Evaluating the effects of remineralizing agents on initial carious lesions

Suwimon Jettanacheawchankit*, Tuksaporn Pongpradit*, Amornrat Euapokai*, Auchchara Eiamsakul*, Kritsa Wongmoon*, Kornchanok Wayakanon§

PRACTICAL IMPLICATIONS OF THIS RESEARCH

- Initial caries are areas of demineralized tooth enamel where physical characteristics, chemical composition, and mechanical properties have been altered, leading to defectiveness.
- Early detection and clinical management are vital to preserving the natural tooth structure; remineralizing agents are promoted as useful tools.
- This study provides oral health professionals with data on the effectiveness of remineralizing agents, contributing to informed decisions for patient care.

ABSTRACT

Background: Initial carious lesions are reversible, and fluoride-containing reagents effectively promote enamel remineralization in these initial lesions. Numerous remineralizing agents are available, some containing fluoride and others containing alternatives because of fluoride toxicity concerns. The objective of this study was to investigate the effects of the following remineralizing agents: fluoride varnish (5% NaF, Duraphat®), casein phosphopeptide–amorphous calcium phosphate paste (CPP-ACP, Tooth Mousse®), and casein phosphopeptide–amorphous calcium phosphate fluoride paste (CPP-ACPF, Tooth Mousse Plus®) on initial carious lesions. **Materials and Methods:** Forty human maxillary premolar teeth were randomized into 4 experimental groups: distilled water, fluoride varnish, CPP-ACP, and CPP-ACPF. All 40 teeth were cut in half mesiodistally. A Vickers microhardness test was performed on the buccal half of each tooth; the lingual half was examined with a spectrophotometer for colour dimension. An Atomic Force Microscope was used to evaluate roughness. During the experiments, microhardness, colour, and roughness were examined 3 times: 1) before treatment; 2) after demineralization; and 3) after remineralization. At each stage, a scanning electron microscope was used to examine surface features and enable energy dispersive X-ray chemical composition analysis. **Results:** Demineralization carried out in preparation for testing of the agents caused enamel hardness to decrease significantly. Enamel roughness increased, although not significantly. After remineralization, each of the 3 remineralizing agents had significantly increased both hardness and lightness (L*), compared to the demineralized enamel. Each agent decreased enamel roughness, but the roughness change was not significant. **Conclusion:** Comparing the 3 remineralizing agents, there were no significant differences between the agents in effectiveness.

RÉSUMÉ

Contexte : Les lésions carieuses initiales sont réversibles et les réactifs qui contiennent du fluorure favorisent efficacement la reminéralisation de l'émail dans ces lésions initiales. Il existe de nombreux agents reminéralisants, dont certains contiennent du fluorure et d'autres contiennent des solutions de remplacement en raison de préoccupations liées à la toxicité du fluorure. L'objectif de cette étude était d'examiner les effets des agents reminéralisants suivants : vernis fluoré (5 % NaF, Duraphat®), pâte de phosphate de calcium amorphe et de phosphopeptide de caséine (CPP-ACP, Tooth Mousse®), et pâte de fluorure de phosphate de calcium amorphe et de phosphopeptide de caséine (CPP-ACPF, Tooth Mousse Plus®) sur les lésions carieuses initiales. **Matériaux et méthodes :** Quarante prémolaires maxillaires humaines ont été randomisées en 4 groupes expérimentaux : eau distillée, vernis fluoré, CPP-ACP et CPP-ACPF. Les 40 dents ont été coupées en 2 dans le sens mésiodistal. Un test de microdureté Vickers a été effectué sur la moitié buccale de chaque dent; la moitié linguale a été examinée à l'aide d'un spectrophotomètre pour déterminer la dimension de la couleur. La rugosité a été évaluée à l'aide d'un microscope à force atomique. Au cours des expériences, la microdureté, la couleur et la rugosité ont été examinées 3 fois : 1) avant le traitement; 2) après la déminéralisation; et 3) après la reminéralisation. À chaque étape, un microscope électronique à balayage a été utilisé pour examiner les caractéristiques de la surface et permettre l'analyse de la composition chimique par rayons X à dispersion d'énergie. **Résultats** : La déminéralisation effectuée en préparation des tests sur les agents a entraîné une diminution significative de la dureté de l'émail. L'émail est devenu plus rugueux, mais pas de manière significative. À la suite de la reminéralisation, chacun des 3 agents reminéralisants a augmenté de manière significative la dureté et la luminosité (L*), par rapport à l'émail déminéralisé. Chaque agent a réduit la rugosité de l'émail, mais le changement de rugosité s'est avéré non significatif. **Conclusion :** Si l'on compare les 3 agents reminéralisants, il n'y avait aucune différence remarquable entre les agents en matière d'efficacité.

Keywords: colour; CPP-ACPF; fluoride varnish; hardness; remineralizing agent; roughness
CDHA Research Agenda category: access to care and unmet needs

*Department of Oral Biology, Faculty of Dentistry, Naresuan University, Phitsanulok, Thailand
§Department of Restorative Dentistry, Faculty of Dentistry, Naresuan University, Phitsanulok, Thailand

Correspondence: Dr. Kornchanok Wayakanon; Kornchanokw@nu.ac.th
Manuscript submitted 5 June 2024; revised 5 September 2024; accepted 20 September 2024

BACKGROUND

Dental caries is the most prevalent oral health problem throughout the world. Dental caries develops when inorganic components of the tooth undergo a demineralization process caused by acidic products from bacterial fermentation of food residue. This process of demineralization is reversible if treated in its early stages, before the alteration of the tooth's physiology causes an actual cavity.

The natural composition of teeth is unique. No restorative material can truly replicate the optimal properties of either enamel or dentin. Therefore, the best response to initial dental caries is to preserve the natural tooth structure as much as possible.[1] To this end, early detection of caries and promotion of remineralization at the carious lesion are invaluable oral health care practices.

Fluoride has been widely accepted as an effective remineralizing agent. Fluoride facilitates the binding of phosphate and calcium ions from saliva and demineralized enamel, which together precipitate as either hydroxyfluorapatite or fluorapatite onto the surface of the demineralized lesions.[2] Both hydroxyfluorapatite and fluorapatite are more resistant to the acidic products of bacterial fermentation compared with hydroxyapatite, the tooth's natural component from which they are converted.[3] In addition to that benefit, fluoride also inhibits the carbohydrate metabolism of the acidogenic bacteria by inhibiting the function of enolase. Fluoride is taken up into the cytoplasm of the bacteria in the protonated form (HF), after which the HF dissociates into H^+ and F^-, and this results in the reduction of enolase activity.[4]

However, the toxicity of fluoride has been an ongoing concern, especially with frequent application. In response, manufacturers have introduced several alternative remineralizing products intended to compete with the effectiveness of fluoride, without fluoride's risk of toxicity, although for a higher purchase price. Among these non-fluoride products, the predominant one in the market has been casein phosphopeptide–amorphous calcium phosphate (CPP-ACP). To date, however, there have been no clear studies of the effectiveness of these alternative products or their effectiveness compared to ordinary fluoride in the physical properties, particularly the colour of remineralized initial carious lesions.

