Abstract: This in vitro study aimed to investigate the efficacy of tannin-fluoride and milk-fluoride mixtures on human enamel erosion after exposure to inappropriately chlorinated pool water. Enamel specimens were immersed in swimming pool water (pH 2.7) for 30 min and in each test reagent for 4 min once a day for 60 consecutive days (group I: control, group II: tannin-fluoride, group III: milk-fluoride, group IV: tannin-fluoride before and milk-fluoride after erosive challenge, and group V: milk containing tannin-fluoride before and after erosive exposure). Surface microhardness was assessed on days 0, 30, and 60. Scanning electron microscopy (SEM) and electron probe microanalysis (EPMA) were performed after treatment of samples for 60 days. Surface microhardness of experimental groups was ranked as follows: group III > group IV-group V > group II > group I (P < 0.05). Moreover, SEM images revealed deposition of substances on erosive enamel surface after treatment with tannin-fluoride and milk-fluoride mixtures. Furthermore, EPMA profiles showed decrease of phosphorus and increase of fluoride content in groups II and IV. In conclusion, we demonstrated that treatment with fluoridated milk with or without tannin-fluoride has protective effects against enamel erosion caused by low-pH swimming pool water.

Keywords: enamel erosion; tannin-fluoride; milk-fluoride; inappropriately chlorinated pool; in vitro.

Introduction

Dental erosion or tooth wear has recently gained more attention since the decline in caries prevalence and increase in tooth longevity. Dental erosion is defined as dissolution of tooth caused by chemical attack that does not involve bacteria (1). Over the past decade, there is growing awareness in prevalence and severity of dental erosion. The etiological factors of dental erosion can be divided into intrinsic (e.g., gastric acids from regurgitation and reflux disorders) and extrinsic (e.g., dietary acids and occupational acid exposure) factors (2). Most important sources of extrinsic factors are primarily from dietary acids. A strong correlation was proposed between dental erosion prevalence and consumption of acidic food and drinks (3). In addition to consumption of acidic foods, swimming in low-pH chlorinated pools has been associated with rapid and severe dental erosion (4). Swimming pools are usually chlorinated to reduce microbial contamination. After chlorine treatment, the excess acidity is neutralized and buffered to maintain recommended pool pH (pH 7.2-7.8) (World Health Organization. Guidelines for safe recreational water environments, 2006). However, in inappropriately maintained swimming pools, prolonged exposure to pool water can be a potential cause of dental erosion (5).

Several approaches have been used to prevent dental erosion. Preventive measures for dental erosion include reduction of acid exposure and enhancement of re-mineralization. To enhance re-mineralization, application of
re-mineralizing agents such as fluoride or nonfluorinated agents is recommended (2,6). High-concentrated fluoride agents have been shown to decrease enamel erosion in vitro and in situ (7-9). The antierosive effect of fluoride is primarily attributed to the formation of calcium fluoride (CaF$_2$)-like material on the dental surfaces (2,10). While fluoride has long been known to have anticariogenic effects, its antierosive potential has remained controversial. An in vitro study revealed that high-concentration fluoride applied before exposure to an acidic beverage can inhibit initial erosion of human enamel (7). Moreover, toothpaste fluoridation has been shown to decrease the development of erosion in situ (11,12). In contrast, several studies have demonstrated that fluorides cannot prevent dental erosion (13,14).

In addition to fluoride application, other nonfluorinated agents have been reported as potential alternatives to reduce dental erosion. Organic components of tea such as tannic acid and catechin enhance the acid resistance of enamel (15). Furthermore, mixture of tannic acid and fluoride forms a coating layer, which protects the enamel from acid attack (16). Modification of acidic beverages by adding calcium and milk has been demonstrated to decrease the erosive potential of acidic drinks (17,18). Milk-derived proteins such as casein phosphopeptides (CPPs) exhibit anticariogenic and re-mineralizing potential (19). CPPs can be complexed with calcium and phosphate to casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), which acts as a vehicle for bioavailable calcium and phosphate ions. CPP-ACP or CPP with the addition of fluoride has been shown to reduce dental erosion (20,21). However, the effect of milk-fluoride mixture and a combination of tannin-fluoride and milk-fluoride on human enamel erosion has not yet been reported. This in vitro study aimed to investigate the combined effects of fluoride, tannic acid, and milk on human enamel erosion caused by low-pH chlorinated pool water.

