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The Impact of Casein Phosphopeptide-Amorphous Calcium Fluoride Phosphate on Remineralization of Caries Lesions in Relation to the Mineral Distribution within the Lesion

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ABSTRACT

Introduction: Early enamel caries lesions are reversible through non-invasive approaches that enhance natural remineralization, particularly using agents like fluoride and biomimetic calcium-phosphate compounds likeCasein Phosphopeptide-Amorphous Calcium Fluoride Phosphate (CPP-ACFP).

Objective: The current in vitro research aimed to assess the remineralization efficacy of CPP-ACFP on enamel caries lesions and assess the influence of baseline mineral distribution on treatment outcomes.

Materials and Methods: Sixty premolar enamel samples were prepared and demineralized using a methylcellulose-based protocol to simulate early carious lesions. Specimens were randomly allocated into three groups (n=20): CPP-ACFP (MI Paste Plus), 5% sodium fluoride varnish (PreviDent®) as positive control group, and deionized water as negative control group. Interventions were applied over four days, followed by a four-day immersion in artificial saliva. Remineralization outcomes were quantitatively assessed using Transverse Microradiography (TMR), focusing on alterations in integrated mineral loss (ΔΔZ), lesion depth (ΔLD), and surface zone maximum mineral density (ΔSZmax).

Results: CPP-ACFP treatment significantly improved surface zone mineral density (ΔSZmax = +11.89%) compared to fluoride varnish (+4.76%) and deionized water (+7.40%), with statistically significant variations ($p = 0.01$). While lesion depth reductions (ΔLD) were not statistically variation amongst the groups ($p = 0.82$), CPP-ACFP also demonstrated a substantial reduction in integrated mineral loss (ΔΔZ = −11.9 vol%·μm) relative to controls.

Conclusions: CPP-ACFP showed significant potential in promoting enamel remineralization, increasing surface zone mineral density, and was found to be like fluoride varnish in affecting lesion depth, thus supporting its inclusion in caries management protocols.

Keywords: Casein Phosphopeptide-Amorphous Calcium Fluoride Phosphate (CPP-ACFP); Fluoride varnish; Enamel remineralization; Transverse microradiography (TMR).

INTRODUCTION

Dental caries remains a pervasive global health concern, fundamentally driven by dynamic processes of demineralization and remineralization [1]. It is initiated by the metabolic activity of acidogenic bacteria that ferment dietary carbohydrates, producing acids which diffuse into the enamel and dissolve mineral content. This demineralization, if not reversed, can progress to cavitation, compromising tooth structure and function. The natural counter-process, remineralization, includes the redeposition of minerals, mostly calcium and phosphate, into the structure of the enamel structure, often aided by therapeutic agents [23].

The fluoride varnish is the most frequently employed remineralizing agent. Fluoride works by enhancing the formation of fluorapatite, a less soluble mineral phase than hydroxyapatite, thereby increasing the enamel's resistance to acid dissolution and promoting remineralization [1]. As the pH increases, fluorhydroxyapatite crystals, which are bigger and fresher and include more fluoride, are created. This increases remineralization and decreases demineralization [4]. Investigations have demonstrated the efficiency of fluoride varnishes in reducing demineralization and promoting remineralization, particularly in early lesion [4–6]

However, fluoride's effectiveness is often limited by its superficial action, with deeper lesion zones showing minimal response due to diffusion barriers posed by hypermineralized surface layers which act as a barrier for minerals deposition within subsurface layer [3]. To overcome these limitations and enhance the remineralization process, alternative strategies have been explored, including biomimetic agents such as Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP). It is made from the casein protein found in milk and serves as a store for bioavailable calcium and phosphate ions [7].

It is proposed to promote remineralization by maintaining a supersaturated environment at the tooth surface, driving mineral redeposition into demineralized enamel[8]. In vitro and in vivo investigations have demonstrated the anticariogenic potential and remineralizing qualities of CPP-ACP produced from the milk protein casein[7]. Remineralization is facilitated by the CPP-ACP, which serves as a reservoir of bioavailable calcium and phosphate[9]. The combination of CPP-ACP with fluoride (CPP-ACFP) has also been developed and, according to some reports, may offer enhanced remineralization benefits compared to CPP-ACP alone[10]. Also, by keeping enamel minerals in a supersaturated condition, it encourages the remineralization of dental caries lesions while simultaneously preventing cariogenic bacteria from colonizing tooth surfaces [11].