The purpose of this study is to investigate the effects of 3 of these remineralizing agents on initial carious lesions. The 3 remineralizing agents are fluoride varnish (5% sodium fluoride [NaF], Duraphat®), casein phosphopeptide–amorphous calcium phosphate paste (CPP-ACP, Tooth Mousse®), and casein phosphopeptide–amorphous calcium phosphate fluoride paste (CPP-ACPF, Tooth Mousse Plus®). The effects of these 3 products on the hardness, colour, roughness, surface characteristics, and chemical composition of artificially demineralized enamels will be determined. The null hypothesis is that none of the 3 remineralizing agents has a greater or lesser effect on enamel properties than the others.

MATERIALS AND METHODS

This study was approved by the Naresuan University Ethics Committee (Approval No. 684/62).

Investigating the effects of remineralizing agents on hardness, colour, and roughness

Forty sound human maxillary premolar teeth (without cracks, restorations or carious lesions) that had been extracted for orthodontic treatment within the previous 3 months and stored in 0.1% thymol solution at room temperature were selected for this study. The 40 teeth were randomly divided into 4 experimental groups (n = 10), one group for each remineralizing agent: fluoride varnish (Duraphat® Varnish, Colgate®, New Zealand); casein phosphopeptide–amorphous calcium phosphate (CPP-ACP) (GC Tooth Mousse®, GC Corporation, Japan); casein phosphopeptide–amorphous calcium phosphate fluoride (CPP-ACPF) (GC Tooth Mousse Plus®, GC Corporation, Japan); and distilled water (control). The ingredients of the 3 remineralizing agents are shown in Table 1.

Each tooth was cut in half in the mesiodistal direction, to separate the buccal half and lingual half. Each piece was mounted onto self-curing acrylic resin, with the enamel surface facing upward, parallel to the horizontal plane. The buccal pieces were used to determine colour properties with a spectrophotometer (VITA Easyshade® V, Bad Säckingen, Germany) and to evaluate roughness with an Atomic Force Microscope (AFM) (Flex-Axiom, Nanosurf, Liestal, Switzerland). The length of the AFM probes (Tab150Al-G, Budgetsensors, Sofia, Bulgaria) is 17 µm, and their radius is less than 10 nm. Enamel roughness was investigated in a sample area of 50 x 50 μm^2. The lingual pieces were used to measure hardness with the Vickers microhardness test (Zwick ZHVµ, West Midlands,

Table 1. Ingredients of the remineralizing agents

Remineralizing agent	Ingredients
Fluoride varnish	5% sodium fluoride, colophonium, ethanol
CPP-ACP	CPP-ACP, D-sorbitol, propylene glycol, silicon dioxide, titanium dioxide, xylitol, phosphoric acid, zinc oxide, sodium saccharin, ethyl p-hydroxybenzoate, magnesium oxide, guar gum, propyl p-hydroxybenzoate, butyl p-hydroxybenzoate, glycerol, water
CPP-ACPF	0.2% sodium fluoride, CPP-ACP, D-sorbitol, propylene glycol, silicon dioxide, titanium dioxide, xylitol, phosphoric acid, ethyl p-hydroxybenzoate, propyl p-hydroxybenzoate, butyl p-hydroxybenzoate, glycerol, water

United Kingdom). Each tooth piece was examined at 3 stages: initial, demineralized, and remineralized. The initial stage examination occurred right after the piece was mounted onto the acrylic resin. The demineralized stage examination happened after the piece had been submerged in demineralizing solution (0.05 M acetic acid, 2.2 mM $CaCl_2$, 2.2 mM KH_2PO_4, and 1 M KOH with pH 4.4) at 37°C for 3 days, with used solution replaced by fresh solution every 24 hours. Before this second examination, the enamel surfaces were washed with de-ionized water. The remineralized stage examination took place after the remineralizing agent had been applied to the specimens for 3 days. These agents were also replaced every 24 hours, and the enamel surfaces were once again washed with de-ionized water before this third examination.

The Vickers microhardness test was performed by applying a 135° square-based diamond indenter. Three separate indentations were made, each with a load of 200 g for 10 s. Colour measurements were obtained using a spectrophotometer, which determined lightness (L*), redness (a*), and yellowness (b*) at all 3 examination stages. The white index (w) was calculated using the following formula[5]:

$$W = [(a^*)^2 + (b^*)^2 + (L-100)^2]^{1/2}$$

Testing for surface characteristics and chemical composition

Because by nature the scanning electron microscope (SEM) destroys any specimen that it examines, an additional group of teeth were prepared for the SEM, paralleling the same treatment applied to the original set of 40 teeth in order to replicate the sample conditions. Twelve teeth (n = 3) were randomly assigned to the different remineralizing agents—fluoride varnish, CPP-ACP, and CPP-ACPF—and distilled water. These teeth were cut in both the mesiodistal and buccolingual directions to obtain 4 pieces. Three of the pieces of each tooth were randomly selected for SEM examination, one piece for each of the 3 examination stages: initial, demineralized, and remineralized. The unselected, unused fourth piece was discarded. After reaching their assigned stage, but before being examined, all tooth pieces were dehydrated at 60° C for 5 days, mounted onto aluminum stubs, and sputter-coated with gold. Each was then examined with the SEM to identify the specimen's surface characteristics and to perform an element analysis using energy dispersive X-ray spectroscopy (EDS/EDX).

Data analysis

After the means and standard deviations of the colour, roughness, and hardness data were calculated, they were analysed for statistically significant differences using one-way ANOVA in SPSS software (SPSS 23.0, SPSS Inc., Chicago, IL, USA). The significance level was set at 0.05. The surface characteristics and elemental analysis are reported as descriptive data.

RESULTS

Hardness of demineralized and remineralized enamel

The effects of the demineralizing solution and remineralizing agents, as seen in each tooth piece examined at the initial, demineralized, and remineralized stages, are shown in Figure 1. The hardness of the demineralized enamel was significantly lower ($p < 0.05$) than that of the initial enamel (Figure 1A). After treatment with the remineralizing agents, the hardness of the enamel increased, but not significantly ($p > 0.05$) compared to the demineralized stage, and the hardness never recovered to the level of the initial stage. As for the control group, enamel hardness in the remineralized stage continued to fall even lower than in the previous demineralized stage, although this second drop in hardness was not statistically significant ($p > 0.05$).

