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Original Article

Influence of Fluoride Casein Phosphopeptide and Tri-Calcium Phosphate on Enamel Recalcification: Surface Modifications and Biofilm Prevention

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ABSTRACT

This research investigated, under in vitro conditions, the protective capability of two enamel-repairing formulations: a varnish composed of β-tricalcium phosphate combined with sodium fluoride (β-TCP-F), and a paste containing casein phosphopeptide–amorphous calcium phosphate supplemented with sodium fluoride (CPP-ACP-F). Enamel samples (n = 120) obtained from extracted human third molars were divided into four experimental sets (30 specimens per group): Group I—sound enamel (control), Group II—artificially demineralized enamel, Group III—demineralized enamel treated with β-TCP-F, and Group IV—demineralized enamel treated with CPP-ACP-F. For 15 consecutive days, Groups II–IV underwent daily pH cycling consisting of 21 h in a demineralizing medium (pH 4.4) and 3 h in a remineralizing medium (pH 7.0). Before each cycle, the treated groups received their respective materials. Fluoride ion release was recorded after every cycle. Surface hardness, surface roughness, contact angle, and *Streptococcus mutans* biofilm accumulation were determined on days 5, 10, and 15. CPP-ACP-F application resulted in a greater increase in surface microhardness (515.2 ± 10.7) compared to β-TCP-F (473.6 ± 12.8). Both agents lowered surface irregularity relative to untreated demineralized enamel; however, after 15 days, β-TCP-F-treated samples retained a roughness of 1.193 µm—still significantly higher than intact enamel—whereas CPP-ACP-F-treated enamel achieved 0.76 µm, matching that of healthy enamel. Contact angle analysis showed enhanced wettability in both experimental groups (β-TCP-F: 71.01°, CPP-ACP-F: 65.24°), approaching or slightly surpassing that of normal enamel. Both β-TCP-F and CPP-ACP-F demonstrated remineralizing and protective potential against enamel demineralization. Nevertheless, CPP-ACP-F provided superior recovery of hardness and smoother surfaces under controlled in vitro conditions. These materials effectively prevented the detrimental surface changes caused by acid challenge on human enamel.

Keywords: Enamel remineralization, β-TCP-F, CPP-ACP-F, Surface properties, Hardness, Roughness, Wettability, Biofilm

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Introduction

The enamel layer forms the outer shell of the tooth crown and represents the densest and most mineralized biological material in the human body. It is primarily composed of approximately 95% inorganic matter, largely crystalline hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), about 1% organic material (mainly proteins and lipids), and nearly 4% water. This unique composition grants enamel remarkable mechanical strength and chemical resistance, enabling it to tolerate high masticatory pressure and variable pH conditions within the oral cavity [1]. Despite its

strength, the mineral component is vulnerable to acid dissolution when the pH drops below 5.5, leading to the leaching of calcium, phosphate, and other essential ions—a process defined as demineralization [2].

Although saliva naturally contributes to enamel recovery by delivering calcium and phosphate ions, this physiological remineralization becomes insufficient when the oral pH remains persistently acidic [3]. Continuous exposure to an acidic environment, commonly linked to dietary sugars and bacterial metabolism, promotes irreversible mineral loss, modifies the surface morphology, enhances bacterial adherence, and increases the likelihood of developing dental caries and erosive defects [4].

According to the World Health Organization, dental caries remains the most prevalent noncommunicable condition worldwide, affecting between 60% and 90% of children and the majority of adults. This disease represents a major financial burden, accounting for 5–10% of total healthcare expenditures in industrialized countries [5]. Because caries originates from enamel demineralization, numerous remineralization strategies have been designed to rebuild or maintain the mineral integrity of enamel. These approaches generally encourage mineral redeposition by triggering nucleation and crystal growth from ion-saturated solutions [6]. Among them, the application of fluoride compounds is the most widely accepted method [7].

Sodium fluoride (NaF) has been extensively used for the remineralization of early enamel lesions; however, its efficacy depends on the surrounding concentrations of calcium and phosphate ions [8]. The absence of sufficient ionic content restricts fluoride's capacity to promote mineral recovery.

In pediatric dentistry, two fluoride-based remineralizing products are particularly prevalent: (a) β-tricalcium phosphate varnish containing sodium fluoride (NaF; 22,600 ppm) and (b) casein phosphopeptide–amorphous calcium phosphate paste enriched with NaF. The casein phosphopeptide component—derived from milk proteins—stabilizes calcium and phosphate ions in aqueous media, improving their interaction with enamel. In both formulations, fluoride enhances mineral uptake and serves as an anticaries agent [9]. Typically, β-TCP-F varnish is applied by dental professionals, whereas CPP-ACP-F paste can be used independently at home. Given the widespread use of both materials, comparative evaluation of their remineralizing effects is necessary to clarify their respective advantages and clinical significance [10].

In β-TCP-F-based varnishes, the material serves as a controlled source of calcium and phosphate ions, regulating their diffusion into the enamel surface and enhancing the remineralization process in synergy with fluoride ions. Within the oral cavity, these ions can combine to favor the formation of fluorapatite crystals, which improve the enamel's ability to resist acid dissolution. Sodium fluoride also stabilizes the formulation by preventing premature reactions between calcium and phosphate during storage. Once the varnish comes into contact with saliva, its outer layer progressively dissolves, ensuring a continuous release of ions that supports sustained remineralization [11, 12]. The U.S. Food and Drug Administration (FDA) has approved the use of dental varnishes for managing tooth sensitivity, particularly in cases of exposed dentin or root surfaces. These varnishes can also be applied inside prepared cavities before placing restorative materials to minimize penetration into the dentin. Fluoride varnishes are acknowledged as reliable treatments not only for dentin hypersensitivity but also for individuals prone to recurrent caries. One of the most frequently used products in pediatric dentistry is the Vanish® β-TCP-F varnish (St. Paul, MN, USA), which is preferred for three main reasons: (1) its capacity to continuously release beneficial ions to nearby tooth surfaces and throughout the oral cavity, (2) its formulation incorporating a modified β-TCP-F complex, and (3) its simplicity and rapid application in clinical settings.