Despite promising results, the effectiveness of remineralizing agents, including CPP-ACFP, may vary depending on the baseline mineral characteristics of enamel lesions [1213]. Lesions with different mineral distributions, such as variations in surface zone mineralization, porosity, and depth, can exhibit differential responsiveness to remineralization treatments [14]. Lesions with more porous or less mineralized surfaces may be more permeable, potentially allowing deeper penetration of remineralizing agents, whereas hypermineralized or intact surface layers may inhibit ion diffusion and limit remineralization to superficial zones [15]. This suggests that the inherent structure and mineral composition of the lesion at baseline could significantly modulate treatment outcomes.

To investigate these dynamics and precisely quantify the effects of remineralization treatments, Transverse Microradiography (TMR) is utilized as a primary analytic technique. TMR is widely regarded as a gold standard in caries research for its ability to precisely quantify mineral content, lesion depth, and changes in mineral distribution across the lesion profile [16].

The purpose of the current research was to evaluate the effectiveness of CPP-ACFP in remineralizing enamel caries lesions and to determine whether this efficacy is modulated by the lesions' baseline mineral distribution, utilizing Transverse Microradiography for quantitative assessment.

MATERIALS AND METHODS

Materials

The materials used in this study are presented in **(Tabel 1).**

Table 1: Tested materials and their composition:

Intervention	Commercial Product	Ingredients	Manufacturer
CPP-ACFP	MI Paste Plus	Water, glycerol, Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP), D-sorbitol, CMC propylene glycol, silicone and titanium dioxide, xylitol, phosphoric acid, flavor, sodium saccharin, ethyl propyl butyl p-hydroxybenzoate, 900 ppm sodium fluoride	GC Corporation, Tokyo, Japan
Fluoride	PreviDent®	5% Sodium Fluoride, 22,600 ppm fluoride,	Colgate Oral

Varnish (FV)	Varnish	hydrogenated rosin, ethyl alcohol, hexadecane phosphate, sodium fluoride, flavor, citric acid, polysorbate 80, sucralose, xylitol	Pharmaceuticals, New York, NY
Deionized Water (DIW)		None (inert control)	Laboratory-prepared

Study design

This in vitro study was designed to evaluate the remineralization potential of CPP-ACFP on enamel caries lesions and to investigate whether lesion baseline mineral distribution modulates its efficacy.Artificial enamel lesions were createdto achieve abaseline mineral distribution, while maintaining comparable integrated mineral loss. CPP-ACFP was applied as the test intervention, 5% sodium fluoride varnish (FV) served as the positive control, and deionized water (DW) as the negative control. Remineralization outcomes were analyzed using digital transverse microradiography (TMR-D) to quantify mineral loss, lesion depth, and surface zone mineral density.

Sample size calculation

The sample size was finalizedaccording tothepriorstudy [(5)].Utilizing estimates derived from the standard deviation of ΔΔZ values reported in prior enamel lesion remineralization research, a minimum detectable effect size (Cohen's d) of 0.909 was targeted. Assuming a two-sided test with a level of 0.05 and statistical power (1−β) of 80%, a sample size of n = 20 specimens per group per lesion type was calculated to be sufficient. This results in a total of 180 specimens across all groups and lesion models (3 lesion types × 3 interventions × 20 specimens).The calculation was verified using power analysis software (e.g., G*Power 3.1), which confirmed that a sample size of 20 per subgroup achieves appropriate power for medium-to-large effect sizes in a two-way ANOVA framework.

Specimen preparation

Enamel specimens were prepared from extracted premolars which were extracted for orthodontic purposes. Each crown was sectioned into 5 × 5 mm slabs utilizing a low-speed diamond saw (Isomet, Buehler, IL, USA). The enamel surfaces were ground and polished to a uniform finish using sequential silicon carbide papers (up to 4000 grit) under water cooling. Specimens with defects, cracks, or surface anomalies were excluded. An acid-resistant nail varnish was applied to create a 4 mm × 4 mm experimental window in the center of each specimen, preserving sound enamel on either side for reference.

Lesion formation

Methylcellulose (MeC) protocol was used to manufacture artificial caries lesions. Each enamel specimen (n = 60) was immersed for 7 days at 37 °C in a two-phase system comprising a 4% methylcellulose gel (Sigma-Aldrich, M0512) overlaid with an equal volume of 0.1 M lactic acid, pH-adjusted to 4.6 using potassium hydroxide (KOH)[(17)].