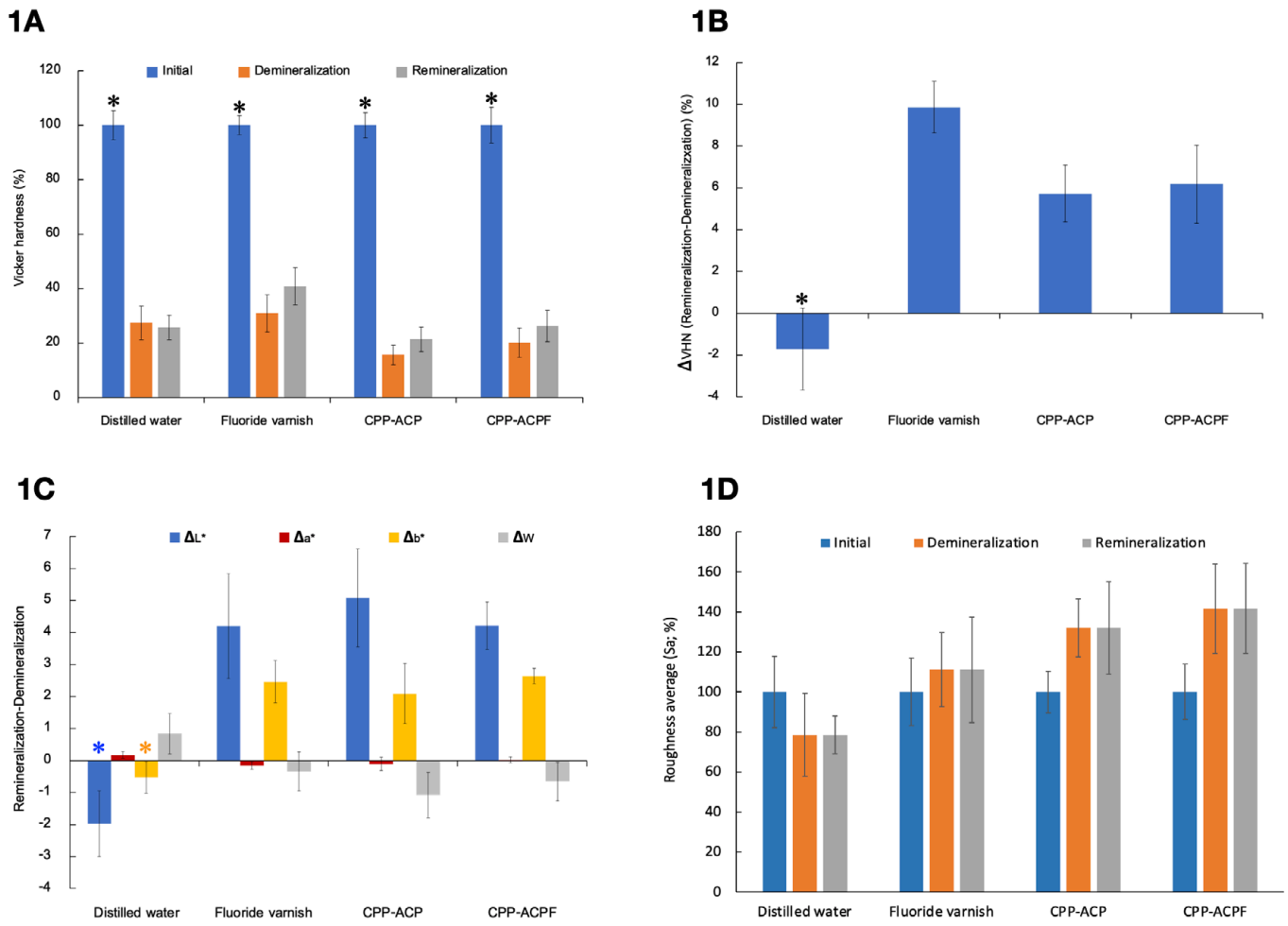
The difference in enamel hardness from the demineralized stage to the remineralized stage in each of the 4 treatments is shown in Figure 1B. Fluoride varnish, CPP-ACP, and CPP-ACPF all increased hardness significantly more than did the distilled water control ($p < 0.001$, $p = 0.003$, and $p = 0.005$, respectively).

Table 2. Mean and standard deviation of lightness (L*), redness (a*), and yellowness (b*) of enamel at the initial, demineralized, and remineralized stages when using different remineralizing agents

	L*			a*			b*		
	Initial	Demineralized	Remineralized	Initial	Demineralized	Remineralized	Initial	Demineralized	Remineralized
Distilled water	77.98 ±0.83[a]	75.58 ±0.86[a]	73.61 ±0.79[b]	1.46 ±0.18[a]	1.93 ±0.13[b]	2.10 ±0.14[b]	25.99 ±1.37[a]	28.94 ±0.88[b]	28.41 ±0.83[b]
Fluoride varnish	78.52 ±0.89[ab]	77.24 ±1.2[a]	81.44 ±1.14[b]	1.12 ±0.17[a]	1.62 ±0.18[b]	1.46 ±0.12[ab]	22.52 ±1.25[a]	26.37 ±0.96[b]	28.83 ±0.78[b]
CPP-ACP	79.01 ±0.93[ab]	75 ±1.17[a]	80.09 ±1.63[b]	1.34 ±0.19[a]	2.07 ±0.21[b]	1.96 ±0.2[ab]	24.88 ±1.26[a]	27.41 ±1.18[b]	29.5 ±1.25[b]
CPP-ACPF	78.27 ±1.15[ab]	75.05 ±1.85[a]	79.26 ±1.22[b]	1.21 ±0.22[a]	2.01 ±0.28[b]	2.03 ±0.22[ab]	24.85 ±1.13[a]	27.98 ±1.13[b]	30.62 ±1.13[b]

[a,b]Lower case characters represent statistically significant difference ($p < 0.05$) within one row of one property

Figure 1. Effects of demineralizing and remineralizing agents and distilled water on enamel hardness, colour, and roughness



1A: Mean enamel hardness during 3 stages of testing. The asterisks indicate that the initial stage result is significantly different from results of the other 2 stages within each remineralization treatment. 1B: Mean change in hardness from demineralized enamel to remineralized enamel, according to the different remineralizing agents. The asterisk shows the statistical difference among the control group and all 3 remineralizing agents. 1C: Mean changes in colour values between demineralized enamel and remineralized enamel. The 2 asterisks for statistical significance indicate that only the (control group) distilled water L* and distilled water b* changes are significantly different from these changes in other remineralizing agents. 1D: Buccal surface topography of the enamel specimens during all 3 stages of treatment.

Colour measurements of demineralized and remineralized enamel

The changes in lightness (L*), redness (a*), and yellowness (b*) of demineralized and remineralized enamel that resulted from the 3 remineralizing agents (and control) were measured by spectrophotometry and are shown in Table 2 and Figure 1C.

Enamel lightness decreased insignificantly as a result of undergoing demineralization. However, use of any of the 3 remineralizing agents not only recovered any lightness lost during demineralization (p = 0.028), but also resulted in a final lightness measurement significantly higher than the lightness of the initial (undamaged) specimen (p = 0.042). In contrast, use of the distilled water control only decreased the lightness further beyond the demineralized measurement.