Proteins such as caseins play a critical role in controlling calcium and phosphate solubility within biological fluids [13]. Without such regulation, excessive concentrations of these ions can cause unwanted calcifications. Therefore, fluoride-enriched pastes containing CPP and ACP promote regulated remineralization, reducing the risk of abnormal mineral deposition. Casein, the predominant milk protein, has a high affinity for calcium and phosphate, forming stable complexes that, upon partial enzymatic hydrolysis, yield CPP fragments [14]. CPP-ACP-F systems have been shown to protect against enamel demineralization and dental erosion [15, 16]. When oral pH declines, these systems release Ca^{2+} and PO_4^{3-} ions, producing an ionically supersaturated layer near the enamel surface [17]. CPP stabilizes this condition within a pH range of 4.5–7.0, encouraging the formation of fluorapatite crystals in the presence of fluoride [18]. Furthermore, CPP-ACP-F products delay biofilm establishment, stimulate calcium phosphate crystallization [17, 19-22], and inhibit mineral loss, thereby preventing enamel softening and decay [23].

At present, numerous commercial products—such as gels, varnishes, and pastes—are designed to facilitate enamel remineralization. Although many display

favorable outcomes, additional experimental data are needed to confirm their clinical reliability and to assess their influence on various enamel surface properties. The present research aimed to compare the performance of two representative formulations: a β-TCP-F-containing varnish and a CPP-ACP-F-based paste, both widely used in dental practice for enamel repair [24].

The purpose of this study was to conduct an in vitro comparative assessment of two commonly applied fluoride-containing agents in pediatric dentistry: a β-tricalcium phosphate varnish with sodium fluoride (β-TCP-F) and a paste comprising casein phosphopeptide–amorphous calcium phosphate with sodium fluoride (CPP-ACP-F). Several laboratory approaches exist to analyze enamel demineralization and remineralization over time, among which the pH cycling model best replicates the alternating acidic and neutral phases of the oral environment [25, 26]. After an initial demineralization phase, enamel specimens were treated daily with either β-TCP-F or CPP-ACP-F and subjected to 21-hour demineralization and 3-hour remineralization cycles for 5, 10, and 15 days. Surface hardness, roughness, and wettability were evaluated at each interval (days 5, 10, and 15) and compared with those of intact enamel. A control group consisting of demineralized samples without treatment underwent identical pH cycling to serve as a negative reference. The fluoride ion concentration in the surrounding solution was monitored daily throughout the experiment. In addition, the potential for biofilm development on each enamel surface was analyzed using *Streptococcus mutans*, a key cariogenic microorganism.

This investigation highlights the differential effectiveness of two fluoride-based remineralization systems in reducing enamel demineralization and biofilm accumulation. The outcomes provide meaningful evidence for dental practitioners in selecting suitable remineralizing therapies based on scientific evaluation.

Materials and Methods

Collection of human dental enamel samples

Enamel fragments were obtained from impacted lower third molars extracted from individuals aged 17–22 who required preventive or orthodontic interventions. Since these teeth had never been exposed to the oral cavity prior to extraction, the likelihood of microbial contamination was minimal. All specimens originated from patients who provided informed consent, with the extractions performed by oral surgeons from several private dental clinics. Tooth collection occurred over a three-month period. Ethical approval was granted by the Committee of the Faculty of Higher Studies (FES) Iztacala under protocol CE/FESI/032022/1499 on 1 January 2022, and all procedures adhered to the Declaration of Helsinki guidelines.

Experimental group preparation

A total of 60 intact third molars without cracks, stains, or structural defects were selected. Each molar was longitudinally divided through the mesiodistal axis using a diamond disk (Brasseler™ 910, <20,000 rpm, Savannah, GA, USA) with constant water cooling, generating 120 enamel surfaces. Any residual periodontal tissue was carefully removed prior to sectioning. The internal cavity of each sample (pulp chamber and canals) was filled with modeling wax to provide a stable backing surface, and all prepared specimens were kept in deionized, distilled water until experimentation.

Following the protocol by Ten Cate [19], a non-fluoridated prophylactic paste (Viarden Lab, Mission, TX, USA) was used to clean each surface. The enamel was then coated with an acid-resistant varnish (Revlon™, New York, NY, USA) on the crown and root, leaving a 3 × 6 mm² central window exposed on the buccal or lingual surface. Thirty samples remained untreated as sound enamel controls (Group I: HE). The remaining 90 samples were exposed for 96 hours to a demineralizing medium (2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4, and 0.05 M CH_3COOH, pH adjusted to 4.4 with 1 M KOH) at 37 °C, as described by Ten Cate and Duijsters [21], which is a well-validated model replicating in vivo mineral loss and recovery processes. Before starting the experiment, each specimen was equilibrated in distilled, deionized water at 36 °C to stabilize its mineral content.

