state funding, and/or other intangible expenses.

In summary, we found that in patients with artificial incipient caries, drinking F-milk in adjunct to FD increased incipient lesion remineralization more than the use of the FD alone and drinking plain milk. Based on our findings, F-milk might be a good vehicle to deliver adjunctive fluoride to high caries rate school adolescents, especially those to whom milk is being distributed by a publicly funded program where the primary source of drinking water is fluoride deficient. The 2.5 ppm fluoride in milk used in our study is equivalent to 0.5 mg fluoride supplement per day as recommended by the American Dental Association for children 3–6 years- and 6–16 years-old whose drinking water has <0.3 ppm and between 0.3 and 0.6 ppm, respectively [38]. Thus, the 2.5 ppm F in 200 ml milk should not be a safety concern.

Acknowledgements

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Disclosure statement

The milk and F-milk were supplied by Wangnamyen Dairy Cooperative limited, Sakaeo, Thailand. There is no conflict of interest.

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