Enamel redness significantly increased after demineralization (p = 0.037). After remineralization, fluoride varnish and CPP-ACP decreased redness, while CPP-ACPF increased redness. However, none of these redness changes were statistically significant compared to the initial or demineralized steps ($p > 0.05$). In contrast, the distilled water control continued to increase the redness past the demineralized measurement.

Enamel yellowness also significantly increased after demineralization (p = 0.034). Yellowness then continued to increase, but only insignificantly, after use of any of the

Table 3. Mean and standard deviation of enamel white index (W) and the ΔE values for colour differences between the 3 experiment stages for the remineralizing agents

	White index			Delta E (ΔE)		
	Initial	Demineralized	Remineralized	Initial–Demineralized	Demineralized–Remineralized	Initial–Remineralized
Distilled water	34.26 ±1.25[a]	38.03 ±0.75[b]	38.87 ±1.03[b]	13.15 ±0.36	4.48 ±0.23	14.81 ±0.45
Fluoride varnish	31.35 ±0.65[a]	34.86 ±1.02[b]	34.52 ±0.9[ab]	12.92 ±1.49	5.52 ±0.82	13.37 ±0.81
CPP-ACP	33.42 ±0.88[a]	37.07 ±1.28[b]	35.99 ±1.32[ab]	12.97 ±0.36	7.67 ±0.67	13.05 ±0.84
CPP-ACPF	33.19 ±1.22[a]	37.91 ±1.35[b]	37.26 ±1.08[ab]	14.3 ±0.48	9.34 ±0.33	13.96 ±0.53

[a,b]Lower case characters represent statistically significant difference ($p < 0.05$) within a row of white index

3 remineralizing agents. Remineralized yellowness was still significantly higher than that of the initial stage ($p < 0.001$). Changes in enamel yellowness in the distilled water control followed the same pattern as the 3 remineralizing agents.

The changes in white index (W) are shown in Table 3. Enamel white index was significantly higher following demineralization ($p = 0.022$). After use of any of the 3 remineralizing agents the white index decreased insignificantly compared to both the initial and demineralized stages ($p > 0.05$). The distilled water control also had a significant increase in white index after demineralization. The slight increase in white index that followed remineralization was insignificant compared to the demineralized stage, but white index after remineralization still remained significantly higher than the initial stage.

Values of ΔE are used to evaluate the distinguishability of colour differences to the human eye. Table 3 shows the ΔE values and standard deviation for colour differences between the 3 experiment stages for the different remineralizing agents. The ΔE values of all the comparisons were higher than 3.7.

Roughness of demineralized and remineralized enamel

The topography of the buccal surface of the teeth was examined using atomic force measurement. Each specimen was examined at all 3 stages (initial, demineralized, and

Figure 2. The arithmetical surface roughness measurement (Sa) at all 3 stages of treatment with the different remineralizing agents, and with distilled water as a control

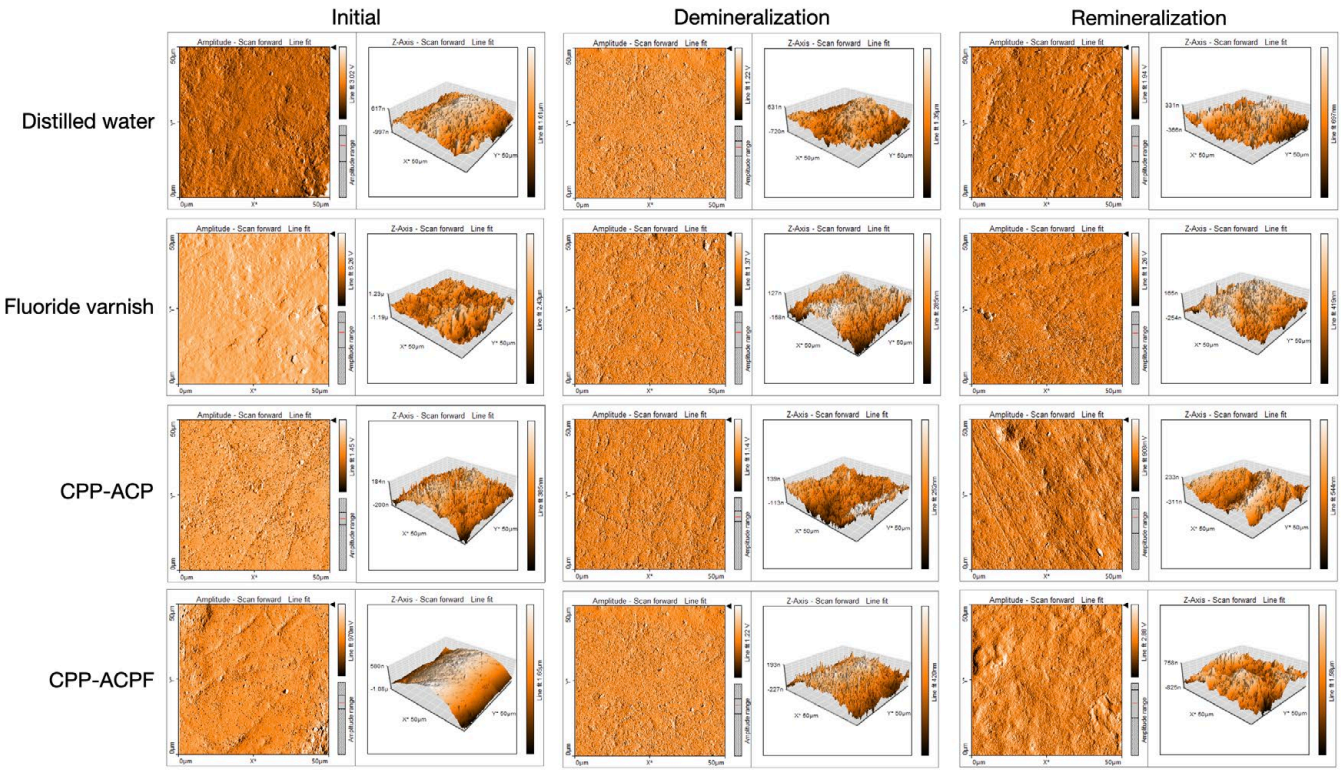


Figure 3. SEM images of natural intact enamel and natural decalcified enamel of the permanent premolar tooth specimen under magnification 4000X and 8000X