Subsequently, 30 demineralized surfaces received a daily application of a fluoride-containing paste with casein phosphopeptide–amorphous calcium phosphate (Mi Paste Plus™, GC IBÉRICA Dental Products, Las Rozas, Madrid, Spain). Another 30 samples were treated with a β-tricalcium phosphate varnish containing sodium fluoride (Clinpro™ White Varnish, St. Paul, MN, USA) according to the manufacturer's directions. The final 30 surfaces remained demineralized and untreated, serving as negative controls.

All treated and untreated samples were exposed to simulated oral pH fluctuations for 5, 10, or 15 consecutive days, alternating between 21 hours in a demineralizing solution (pH 4.4) and 3 hours in a remineralizing medium (pH 7.0) **(Figure 1)**. Each day, fresh solutions were prepared. Ten specimens from

each group were randomly selected at each time interval for surface analysis, including hardness, roughness, and biofilm adherence. These timeframes correspond to approximately 1.2, 2.5, and 3.8 months of remineralization under clinical conditions, assuming weekly treatment application for severe enamel lesions [27, 28].

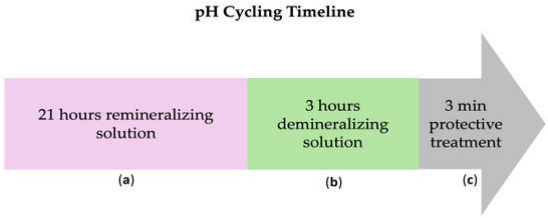


Figure 1. Diagram of the daily pH cycle:
(a) 21 h in remineralizing solution (1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4, 0.15 mM KCl, adjusted to pH 7.0 with 1 M KOH);
(b) 3 h in demineralizing solution (2.2 mM Ca, 2.2 mM P, and 5.0 mM CH_3COOH, pH 4.4);
(c) 3 min treatment exposure to either β-TCP-F varnish or CPP-ACP-F paste for treated groups

Finally, samples were divided into three experimental groups **(Figure 2)**:
Group II: Demineralized, untreated enamel (IL; *n* = 30)
Group III: Demineralized enamel treated with β-TCP-F varnish (*n* = 30)
Group IV: Demineralized enamel treated with CPP-ACP-F paste (*n* = 30)

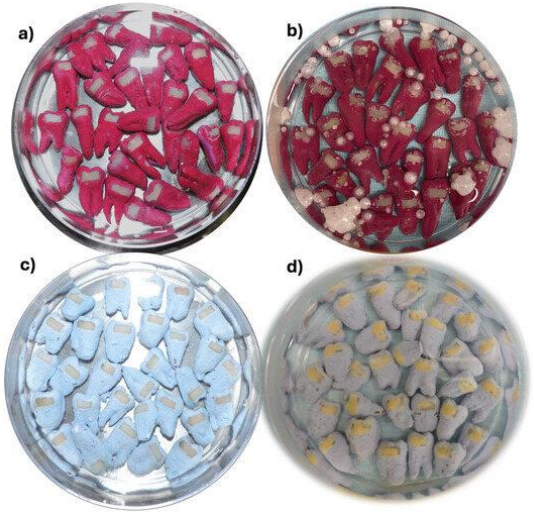


Figure 2. Representative enamel surfaces before and after treatments: (a) untreated baseline; (b) following β-TCP-F varnish; (c) untreated; and (d) after CPP-ACP-F paste application

Determination of fluoride ion concentration during pH cycling

Fluoride levels (ppm) within both demineralizing and remineralizing media were analyzed each day throughout the pH cycling experiment. Measurements were carried out using a fluoride ion-selective electrode (F-ISE) (Orion Star A-214®, Orion Research, Austin, TX, USA) linked to a pH/Ion 450/M® fluorimeter (Techno Scientific, Waltham, MA, USA). To calibrate the electrode, a standard curve covering concentrations between 0.25 and 2 µg/mL was produced using Tissab II® buffer (Orion, Techno Scientific, Waltham, MA, USA). This reagent ensured the stability of ions, increased the pH of the medium, and liberated fluoride ions that were chemically bound to metal cations such as calcium, iron, or aluminum, thereby eliminating interference.

All determinations were conducted at room temperature in the same buffer solution. Fluoride concentration in the test samples was derived by correlating the millivolt readings with the calibration curve in the range of 0.25–2 µg/mL. For each reading, the test solutions were transferred individually into glass containers (Sigma-Merck, Darmstadt, Germany) and continuously stirred magnetically at approximately 25 °C until a stable signal was achieved. After each reading, the electrodes were rinsed with deionized water and dried with Sanitas™ absorbent tissues (Madrid, Spain). To ensure precision and consistency (±2%), new calibration curves were generated daily before measurement.

Vickers microhardness measurement

Hardness testing was conducted on ten enamel specimens per group after 0, 5, 10, and 15 days of exposure to the pH cycle. The test employed a Nano-Microindenter (NANOVEA Inc., Irvine, CA, USA) coupled with Nanovea Micro Indentation Tester software (v1.8.3). Each indentation was made with a 20× objective, the tip positioned perpendicular to the enamel surface. Test parameters were set as follows: approach speed—50 µm/min, contact load—50 mN, maximum load—10 N, loading rate—5 N/min, and unloading rate—5 N/min, with ten indents per specimen. The Vickers Hardness Number (VHN) was automatically computed using the dedicated NANOVEA software [29].

Surface roughness characterization

Surface topography was examined with a ZYGO Nexview 3D optical profilometer (Zygo, Middlefield, CT, USA), which operates without physical contact. The average roughness (Ra), representing the mean deviation of the surface profile from the central baseline, was used to quantify enamel texture. On each 3 × 6 mm² enamel window, six random Ra readings

were collected, and the mean value was used for comparison. Measurements were obtained after 0, 5, 10, and 15 days of cycling. Statistical differences were determined by comparing treated samples with Group I (Healthy Enamel; HE) and Group II (Initial Lesion; IL).