Molar crowns were cut into four portions—mesial, distal, buccal, and lingual—by using a water-cooled diamond disk. The specimens were inspected under a stereomicroscope to exclude those with observable defects from the study. Each portion was then embedded in acrylic resin (Meliodent, Bayer Dental, Leverkusen, Germany) in planar parallel molds. The outer surface of enamel samples was ground under constant tap water cooling by using a rotating polishing machine (Jean Wirtz Phoenix 4000, Jean Wirtz GmbH & Co. KG, Dusseldorf, Germany) with silicon carbide papers of 600, 1,200, 1,500, and 2,000 grit. The specimens were ground to obtain a window of approximately 2 × 2 mm of enamel surface and stored in normal saline solution at room temperature for later use (22).

**Test solutions**
The erosive agent was collected from an inappropriately chlorinated swimming pool water (pH 2.7), and the pH was measured daily before use. Tannic acid (Sigma-Aldrich, St. Louis, MO, USA) and sodium fluoride (Merck Ltd., Bangkok, Thailand) were used to prepare the test solutions. Free fluoride ion concentration used in this study was roughly equivalent to the concentration of fluoride in tooth pastes. The test solutions were as follows: i) tannin-fluoride mixture (TF) contains 1% (w/v) tannic acid and 10,000 ppm F (pH 6.0); ii) milk-fluoride mixture (MF) contains 1,100 ppm F in pasteurized milk (Meiji, Bangkok, Thailand); and iii) tannin-fluoride in milk (TFM) contains 1% (w/v) tannic acid and 1,100 ppm F in pasteurized milk (pH 7.0).

**Cycles of de- and re-mineralization**
The duration of exposure to swimming pool water (exposure time) and the duration of immersion in the treatment solution (immersion time) were arbitrarily set. According to personal interviews, we found that most swimmers swim at least 30 min per workout. Hence, the exposure time was set to 30 min. The immersion time would be roughly equivalent to the time used for 1.23% acidulated phosphate fluoride gel application. Each aforementioned portion of a tooth specimen was randomly distributed into 5 groups (n = 14 for surface microhardness measurement and n = 3 for EPMA analysis) designated as follows (Table 1); group I (Ero): exposure to swimming pool water (Ero) (pH 2.7) for 30 min; group II (TF/Ero/TF): exposure to TF for 4 min before and after exposure to swimming pool water for 30 min; group III (MF/Ero/MF): exposure to MF for 4 min before and after exposure to swimming pool water for 30 min; group IV (TF/Ero/MF): exposure to TF for 4 min before exposure to swim-

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**Materials and Methods**

**Specimen preparation**
Noncarious human third molars and premolars were collected from the Dental Hospital, Faculty of Dentistry, Prince of Songkla University, Songkhla, Thailand. Tooth collection was approved by the Ethics Committee (MOE 0521.2.03/863), and written informed consent was obtained from all donors. Teeth were stored in 0.1% thymol in phosphate-buffered saline. Premolar crowns were used for electron probe microanalysis (EPMA), and molar crowns were used for surface microhardness measurement and scanning electron microscopy (SEM).
ming pool water for 30 min; the specimens were then exposed to MF for 4 min; and group V (TFM/Ero/TFM): exposure to milk containing tannin-fluoride (TFM) for 4 min before and after exposure to swimming pool water for 30 min.

All processes were performed once a day at room temperature, approximately 25ºC, for a total of 60 days. Between exposures, samples were stored in artificial saliva (23) (1.1 mM CaCl$_2$.2H$_2$O, 4.59 mM K$_2$HPO$_4$, 2.39 mM KH$_2$PO$_4$, 8.38 mM KCl, 0.29 mM MgCl$_2$.6H$_2$O, 6.57 mM methyl-p-hydroxybenzoate, and 10 g/L ultra-low viscosity sodium carboxymethylcellulose [CMC], pH 7). The reagents used and the artificial saliva were changed daily. At the end of the cycling period, all specimens were washed with deionized water and blot-dried.

**Surface microhardness measurement**

Surface microhardness was measured using a microhardness tester (Buehler Micromet II, Buehler Ltd., Lake Bluff, IL, USA) with a Vickers diamond head at a load of 200 g for 10 s (22,24). Four well-formed indentations on each specimen were averaged to obtain mean microhardness. The measurement was made after polishing (as baseline) and then after exposure to each treatment on days 30 and 60.

**Scanning electron microscopy**

SEM analysis was performed on day 60 of the immersion cycles. Specimens were mounted and sputter coated with gold in an SPI module sputter coater (Structure Probe, Inc., West Chester, PA, USA). The specimens were examined and photographed using a JEOL JSM-5800LV scanning electron microscope (JOEL Ltd., Tokyo, Japan) operating at 20 kV.