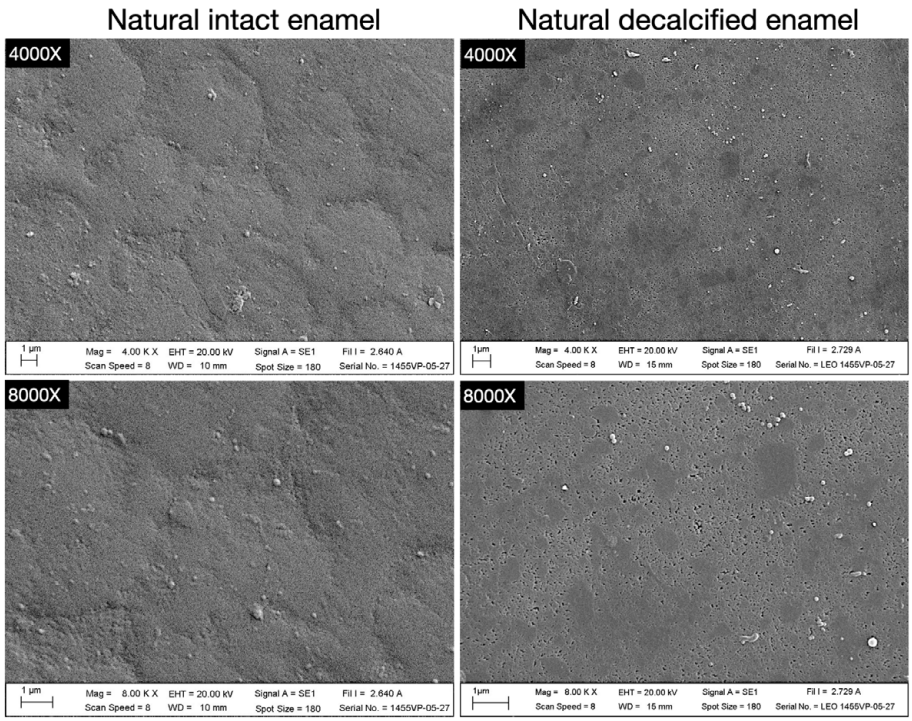


Figure 4. SEM images showing the appearance of the enamel surfaces during treatment with the different remineralizing agents at all 3 stages

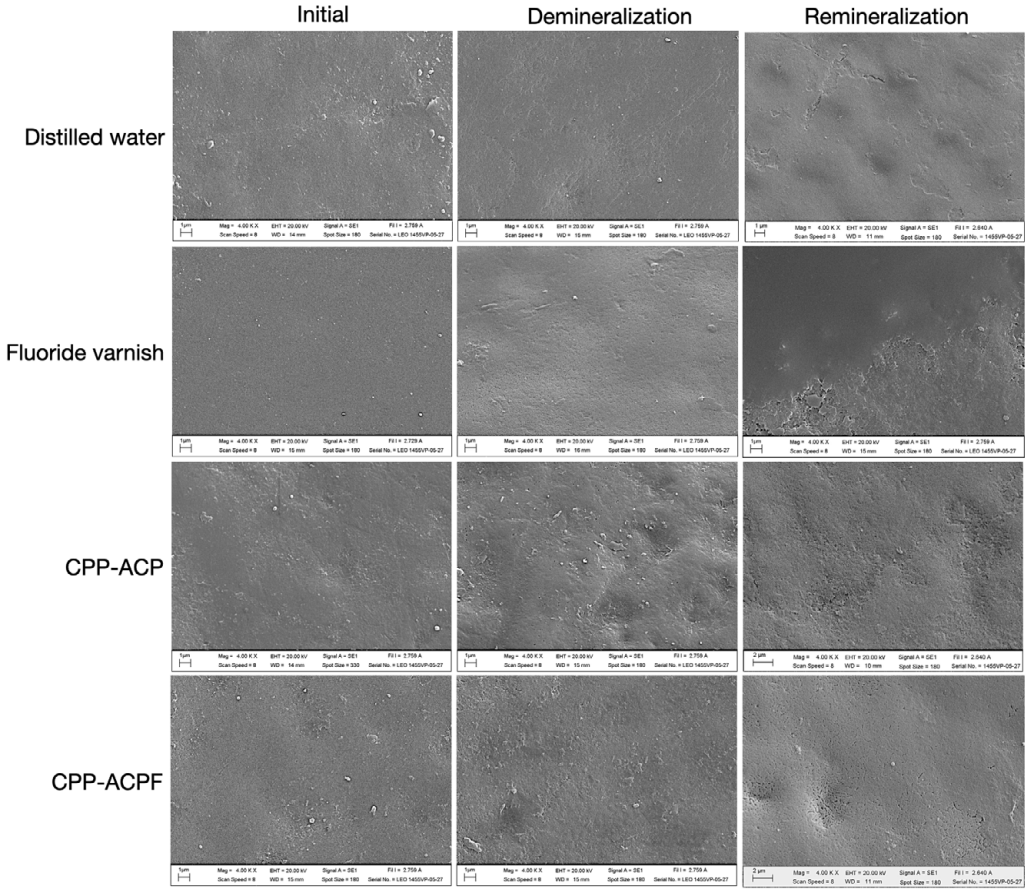


Table 4. The percent content of minerals in the enamel during all 3 stages of treatment

		Natural tooth	Distilled water	Fluoride varnish	Fluoride varnish	CPP-ACP	CPP-ACPF
C	Initial (%)	10.3 ±1.2	18.84 ±6	12.83 ±0.4	12.83 ±0.4	15.32 ±3.52	14.93 ±2.3
	Demineralized (%)	10.5 ±1	18.67 ±6.6	12.18 ±2.4	12.18 ±2.4	23.11 ±11.6	22.82 ±11.7
	Remineralized (%)	-	15.60 ±1.4^{ABC}	18.39 ±0.7^{C}	60.81 ±5.8^{Aa}	15.66 ±1.1^{BC}	12.79 ±0.3^{B}
O	Initial (%)	21.7 ±6.8	13.42 ±3.9	16.28 ±0.7	16.28 ±0.7	22.63 ±0.7	21.63 ±2.2
	Demineralized (%)	14.8 ±4.9	21.19 ±3.6	21.6 ±1.6	21.6 ±1.6	18.85 ±2.5	19.66 ±0.7
	Remineralized (%)	-	23.99 ±2.3^{CB}	14.53 ±1.2^{A}	12.39 ±1.7^{A}	17.96 ±1.2^{ABC}	24.31 ±1.2^{B}
F	Initial (%)	1.5 ±1.1	2.48 ±0.6	1.13 ±0.7^{b}	1.13 ±0.7	2.78 ±1.1	2.98 ±0.1
	Demineralized (%)	1.8 ±0.7	2.38 ±0.2	1.98 ±1.5^{ab}	1.98 ±1.5	1.65 ±0.9	2.06 ±0.1
	Remineralized (%)	-	2.15 ±0.6^{B}	6.5 ±0.4^{Aa}	1.97 ±0.5^{B}	1.77 ±0.4^{B}	2.29 ±0.2^{B}
P	Initial (%)	23.5 ±2.1	24.26 ±3.3	25.78 ±4.6^{b}	25.78 ±4.6^{b}	22.77 ±1.1	22.93 ±1.4
	Demineralized (%)	25.3 ±1.4	20.43 ±3.2	23.34 ±1.4^{ab}	23.34 ±1.4^{ab}	19 ±4.8	20.54 ±3.7
	Remineralized (%)	-	20.91 ±0.4^{ABC}	18.78 ±0.4^{ACa}	8.91 ±1.7^{ACa}	22.45 ±0.3^{B}	22.25 ±0.3^{B}
Ca	Initial (%)	42.9 ±6.1	41 ±7.4	43.97 ±0.6	43.97 ±0.6^{b}	36.49 ±3.7	37.52 ±2.0
	Demineralized (%)	47.51 ±3.9	37.32 ±7.2	40.9 ±4.2	40.9 ±4.2^{ab}	34.4 ±3.9	34.94 ±7.3
	Remineralized (%)	-	37.35 ±1.4^{AB}	41.78 ±2.2^{B}	15.90 ±4.8^{Aa}	42.16 ±1.4^{B}	38.38 ±0.9^{B}