Surface wettability

Contact angle tests were performed to assess surface wettability with a Dataphysics OCA-15EC goniometer (DataPhysics Instruments, Filderstadt, Germany). A 4 µL droplet of deionized water was placed on each enamel surface (3 × 6 mm²), and the static contact angle was measured one second after stabilization using SCA20_U software (v5.0.38, build 5038). For each specimen, 20 measurements were recorded — 10 readings from the left and 10 from the right sides of the droplet — and the average value was calculated for analysis.

Scanning electron microscopy (SEM) analysis

Morphological assessment of enamel samples was performed with a field emission scanning electron microscope (FE-SEM, JEOL JSM-7600F, JEOL Ltd., Tokyo, Japan). Prior to imaging, all samples were coated with a thin carbon layer using a vacuum deposition unit to ensure electrical conductivity and minimize imaging artifacts. Microscopic observation was conducted under high-vacuum conditions at an accelerating voltage of 20 kV and 5000× magnification. The secondary electron detector was used to capture detailed, high-resolution micrographs that accurately represented surface topography.

Assessment of Streptococcus mutans biofilm development

The ability of the cariogenic microorganism Streptococcus mutans (S. mutans; 25175™, ATCC®, Manassas, VA, USA) to establish biofilms on enamel surfaces pretreated with β-TCP-F or CPP-ACP-F for 5, 10, and 15 days was determined using the MTT colorimetric method (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). A pure bacterial culture was isolated from HK agar plates composed of Trypticase Soy Agar, Brain Heart Infusion Agar, and Yeast extract (BBL®), enriched with 1% v/v menadione, 1% v/v hemin (both from Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), and 5% defibrinated sheep blood (Microlab Laboratory S.A. de C.V., Tlalnepantla, Mexico). The obtained colonies were then suspended in Mycoplasma Broth Base (BBL®, Sigma, Saint Louis, MO, USA), which contained the same supplements of 1% menadione and 1% hemin. The bacterial suspension was standardized to 1×10^9 cells/mL by adjusting its optical density to 1 at 600 nm using a BioPhotometer D30 spectrophotometer (Fisher Scientific, 28108, Alcobendas, Madrid, Spain). Subsequently, autoclave-sterilized enamel discs (three from each experimental group) were positioned in 24-well culture plates, and 2 mL of the prepared bacterial suspension (1×10^7 cells/mL) was added to each well. Healthy enamel (Group I: HE) and demineralized enamel (Group II: IL) acted as the positive and negative controls, respectively.

All inoculated specimens were incubated under anaerobic conditions at 37 °C and 80 rpm for 24 hours in an IKA® KS 130 basic orbital shaker (Staufen, Germany). After incubation, samples were carefully washed twice with sterile double-distilled water (dd-H_2O) to remove unattached cells and were then transferred into a fresh culture plate. Each specimen was treated with a 1:10 mixture of MTT reagent and broth medium and incubated for 3 hours at 37 °C and 80 rpm, protected from light and oxygen.

Following incubation, the formazan crystals formed by metabolically active bacteria adhering to the enamel were solubilized using a 1:1 mixture of 99% 2-propanol (ISO grade) and dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). The absorbance of the resulting solution was measured at 570 nm using a Filter-Max F5 multi-mode microplate reader (Molecular Devices, San Jose, CA, USA). The measured optical density reflected the viable bacterial population attached to the surfaces, thereby indicating the extent of biofilm formation on each specimen type.

Statistical evaluation

Normality of the data sets—fluoride ion release (ppm), surface hardness, and roughness—was tested using the Shapiro–Wilk method. Since the data for F-ISE and hardness did not follow a normal distribution, a Kruskal–Wallis non-parametric test was used, and pairwise differences were examined through the Wilcoxon Rank and Dunn's post hoc analyses. Surface roughness comparisons were analyzed with a one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test.

All bacterial assays were conducted in triplicate, and the findings were expressed as mean (ME) ± standard error of the mean (SEM). Intergroup significance was determined via one-way ANOVA with Tukey's multiple comparison procedure, where $p < 0.05$ denoted statistical significance. All computations and graphical representations were performed using

GraphPad Prism 8.0® software (225 Franklin Street, Floor 26, Boston, MA, USA).

Results

Fluoride ion levels in demineralizing and remineralizing media

The total fluoride ion content (ppm) measured in the remineralization and demineralization solutions throughout the 15-day pH cycling protocol for the experimental enamel surfaces is presented in **Figure 3**. Surfaces treated with β-TCP-F consistently released more fluoride ions into both solution types than those treated with CPP-ACP-F. In addition, the remineralizing medium (pH 7, 21 h immersion) exhibited higher fluoride concentrations than the demineralizing medium (pH 4.4, 3 h immersion).