**Electron probe microanalysis**

After exposure to cycles of de- and re-mineralization on day 60, crowns of premolars were separated from roots and buccolingually sectioned by using a water-cooled diamond saw (IsoMet 4000, Buehler Ltd.). Specimens were then embedded in acrylic resin blocks and ground flat by using silicon carbide paper. Subsequently, the specimens were carbon coated and scanned to measure relative percentage weights of calcium, phosphorus, and fluoride. Scans were conducted at 2-μm interval over a total distance of 100 μm from the enamel-resin interface with an electron probe microanalyzer (JXA-8100, JOEL Ltd.), at an acceleration voltage of 15 kV and a beam current of 20 nA.

**Statistical analysis**

Normality and homogeneity of the samples were tested using the Kolmogorov-Smirnov test. The mean surface microhardness at a given time point among groups was compared using one-way analysis of variance. The Tukey’s honestly significant differences (HSD) multiple comparison test was used to compare the intergroup difference. In all tests, the level of significance was set as $P = 0.05$.

**Results**

**Surface microhardness**

The mean initial enamel hardness measured at baseline ranged from 329.09 ± 16.62 to 339.09 ± 14.70 Vickers units (Table 2). Surface microhardness in groups II (TF/Ero/TF), IV (TF/Ero/MF), and V (TFM/Ero/TFM)
progressively decreased over time and was significantly decreased at 60 days in comparison with measurements at baseline and 30 days ($P < 0.05$). However, this hardness in groups I (Ero) and III (MF/Ero/MF) decreased significantly up to 30 days of treatment ($P < 0.05$) and then remained unchanged.

On comparing differences in surface microhardness between groups after 30 days of treatments by using Tukey HSD multiple comparison, no significant differences were noted between groups III, IV, and V ($P > 0.05$) but groups I and II revealed significant differences in microhardness from other groups ($P < 0.05$). Surface microhardness after 30 days of treatment was ranked and grouped from the highest to lowest as follows: III > IV, V > II > I ($P < 0.05$).

**Electron probe microanalysis**

EPMA profiles of mineral content in the enamel surface are presented in Figs. 1-3. After 60 cycles, group II (TF/Ero/TF) showed slightly decreased phosphorus concentrations (Fig. 1) and a remarkable elevation of fluoride profile (approximately 5 µm from the outer surface; Fig. 2). Treatment with tannin-fluoride mixture before and fluoridated milk after the erosive challenge (group IV, TF/Ero/MF) resulted in marked reduction of phosphorus levels and slight increase in fluoride concentrations up to 100 µm from the outer surface (Figs. 1, 2). No notable differences were found in calcium profiles of all groups (Fig. 3).

**Scanning electron microscope**

Panels of SEM micrographs of enamel specimens treated with various test solutions are shown in Figs. 4 and 5. SEM images revealed structural loss and microcracking after exposure to erosive challenge (Fig. 4B). Irregular shapes of deposits and microcracks were found on enamel surfaces treated with tannin-fluoride before and after exposure to pool water (group III: TF/Ero/TF, Fig. 4C). Surfaces treated with MF mixture before and after erosive exposure revealed precipitates with a globular appearance (Fig. 4D). In addition, such precipitates were found in groups IV (TF/Ero/MF, Fig. 4E) and V (TFM/Ero/TFM, Fig. 4F) with more irregular-shaped deposits. In all experimental groups, SEM micrographs of longitudinal sections showed deposit layers with varied thickness on the enamel surface (Fig. 5). The deposit layer in group III (MF/Ero/MF, Fig. 5D) was approximately 8-µm thick, whereas thinner deposits (ranging 1-4 µm) were found in other groups (Fig. 5 C, E, F).
Discussion

The present study aimed to examine the in vitro antierosive effects of tannin-fluoride and fluoridated milk on enamel exposed to inappropriately chlorinated swimming pool water. In recent years, extensive research has been conducted on antierosive agents, including fluoride, in various formulations (6,25,26). Fluoride has been successfully used for caries control. CaF₂, or CaF₂-like materials, precipitate on tooth surfaces when compounds containing fluorides are applied, which might serve as a local reservoir of fluoride and induce re-mineralization (27,28). However, the influence of CaF₂ on prevention of dental erosion has not been conclusively demonstrated. Unlike incipient caries lesions, enamel erosion occurs on the surface. Therefore, fluoride primarily acts to re-harden the softened enamel. In order to protect the tooth surface completely, it has been suggested that the CaF₂-like layer should be dense enough to build up a physical barrier, which protects the underlying enamel from acid attack, and it should be sufficiently stable against erosive dissolution (6). Although several studies have shown that application of high-concentrated fluoride can increase acid resistance of human enamel (7, 29, 30), a study demonstrated that fluoride does not provide protective effects against erosion from carbonated soft drinks (13).