Note: The underlined figures are for fluoride varnish-treated enamel portions that did not have the varnish residue removed.
^{A,B,C}Upper case characters represent statistically significant difference ($p < 0.05$) within a row.
^{a,b}Lower case characters represent statistically significant difference ($p < 0.05$) between values for a single element across the 3 stages.

remineralized [Figure 2]), and the arithmetical surface roughness measurement (Sa) was also taken. The surface roughness of the demineralized enamel increased insignificantly compared to the initial enamel. After remineralization, the enamel roughness remained at a level similar to the demineralized enamel (Figure 1D).

Surface characteristics and chemical composition of demineralized enamel

As a reference to better understand the surface and chemical analyses of the teeth in this study, an extracted tooth unconnected to this study that had undergone natural decalcification, and therefore displayed the characteristic subsurface lesions that result, was observed under SEM. The surface of the naturally decalcified enamel had no cracks or cavities under an optical microscope. However, examination under 4000X and 8000X magnification revealed that the surface of the decalcified enamel was covered with rather large porosities, as seen in Figure 3.

The surface characteristics of the initial, demineralized, and remineralized stage enamel were observed under SEM, and the resulting images are presented in Figure 4. Elemental analysis of each enamel surface was carried out at the time of surface observation, and the results of the elemental analyses are shown in Table 4. After demineralization, the enamel surface looked rougher, with small porosities covering the surface, similar to the natural decalcified enamel.

After the fluoride varnish was applied and then rinsed, a covering of varnish remained deposited on the enamel surface by design. This varnish deposit was protective for clinical purposes, but it also interfered with elemental analysis of the enamel surface. Therefore, the varnish deposit was removed from part of each fluoride varnish treated specimen in order to collect elemental analysis data both with and without varnish deposit on the surface. The other remineralizing agents did not leave any deposit after rinsing.

The elemental analyses revealed that the top 2 to 5 micrometers of the enamel specimens consisted mainly of calcium and phosphorus. Calcium and phosphorus were the main components of the enamel at all 3 stages, regardless of the remineralizing agent.

After the demineralized enamel was treated with the remineralizing agents, the percent composition of fluoride on the enamel surface increased for both the agents containing fluoride (fluoride varnish and CPP-ACPF), but only the increase from the fluoride varnish after deposit removal was significant. When the varnish deposit was not removed, the increase in the carbon percentage (to 69.3 ±8) was significant, but the increase in fluoride percentage was not. The increase in fluoride percentage resulting from CPP-ACPF was also insignificant.

DISCUSSION

Enamel is the hardest tissue in the human body due to its 95% to 97% mineral content, which protects teeth from physical and chemical damage.[6] Enamel that is interior to the outermost area of the tooth has a microstructure consisting of geometric prism structures, known as rods, and interprismatic structures, known as inter-rods. At the nanoscopic level, both the rods and inter-rods are highly organized structures of hydroxyapatite crystals.[7] These crystals are hexagonal in shape with a diameter of approximately 30 nm, and they are arranged with all their c-axes parallel.[8] In contrast, enamel located in the outermost area of the tooth has no prisms. This enamel is normally found in the occlusal, fissural, and cervical regions of all deciduous teeth and 70% of permanent teeth. This layer is 15 µm to 30 µm thick.[9] The structure of this region has 2 components: 1 with a scale-like appearance, and the other with a laminated appearance. The crystallites of the scale-like prismless enamel are arranged parallel to each other and their c-axis is perpendicular to the striae of Retzius, while the crystals of the laminated prismless enamel are perpendicular to the enamel surface.[10-11] The prismless enamel has more negative birefringence[12] and stronger resistance to demineralization than the prismatic enamel. This layer functions as a barrier against the acid dissolution that can result in dental caries.[13]

In all previous studies encountered by the current authors, a portion of the tooth sample was flattened by grinding in preparation for the microhardness test, in the belief that a flat surface is necessary for an accurate test result. Unfortunately, this grinding unavoidably removes the prismless layer that plays an important role in resisting acid challenges. In the current study, the demineralization procedure was designed and performed with the goal of mimicking the natural in vivo demineralization process. Therefore, the tooth samples were not flattened by grinding or any other means, and the prismless enamel was thus preserved, keeping the laboratory procedures of the study closer to actual clinical circumstances. Regarding concern that the natural contour of the tooth will prevent an accurate microhardness test, the current authors make the following observations. The diameter of the Vickers hardness test indenter is 0.5 µm, which is quite small. On that tiny scale, the natural contour of the tooth can be considered negligible and inconsequential, just as a person standing on Earth will generally perceive the Earth as more or less flat, with the Earth's natural curve becoming negligible and inconsequential for purposes in the person's immediate area. When performing the hardness test, the tiny indenter is placed somewhere near the apex of the most convex surface of the sample, and the point of contact at that scale will for all practical purposes be flat. This can be confirmed by the fidelity of the indentations to the shape of the indenter itself. Therefore, testing the enamel surface without preparatory flattening is considered to have no influence on the outcome of the hardness measurement.

The chemical composition as well as the physical and mechanical properties of enamel vary from one person to the next, from one tooth to the next, and even from one area of a tooth to the next. Because the tooth specimens in this study were collected from different patients, their exact chemical composition as well as physical and mechanical properties are expected to be different. Since the purpose of this study is to observe the series of changes that occur when tooth samples undergo the demineralization and remineralization procedures, using and following the same enamel pieces all the way through the study is vital in order to be able to compare before and after figures within the same sample at each experiment stage.

The most familiar properties of colour are hue, chroma, value, and translucency.[14] Value, also called brightness, is the amount of light returned from an object,[15] and this can also be referred to as the lightness (L*) of a colour[16]. Lightness can be measured independently of hue as grayscale. The relationship between lightness and chroma is a negative correlation. Lightness is a dominant colour dimension which determines 75% of shade selection.[14] White index (W) is a quantified measurement of the common idea of "whiteness." When comparing 2 values of W, the higher value is "whiter." White index represents the amount of visible light reflection compared to the complete reflection of all light spectra and is a function of lightness, hue, and chroma, each of which contribute to the total perception of whiteness.[17] Thus the value of W can be calculated from the 3 fundamental dimensions of colour: L*, a*, and b*. The current study utilizes L*, a*, and b*, along with W as a concomitant perception of colour.