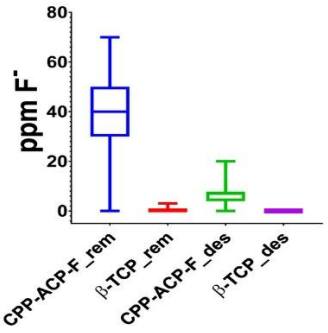
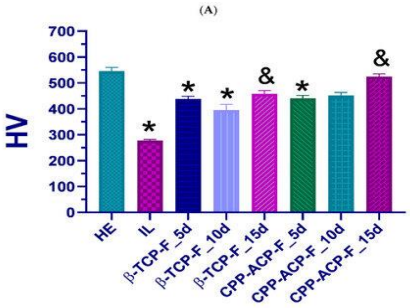


Figure 3. Cumulative release of fluoride ions from enamel surfaces treated with varnish and paste over the 15-day pH cycling treatment, shown as a box-and-whisker plot

Vickers hardness

The average Vickers hardness (HV ± SD) for all groups, measured at 5, 10, and 15 days, is summarized in **Table 1**. Representative indentations and statistical significance are displayed in **Figure 4**.



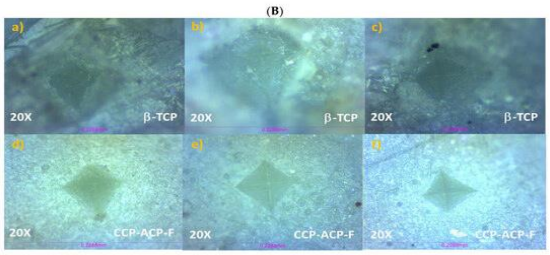


Figure 4. (A) Vickers hardness values of enamel specimens in each group. *p < 0.05 versus Group I (HE, healthy enamel); &p < 0.05 versus Group II (IL, initial lesion).* (B) Representative surface indentations: (a–c) β-TCP-F treatment at 5, 10, and 15 days; (d–f) CPP-ACP-F treatment at 5, 10, and 15 days

Table 1. Vickers hardness (mean ± SD) of human enamel specimens

Study Cohorts	Surface Hardness (HV)
	Initial (0 Days)
Cohort A: HE	533.8 ± 14.8 HV
Cohort B: IL	281.0 ± 04.1 HV
Cohort C: β-TCP-F	
Cohort D: CPP-ACP-F	

Group I: HE (healthy enamel), Group II: IL (initial lesion), Group III: β-TCP-F (β-tricalcium phosphate + NaF), Group IV: CPP-ACP-F (casein phosphopeptide–amorphous calcium phosphate + NaF).

Hardness of untreated enamel (Group I) was 533.8 ± 14.8 HV, which decreased to 281.0 ± 04.1 HV after artificial demineralization (Group II: IL). Surfaces treated with β-TCP-F or CPP-ACP-F demonstrated a recovery in hardness, ranging from 406.3 to 515.2 HV during pH cycling. The increase reached statistical significance only after 15 days, with β-TCP-F achieving 473.6 ± 12.8 HV and CPP-ACP-F achieving 515.2 ± 10.7 HV.

Surface roughness

Surface roughness (Ra ± SD) values for all groups are provided in **Table 2**, and typical profilometry images are shown in **Figure 5**. Initially demineralized enamel (Group II: IL) exhibited higher roughness (Ra = 1.33 ± 0.14 µm) than healthy enamel (Group I: HE, Ra = 0.88 ± 0.08 µm).

After 5 days of β-TCP-F treatment, roughness remained similar to the untreated lesion (Ra = 1.358 ± 0.25 µm). Continued treatment gradually reduced roughness, reaching Ra = 1.29 ± 0.13 µm at 10 days and Ra = 1.193 ± 0.14 µm at 15 days. In contrast, CPP-ACP-F significantly decreased roughness from day 5 (Ra = 0.72 ± 0.05 µm) and maintained this low value through day 15 (Ra = 0.76 ± 0.05 µm), approximating healthy enamel levels.

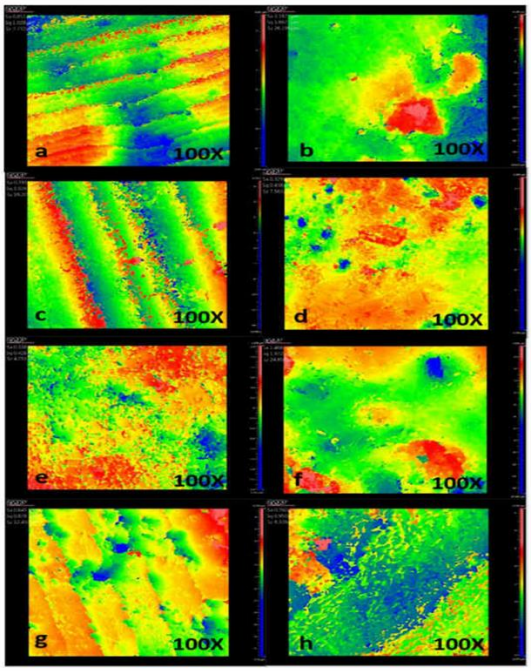


Figure 5. Representative 3D profilometry images: (a) HE; (b) IL; (c–e) CPP-ACP-F at 5, 10, 15 days; (f–h) β-TCP-F at 5, 10, 15 days

Table 2. Surface roughness (Ra ± SD) of enamel specimens

Study Cohorts	Surface Roughness (µm)
	Initial (0 Days)
Cohort A: HE	0.88 (±0.08)
Cohort B: IL	1.33 (±0.14)
Cohort C: β-TCP-F	
Cohort D: CPP-ACP-F	

Ra = arithmetic mean roughness. Group I: HE; Group II: IL; Group III: β-TCP-F; Group IV: CPP-ACP-F.
Statistical differences were determined using one-way ANOVA.