In the present study, we demonstrated that inappropriately chlorinated pool water causes dental erosion. On immersion in pool water, enamel hardness was markedly decreased after 30-day cycles but remained unchanged thereafter. It is known that the amount of mineral loss caused by erosion depends on pH, type of acid, buffering capacity, and length of exposure time. In this study, enamel hardness after immersion in pool water (group I) on day 60 was not significantly different from that on day.
30. In contrast, our previous study reported significant differences in enamel hardness between days 30 and 60 when enamel was immersed in orange juice (pH 3.74) (22). It is possible that the type of acid used in this investigation did not have additive effects with longer exposure time.

Treatment of fluoridated milk and tannin-fluoride promotes acid resistance in human enamel after in vitro exposure to low-pH swimming pool water. However, treatment with fluoridated milk or combination of tannin-fluoride and milk-fluoride provided additional protective effects against enamel erosion than did tannin-fluoride alone. After 60 treatment cycles, enamel treated with fluoridated milk both before and after exposed to low-pH swimming pool water showed thickest deposit layers on the surface and greatest surface microhardness compared with all other groups. Moreover, after 30 days, microhardness of this group remained unchanged. A possible explanation is that fluoridated milk contains high amount of calcium, phosphorus, and fluoride, which enhances re-mineralization of softened enamel; this finding is consistent with a previous report, wherein a combination of calcium, phosphate, and fluoride exhibited significantly greater protective potential than calcium, phosphate, or fluoride alone (31). Furthermore, other components in milk such as phosphoprotein/phosphopeptide-stabilized calcium phosphates possess re-mineralizing potential by providing calcium and phosphate (32). Casein in milk may reduce hydroxyapatite dissolution by binding to the hydroxyapatite surface and forming a thin layer; this thin layer was proposed to function as a diffusion barrier, which restricted H+ access to the tooth surface and prevented further de-mineralization of enamel (33).

Several studies have demonstrated the antierosive potential of tannin (34,35). It is possible that tannin inhibits de-mineralization through interaction with the organic matrix of enamel. Changes in organic matrix network in the inter- and intra-prismatic spaces could modify the diffusion pathway inside the tooth structures, thereby affecting the process of enamel de-mineralization (36,37). Our findings are consistent with a previous study wherein tannin-fluoride was reported to promote enamel re-mineralization (16). Another study reported inhibitory effects of tannin-fluoride on enamel de-mineralization (15). A possible mechanism is that tannin may bridge bonds between calcium phosphorus and other organic substances, resulting in the formation of insoluble compounds on the enamel surface. Unlike CaF2, insoluble substances are highly resistant to acid attack and water washing (16). Even though tannin can inhibit enamel erosion, prolonged exposure of solutions containing tannin cause tooth discoloration. In the present study, in comparison with initial hardness, distinct loss of enamel hardness were observed in all groups. The acid-induced enamel mineral loss that occurs during the erosion process is much greater than that caused by the caries process. This means that the anti-de-mineralization mechanisms of fluoride, tannin, and/or milk proteins are unlikely to function as efficiently as they do during caries pathogenesis.

Analysis of mineral content revealed considerable elevation of fluoride adjacent to the outer surface in group II (TF/Ero/TF). This could probably be attributed to the high concentration of fluoride (10,000 ppm) used in this group. The highest fluoride content was observed in the deposit layer, which was approximately 5-μm thick. As mentioned earlier, tannic acid could function as a bridge between calcium, phosphate, and fluoride, creating a deposit layer on the surface. Tannic acid could have maintained fluoride concentrations in this layer as well. In addition to group II, slight increase in fluoride concentrations was found within 100 μm of the outermost layer in group IV (TF/Ero/MF). Interestingly, the increase in fluoride concentrations was associated with reduction of phosphorus level. We speculated that tannin might have interacted with phosphorus and form a tannin-phosphorus complex (38). A previous study demonstrated formation of phosphate-containing CaF2 on enamel treated with acidified solution with high concentration fluoride (10). The globular precipitate was called CaF2-like material because it was contaminated with phosphate and its morphology was different from pure CaF2. It is likely that CaF2-like deposits were formed on the surface enamel of group IV, as observed in SEM micrographs.

In conclusion, we demonstrated that treatment with fluoridated milk alone and combination of fluoridated milk and tannin-fluoride provides protective effects against enamel erosion caused by low-pH swimming pool water. However, application of fluorinated milk both before and after erosive exposure provides the greatest protective effects. Additional in situ studies are warranted to determine whether application of fluoridated milk or tannin-fluoride is effective in reducing dental erosion.

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Conflict of interest
The authors have no potential conflict of interest to declare with respect to the authorship and/or publication of this article.

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