In the current study, the whiteness index of the demineralized enamel significantly increased. The effect was that the demineralized teeth became excessively white in subjective appearance and thus lost their natural look. Use of any of the 3 remineralizing agents (but not the water control) succeeded in improving the subjective appearance of the demineralized teeth, not by lowering whiteness but by increasing lightness. However, none of the remineralizing agents completely recovered the natural appearance of the initial teeth. The 3-day period that the remineralizing agents were in contact with the teeth allowed all 3 agents to ameliorate the appearance of the demineralized enamel, and a treatment period of additional length might yield even more pronounced results in future studies.

The ΔE values that were calculated in this study are used to evaluate the perceptibility of colour differences. Given any 2 objects of different colour, the difference between the 2 colours can be calculated as a ΔE value. Importantly, if the value of ΔE is greater than 1, this means that the colour difference in question can be perceived by the human eye as long as the 2 colours are displayed side by side.[18] If ΔE

is greater than 3.3, this means that the human eye can detect the colour difference even when the 2 colours are not seen side by side.[19] When ΔE is greater than 3.7, this means that the 2 colours are quite dissimilar and of little relation. In this case, the colour difference between the observed subjects is obvious to the human eye.[20] In the current study, the ΔE values calculated between all 3 stages of all 3 remineralizing agents were all greater than 3.7. Therefore both the demineralization and remineralization processes alter the enamel colour to an extent that people can obviously see.

Regarding the SEM images, the demineralized enamel revealed tiny porosities all over its surface, which together appear similar to the subsurface lesion seen in the naturally demineralized reference tooth. The area of this lesion has lower mineral composition compared to the intact enamel.[21] It is believed that the porosities on the enamel surface may provide access for the demineralizing solution to get into the enamel and dissolve some amount of minerals, thus creating the subsurface lesion. This in turn affects the refractive index of the enamel surface, leading to the tooth colour alteration. In 2019, the scattering coefficient was observed at wavelength 543 nm to 1060 nm in both intact and demineralized enamels. The latter had 2 times the scattering coefficient (8.46 mm^{1-}) compared to the intact enamel (4.60 mm^{1-}) when 37% phosphoric acid was applied for 120 seconds, and as the application time increased, so did the scattering coefficient.[22] The colour alteration in demineralized enamel is likely the result of this increase in the porous enamel's scattering coefficient.

Energy dispersive X-ray spectroscopy (EDX or EDS) is used in conjunction with SEM for element analysis. Electrons from the X-ray photons hit atoms in the observed material, causing the material's electrons to be ejected, and the electron vacancy is then filled by electrons from the higher shell. The resulting energy difference causes X-ray energy to be emitted. That X-ray energy is used to characterize the elements from which it is emitted.[23] The depth of EDS analysis depends on the strength of the primary X-ray beam energy sent to stimulate the material. The current study employed a beam energy of 20 kV. At this strength, the electrons can reach a depth of approximately 1 µm.[24] Thus, the element analysis using EDX in this study was investigated within that depth of the enamel surface.

In previous studies, the amount of calcium and phosphate significantly decreased in demineralized enamel in both the surface area[25] and the subsurface area,[21] reflecting the same results found here. No previous study is known to have observed the amount of fluoride in enamel after undergoing demineralization. As mentioned earlier, application of the fluoride-containing remineralizing agents in the current study caused the percentage of fluoride in the enamel to increase, and particularly so in the case of the fluoride varnish. The fluoride percentage increase from fluoride varnish was significantly higher than any of the other remineralizing agents. This higher percent increase may be a result of the fact that the fluoride varnish contains more fluoride (22,500 ppm fluoride ions) than CPP-ACPF (900 ppm fluoride ions). The hardness test in this study showed that the fluoride varnish also produced the highest hardness result, followed by the CPP-ACPF, and then the CPP-ACP. This might be because the first 2 reagents contain fluoride, so they can form fluorapatite or hydroxyfluorapatite, whereas CPP-ACP contains no fluoride and so can only form hydroxyapatite, which is not as hard as fluorapatite or hydroxyfluorapatite.

It is noteworthy that both CPP-ACP and CPP-ACPF possess an advantageous ability that fluoride varnish does not. CPP-ACP and CPP-ACPF contain peptide (CPP) in an alkaline supersaturated calcium phosphate solution (ACP). One molecule of this peptide can bind as many as 21 calcium ions or 14 phosphorous ions. The purpose of the peptide is to prevent the calcium and phosphate ions in the solution from precipitating into calcium phosphate, because the precipitate form is of no clinical use.[26] In contrast, the peptide-separated calcium and phosphate ions in the form of CPP-ACP or CPP-ACPF are able to enter carious lesions in the enamel to form hydroxyapatite or hydroxyfluorapatite. In this way, CPP-ACP or CPP-ACPF can deliver calcium and phosphate ions to the enamel due to the different concentration gradient.[26-27] In the acid condition, the peptide could maintain high concentrations of calcium and phosphate in the lesions.[26-29]

The roughness of the demineralized enamel in this study increased insignificantly compared to the natural enamel, which corresponds to the results of previous studies.[22,30-31] The AFM micrographs show many tiny peaks for both the demineralized and remineralized teeth. Each peak represents a porosity that the scanning probe investigated. The higher the peak, the deeper the porosity. The AFM probe is able to characterize the porosities because the probe diameter is smaller than 20 nm, while the diameter of the enamel porosities ranges from approximately 21.27 nm to 191.50 nm. The enamel specimens in this study were submerged in pH4.4 demineralizing solution for 3 days, and the AFM probe determined that the resulting depth of the porosities was in the range of 100 nm to 200 nm (data not shown). By contrast, Yu et al.[32] submerged enamel pieces in a pH5.0 acetate buffer demineralizing solution for 21 days and found that the resulting average depth of the porosities in the demineralized enamel was approximately 134 µm. This difference in porosity depth seems to indicate that the type of acid used and the duration of acid exposure influence the degree of demineralization. After applying the remineralizing agents for 3 days in the current study, the roughness of the remineralized enamel was still similar to that of the demineralized enamel, and there was no significant difference in roughness between the different remineralizing agents. Thus all 3 remineralizing agents were able to increase the hardness of the demineralized

enamel at the molecular level through formation of fluorapatite or hydroxyfluorapatite, although surface texture was not improved.

This study has limitations due to its laboratory setting, which does not fully represent the oral environment. Salivary factors, including flow rate, buffer systems, and calcium and phosphate concentrations, may further reduce demineralization and enhance remineralization. The SEM and EDS mineral measurement processes led to the loss of specimens, preventing consecutive testing. This study cannot continuously use the same specimens for 3 stages (before treatment, after demineralization, and after remineralization). Therefore, to control for potential variability, 3 EDS mineral measurements were conducted on different samples from the same tooth.