Wettability

The hydrophilic properties of enamel surfaces were evaluated through water contact angle (WCA) measurements, presented as mean ± SD in Table 3 and illustrated in **Figure 6**. All tested enamel specimens, including controls and treated groups, displayed water-attracting behavior, indicated by WCA values under 90°. Differences, however, were apparent among groups. Healthy enamel (Group I: HE) demonstrated the lowest WCA (73.65°), showing higher affinity for water than demineralized enamel (Group II: IL), which had a WCA of 80.82°.

Treatment of demineralized enamel with fluoride-containing agents increased surface wettability to levels comparable to healthy enamel. Specifically, β-TCP-F-treated enamel gradually decreased in WCA over time, reaching 71.01° at 15 days. In contrast, CPP-ACP-F-treated enamel initially dropped to 60.42° after 5 days, then gradually rose to 65.24° by the end of the 15-day treatment period, indicating a dynamic modification of surface hydrophilicity.

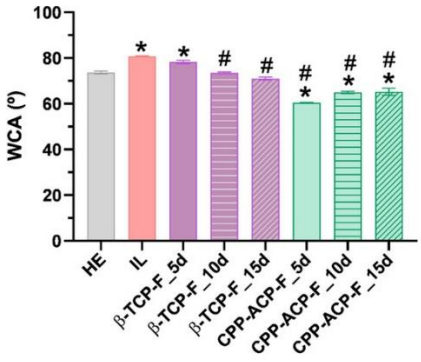


Figure 6. Water contact angles (°) for enamel surfaces across experimental groups. Symbols * and # indicate statistically significant differences ($p < 0.05$)

Table 3. WCA (°) values for enamel specimens (mean ± SD)

Study Cohorts	Wettability Angle (°)
	Initial (0 Days)
Cohort A: HE	73.65 ± 0.72
Cohort B: IL	80.82 ± 0.15
Cohort C: β-TCP-F	
Cohort D: CPP-ACP-F	

Group I: HE (healthy enamel), Group II: IL (initial lesion), Group III: β-TCP-F (β-tricalcium phosphate + NaF), Group IV: CPP-ACP-F (casein phosphopeptide–amorphous calcium phosphate + NaF).

Scanning electron microscopy

Surface morphology observed via SEM revealed bright particles of 1–3 µm on healthy enamel. Demineralized enamel (IL) exhibited larger dark regions and microcracks. After 15 days of CPP-ACP-F treatment, enamel displayed pores approximately 5 µm in diameter, with chains of 0.5 µm particles likely representing residual paste adhering to the surface. This irregular surface topography may facilitate bacterial adhesion due to the increased available area.

Enamel treated with β-TCP-F for 15 days appeared smoother and largely pore-free, displaying a striated pattern consistent with brush application. Small spherical residues were also visible. This even surface likely contributed to the observed increase in hardness and reduced bacterial colonization **(Figure 7)**.

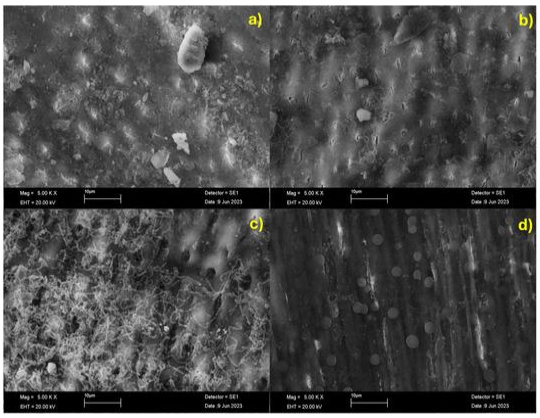


Figure 7. SEM images of (a) healthy enamel, (b) initial lesion, (c) CPP-ACP-F-treated enamel (15 days), and (d) β-TCP-F-treated enamel (15 days)

Biofilm formation

Biofilm formation by S. mutans was quantified via the MTT assay (absorbance at 570 nm), as shown in **Figure 8**. Demineralized enamel (IL) showed significantly higher bacterial adhesion compared to healthy enamel (HE).

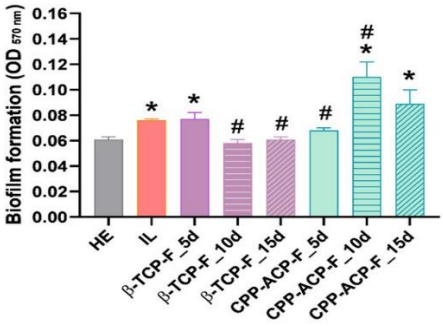


Figure 8. S. mutans biofilm formation on enamel surfaces after 5, 10, and 15 days of treatment with β-TCP-F or CPP-ACP-F. $p < 0.05$ versus HE; # $p < 0.05$ versus IL

For β-TCP-F, no reduction in biofilm formation was detected at 5 days, indicating minimal protective effect at this stage. By 10 and 15 days, a marked decrease in bacterial colonization was observed, comparable to healthy enamel. Conversely, CPP-ACP-F demonstrated strong anti-biofilm activity after 5 days, but this effect diminished at 10 and 15 days, suggesting limited durability of protection (Table 4).