CONCLUSION

This study found that the 3 investigated remineralizing agents (fluoride varnish, CPP-ACP, and CPP-ACPF) are all able to significantly improve the hardness of demineralized enamel. They are also able to significantly improve the appearance of demineralized enamel by increasing the lightness of the tooth. Comparing the 3 agents, no significant difference in effectiveness was found among them.

ACKNOWLEDGMENTS

Editing assistance was kindly provided by Mr. Paul Freund of Naresuan University Writing Clinic (DIALD).

CONFLICTS OF INTERESTS

The authors have declared no conflicts of interest.

REFERENCES

1. Frencken JE, Peters MC, Manton DJ, Leal SC, Gordan VV, Eden E. Minimal intervention dentistry for managing dental caries—a review: report of a FDI task group. *Int Dent J*. 2012;62(5):223–43.
2. Ten Cate JM. Current concepts on the theories of the mechanism of action of fluoride. *Acta Odontol Scand*. 1999;57(6):325–29.
3. Featherstone JD. Dental caries: a dynamic disease process. *Aust Dent J*. 2008;53(3):286–91.
4. Hamilton IR. Biochemical effects of fluoride on oral bacteria. *J Dent Res*. 1990;69:660–83.
5. Joiner A, Hopkinson I, Deng Y, Westland S. A review of tooth colour and whiteness. *J Dent*. 2008;36(1):S2–S7.
6. Chen HF, Tang ZY, Liu J, Sun K, Chang SR, Peters MC, et al. Acellular synthesis of a human enamel-like microstructure. *Adv Mater*. 2006;18(14):1846–1851.
7. Vila Verde A, Ramos MM, Stoneham AM. The role of mesoscopic modelling in understanding the response of dental enamel to mid-infrared radiation. *Phys Med Biol*. 2007;52(10):2703–2717.
8. Robinson C, Connell SD. Crystal initiation structures in developing enamel: possible implications for caries dissolution of enamel crystals. *Front Physiol*. 2017;8:405–409.
9. Ripa LW, Gwinnett AJ, Buonocore MG. The "prismless" outer layer of deciduous and permanent enamel. *Arch Oral Biol*. 1966;11(1):41–48.
10. Fava M, Watanabe IS, Fava De Moraes F, Sucasas da Costa L. Prismless enamel in human non-erupted deciduous molar teeth: a scanning electron microscopic study. *Rev Odontol Univ São Paulo*. 1997;11(4):239–43.
11. Gwinnett AJ. The ultrastructure of the "prismless" enamel of deciduous teeth. *Arch Oral Biol*. 1966;11(11):1109–1115.
12. Gwinnett AJ. The ultrastructure of the "prismless" enamel of permanent human teeth. *Arch Oral Biol*. 1967;12(3):381–87.
13. Kuroiwa M. Acid resistance of surface "Prismless" enamel in human deciduous and permanent teeth. *Showa Univ J Med Sci*. 1990;2(1):31–44.
14. Chu SJ. The science of color and shade selection in aesthetic dentistry. *Dent Today*. 2002;21(9):86–89.
15. Sikri VK. Color: Implications in dentistry. *J Conserv Dent*. 2010;13(4):249–55.
16. Ragain J. A review of color science in dentistry: The process of color vision. *J Dent Health Oral Disord Ther*. 2016;4(1):1–5.
17. Luo W, Westland S, Ellwood R, Pretty I, Cheung V. Development of a whiteness index for dentistry. *J Dent*. 2009;37(1):21–26.
18. Kuehni RG, Marcus RT. An experiment in visual scaling of small color differences. *Color Res Appl*. 1979;4(2):83–91.

19. Ruyter IE, Nilner K, Moller B. Color stability of dental composite resin materials for crown and bridge veneers. *Dent Mater*. 1987;3(5):246–51.

20. Johnston WM, Kao EC. Assessment of appearance match by visual observation and clinical colorimetry. *J Dent Res*. 1989;68(5):819–22.

21. Hayashi O, Chiba T, Shimoda S, Momoi Y. Demineralization and remineralization phenomena of human enamel in acid erosion model. *J Hard Tissue Biol*. 2016;25(1):27–34.

22. Tsai MT, Wang YL, Yeh TW, Lee HC, Chen WJ, Ke JL, Lee YJ. Early detection of enamel demineralization by optical coherence tomography. *Nature Research Scientific Reports*. 2019;9:1–9.

23. Sakoolnamarka R, Burrow MF, Swain M, Tyas MJ. Microhardness and Ca:P ratio of carious and Carisolv™ treated caries-affected dentine using an ultra-micro-indentation system and energy dispersive analysis of x-rays—A pilot study. *Aust Dent J*. 2005;50(4):246–50.

24. Wassilkowska A, Czaplicka-Kotas A, Zielina M, Bielski A. An analysis of the elemental composition of micro-samples using EDS technique. *Technical Transactions Chemistry* (Poland). 2014:133–48.

25. Hegde MN, Shetty S, Pardal D. Remineralization of enamel sub-surface lesion using casein phosphopeptide amorphous calcium phosphate (CPP-ACP)-a quantitative energy dispersive X-ray analysis (EDAX). *J Conserv Dent*. 2007;10(1):19–25.

26. Reynolds EC. Remineralization of enamel subsurface lesions by casein phosphopeptide-stabilized calcium phosphate solutions. *J Dent Res*. 1997;76(9):1587–1595.

27. Reynolds EC. Anticariogenic complexes of amorphous calcium phosphate stabilized by casein phosphopeptides: a review. *Spec Care Dentist*. 1998;18(1):8–16.

28. Reynolds EC, Cai F, Shen P, Walker GD. Retention in plaque and remineralization of enamel lesions by various forms of calcium in a mouthrinse or sugar-free chewing gum. *J Dent Res*. 2003;82(3):206–211.

29. Shen P, Cai F, Nowicki A, Vincent J, Reynolds EC. Remineralization of enamel subsurface lesions by sugar-free chewing gum containing casein phosphopeptide-amorphous calcium phosphate. *J Dent Res*. 2001;80(12):2066–2070.

30. Danesh G, Podstawa PKK, Schwartz CE, Kirschneck C, Bixhang M, Arnold WH. Depth of acid penetration and enamel surface roughness associated with different methods of interproximal enamel reduction. *PLoS One*. 2020;15(3):1–12.

31. Mohamed AM, Hung WK, Jen LW, Nor MM, Hussaini HM, Rosli TI. In vitro study of white spot lesion: maxilla and mandibular teeth. *Saudi Dent J*. 2018;30(2):142–50.

32. Yu OY, Mei ML, Lo ECM, Chu CH. Effects of fluoride on two chemical models of enamel demineralization. *Materials*. 2017;10(11):1245–1253.