Table 4. Comparative physicochemical data for β-TCP-F and CPP-ACP-F-treated enamel

Treatment & Time	Fluoride Content (ppm) *	Surface Hardness (HV) &	Surface Texture (µm) +	Wettability Angle (°) #
β-TCP-F 5 Days	--	448.2 ± 09.8	1.358 ± 0.25	78.26 ± 0.72
β-TCP-F 10 Days	--	406.3 ± 22.1	1.29 ± 0.13	73.54 ± 0.40
β-TCP-F 15 Days	--	473.6 ± 12.8	1.193 ± 0.14	71.01 ± 0.69
CPP-ACP-F 5 Days	--	448.1 ± 10.9	0.72 ± 0.05	60.42 ± 0.18
CPP-ACP-F 10 Days	--	464.6 ± 11.6	0.68 ± 0.01	63.95 ± 0.21
CPP-ACP-F 15 Days	72 sol rem	515.2 ± 10.7	0.76 ± 0.05	65.24 ± 1.57

*: cumulative fluoride release, NaF, ppm over 15 days
&: Vickers hardness, HV
+: surface roughness, Ra (µm)
#: water contact angle, WCA (°)*

Discussion

The present investigation aimed to assess the enamel remineralization capabilities of two fluoride-containing treatments. In vitro experiments play a critical role in generating preliminary data, allowing researchers to predict the potential clinical performance of novel materials, delivery vehicles, or therapeutic strategies [30,31]. Such models provide insights into bioequivalence and offer a controlled setting to estimate how a product may behave in clinical conditions.

Recent progress in remineralization therapy has focused on maintaining prolonged supersaturation of calcium, phosphate, and fluoride ions at demineralized sites, thereby creating stable delivery systems to support mineral deposition. Cyclical pH models are commonly used to evaluate these approaches, as they closely replicate the fluctuating pH environment found in the oral cavity [23,25]. These models alternate between demineralizing solutions (pH 4.4, 21 h) and remineralizing solutions (pH 7.0, 3 h) [18,21], making them a widely accepted in vitro method for simulating enamel mineral loss and recovery [26]. The present study adopted this well-characterized cyclical pH model due to its precision, reduced variability, and lower sample size requirements. It allowed for the controlled investigation of two widely used fluorine-based agents in pediatric dentistry: β-TCP-F and CPP-ACP-F, delivered as Clinpro™ White Varnish (22,600 ppm NaF) and Mi Paste Plus™ (900 ppm NaF), respectively. Their effects were evaluated on human enamel over 5, 10, and 15 days.

Amorphous calcium phosphate (ACP) functions as a key precursor in mineral formation for both teeth and bones. As a hydroxyapatite precursor, ACP provides calcium and phosphate ions in highly hydrated clusters, which can be effectively delivered to repair enamel defects. When combined with casein phosphopeptide (CPP), these ions are stabilized and guided to the tooth surface. CPP molecules, carrying four to seven phosphate groups, bind ACP nanoclusters, enhancing their bioavailability. The inclusion of fluoride further facilitates mineral uptake by enamel [9].

In comparison, β-tricalcium phosphate (β-TCP-F), present in Clinpro™ White Varnish, releases calcium, phosphate, and a high fluoride concentration (22,600 ppm, equivalent to 50 mg) upon contact with saliva, which gradually breaks down the varnish matrix. The formulation also contains xylitol, known to reduce biofilm accumulation by inhibiting Streptococcus mutans and Lactobacillus acidophilus. While primarily indicated for dentin hypersensitivity, xylitol's effects on initial caries lesions are under evaluation. Some studies suggest that polyol compounds like xylitol or sorbitol can form Ca^{2+}-polyol complexes in saturated calcium sulfate solutions, potentially increasing calcium bioavailability and promoting deeper enamel remineralization. However, the precise concentration of xylitol in Clinpro™ White Varnish is not disclosed by the manufacturer [32]. The different modes of action of CPP-ACP-F and β-TCP-F were thus directly compared in this study using the in vitro cyclical pH model.

To replicate oral dynamics, fluoride ion concentrations in the demineralizing and remineralizing solutions were measured daily over the 15-day cycle. This procedure provides a perspective on systemic fluoride exposure resulting from saliva containing residual agents, a consideration particularly relevant for pediatric patients. Our measurements revealed that the average fluoride content in the demineralizing solutions for β-TCP-F was 40 ppm, substantially higher than that for CPP-ACP-F, reflecting the much higher fluoride load in the varnish compared to the paste (22,600 vs. 900 ppm). While the elevated fluoride may enhance in vitro remineralization, it also raises clinical concerns. Excess fluoride intake, particularly in children aged 3–8 years, may increase the risk of dental fluorosis during permanent tooth development [21].

Previous in vitro studies using a 96 h demineralization model did not report these differences and found comparable outcomes between β-TCP-F and CPP-ACP-F, though they used different ion concentrations (30–90 mM) [33]. These variations highlight the need for future studies to investigate how altering treatment concentrations might influence fluoride levels and the effectiveness of enamel remineralization.

Currently, the capacity of remineralizing agents to treat early-stage carious lesions in vitro [18,26] and to mitigate erosive enamel wear [26] is well established. Although previous studies support the effectiveness of CPP-ACP-F in promoting dental remineralization—especially when combined with fluoride—our study provides a distinct contribution by examining the specific surface alterations induced on artificially demineralized human enamel following treatment. To thoroughly assess remineralization efficacy, both surface roughness and hardness were measured, as these parameters are critical indicators of enamel structural integrity and functional resilience [34].

Vickers hardness testing was employed to quantify the enamel's resistance to indentation under a standardized 10 N load across all groups. Baseline measurements confirmed that healthy enamel exhibited significantly higher hardness compared to demineralized enamel, reflecting the loss of mineral ions during demineralization and the consequent weakening of the enamel matrix. After applying CPP-ACP-F and β-TCP-F to demineralized enamel and exposing the samples to cyclical mineralizing–demineralizing pH conditions for 5, 10, and 15 days, a partial recovery of hardness was observed. The CPP-ACP-F-treated specimens demonstrated a slightly greater improvement in hardness than those treated with β-TCP-F, suggesting a modestly superior effect in restoring the enamel's mechanical properties.

These findings, which show partial recovery of enamel hardness with both treatments and a slightly stronger effect with CPP-ACP-F, are consistent with the results of Bhat *et al.* [34], who also reported CPP-ACP-F as more effective. While methodological differences exist—Bhat *et al.* utilized a 72 h demineralization period followed by 28 days of treatment, and measured microhardness—the overall agreement indicates that both microhardness and macrohardness are reliable indicators of enamel resistance to indentation. This convergence reinforces the understanding of the remineralizing potential of these agents.

Regarding surface roughness, healthy enamel displayed a lower Ra value (0.88 μm) than demineralized enamel (1.33 μm), reflecting surface erosion caused by acidic exposure (pH = 4.4) during demineralization [4]. Treatment with CPP-ACP-F for 15 days resulted in a substantial reduction of roughness to Ra = 0.76 μm, reaching values comparable to healthy enamel and indicating effective remineralization. Conversely, β-TCP-F treatment produced only a moderate reduction in roughness (Ra = 1.193 μm),

suggesting a relatively lower remineralization effect [22]. These improvements in roughness can be attributed to ionic deposition and adsorption onto the irregular enamel surfaces, filling defects created during demineralization.

Surface wettability, an important property influenced by roughness and surface energy, was assessed using deionized water. All experimental surfaces demonstrated a hydrophilic character (WCA < 90°), implying potential enhanced resistance to acidic challenges. Using Vogler's threshold of WCA 65° [35] to distinguish biologically hydrophilic versus hydrophobic surfaces, only the CPP-ACP-F-treated surfaces fell slightly below this limit, classifying them as hydrophilic. This hydrophilicity may correlate with bacterial colonization patterns, as surface wettability—modulated by microroughness, chemical composition, and intrinsic surface energy—affects adhesion [36]. Higher microroughness and hydrophilicity generally facilitate bacterial attachment.

As anticipated, Streptococcus mutans biofilm formation was greater on demineralized enamel than on healthy enamel. After 15 days of β-TCP-F treatment, biofilm levels on previously demineralized surfaces approached those of healthy enamel, likely due to the restoration of surface properties, including wettability. In contrast, CPP-ACP-F-treated enamel initially exhibited comparable anti-biofilm effects at 5 days, but this protection diminished at 10 and 15 days. The more hydrophilic nature of CPP-ACP-F-treated surfaces may have promoted initial bacterial adhesion and subsequent proliferation of S. mutans. It should be noted that bacterial colonization is a complex phenomenon influenced by factors such as cell size, cell envelope composition, and environmental conditions (pH, temperature, ions, and biomolecules).

Consistent with previous research [16, 37], the results from this study show that both CPP-ACP-F paste and β-TCP-F varnish reduce enamel susceptibility to acidic attacks and support the remineralization of surface enamel, thereby preventing early-stage caries. In a similar investigation, Tibba *et al.* [38] compared Silver Diamine Fluoride (SDF, Riva Star) and sodium fluoride varnishes. They reported that Riva Star improved enamel hardness and that both varnishes lowered oral biofilm formation by 25–35%. In our study, β-TCP-F applied for 10 and 15 days and CPP-ACP-F applied for 5 days protected demineralized enamel surfaces from early colonization by S. mutans, reaching levels of biofilm formation comparable to healthy enamel. This suggests that both treatments can inhibit initial bacterial adhesion, although the duration and mechanisms of their effects differ from SDF and conventional NaF varnishes.

Hardness measurements confirmed that applying fluoride-based remineralizing agents to demineralized enamel markedly increased resistance to indentation. The CPP-ACP-F paste mimics the mineralization function of natural saliva by supplying calcium and phosphate ions that deposit on the enamel surface, restoring structural integrity. Nevertheless, fully replicating the complexity of in vivo biofilm interactions and ionic flux in an in vitro environment is challenging. The lack of a mature biofilm and the artificially controlled supersaturation of ions could have influenced the observed remineralizing effect of CPP-ACP-F [39].

Although CPP-ACP-F can simulate saliva's effect, real oral conditions are far more complex, with biofilms actively modifying ion distribution. The laboratory conditions of this study may have increased ion availability, potentially enhancing the apparent efficacy of CPP-ACP-F compared to natural oral environments. These limitations highlight the need for caution when extrapolating in vitro results. To gain more clinically relevant insights, future research should incorporate in vivo models or controlled clinical trials that account for saliva variability, diet, oral hygiene, and microbiota dynamics.

Conclusions

The present study demonstrates that both β-TCP-F varnish and CPP-ACP-F paste can partially reverse the effects of experimental enamel demineralization. Both agents increased enamel hardness to levels similar to healthy enamel. Regarding surface roughness, only the CPP-ACP-F paste restored values to those comparable to intact enamel. Microbiological testing revealed that β-TCP-F varnish maintained long-term protection against S. mutans biofilm, whereas the effect of CPP-ACP-F paste was significant only during the early phase of treatment. Fluoride ion measurements suggest that caution is needed with β-TCP-F varnish, as its high fluoride release may limit frequent use in young children to avoid the risk of dental fluorosis.

Overall, these remineralizing treatments are effective in preventing enamel deterioration caused by demineralization and could be recommended for high-caries-risk patients or as preventive measures. Considering the widespread prevalence of dental caries, understanding and validating the efficacy of remineralizing products is critical for evidence-based dental care. This study contributes further support for the use of β-TCP-F and CPP-ACP-F in managing early enamel lesions.

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