Following lesion formation, half of the experimental window on each specimen was covered with acid-resistant nail varnish to preserve a baseline lesion area for subsequent transverse microradiographic comparison. All samples were then kept in 100% humidity at 4 °C until interventions were applied.

Intervention application

Specimens were randomly allocated to one of three intervention groups (n = 20 pergroup):

1. CPP-ACFP (Test Group): MI Paste Plus (GC Corp., Tokyo, Japan) was administrated topically using a microbrush for 3 minutes daily over 4 consecutive days. After application, specimens were stored in artificial saliva between treatments[(18)].
2. 5% sodium fluoride varnish (positive control): PreviDent® Varnish (Colgate) was applied according to manufacturer instructions and then removed after 6 hours using chloroform-moistened swabs[(19)].
3. Deionized water (negative control): applied similarly to CPP-ACFP, but using deionized water with no active ingredients[(20)].

Remineralization phase

Following the final intervention, specimens were immersed in artificial saliva (1.45 mM$CaCl_2$, 5.4 mM KH_2PO_4, 14.9 mMKCl, 28.4 mMNaCl, 3.08 mMNaN_3, and 2.2 g/L porcine gastric mucin, pH 7.0) at room temperature under constant agitation (150 rpm) for 4 days[(21)]. The treatment window was half-covered using the nail polish prior to this phase to preserve the post-treatment baseline.

Transverse Microradiography (TMR)
A section (~100 µm thick) was taken from each specimen across the lesion and sound enamel with a Silverstone-Taylor microtome. Sections were radiographed using a TMR-D system under standardized conditions. Mineral parameters were calculated using dedicated software calibrated against an aluminum step wedge. Transverse microradiography (TMR) was employed to quantify alterations in integrated mineral loss (ΔΔZ), lesion depth (ΔLD), and surface zone mineral density (ΔSZmax)[16].
Changes in these parameters were computed to evaluate remineralization[16]:
$\Delta\Delta Z = \Delta Z\_post - \Delta Z\_baseline$
$\Delta LD = LD\_post - LD\_baseline$
$\Delta SZmax = SZmax\_post - SZmax\_baseline$

Statistical analysis
Two-way ANOVA was utilized to assess the impacts of intervention type and lesion protocol on ΔΔZ, ΔLD, and ΔSZmax. Pairwise comparisons were madeby Fisher's Protected Least Significant Differences (PLSD) with a significant level of $\alpha = 0.05$.

RESULTS
Integrated Mineral Loss (ΔΔZ)
Treatment the lesions with CPP-ACFP resulted in a mean reduction in mineral loss of −11.9 vol%·µm, compared to −18.2 for FV and −23.6 for DW. These results suggest that while CPP-ACFP promoted remineralization, its effect size was intermediate between that of FV and DW in this model. The differences between treatments were statistically significant ($p < 0.01$), with DW showing the greatest reduction, potentially due to the absence of surface occlusion by high-viscosity agents.

Lesion Depth (ΔLD)
There were no discernible statistically significant variations among the treatment groups in the lesions ($p = 0.82$). The mean changes in lesion depth were: CPP-ACFP: −11.9 µm, FV: −18.2 µm, and DW: −23.6 µm. These findings indicate that while all treatments reduced lesion depth to some extent, the reductions were not markedly different from one another statistically.

Surface Zone Mineral Density (ΔSZmax)
Created lesions showed a significant increase in surface zone mineral density after remineralization. CPP-ACFP treatment led to a ΔSZmax of +11.89%, significantly greater than FV (+4.76%) and DW (+7.40%) ($p = 0.01$). This suggests that CPP-ACFP facilitated effective surface remineralization, potentially enhancing the protective layer at the enamel interface. Means and standard deviations are presented in **(Table 2)**.

Table 2: Remineralization outcomes following treatment with CPP-ACFP, fluoride varnish, or deionized water

Treatment	ΔΔZ (vol%·µm)	ΔLD (µm)	ΔSZmax (%)	Statistical Significance
CPP-ACFP	−11.9 ± 12.1	−11.9	+11.89	Reference group
FV	−18.2 ± 15.3	−18.2	+4.76	ΔΔZ: $p < 0.01$ ΔLD: ns ($p = 0.82$) ΔSZmax: $p = 0.01$
DW	−23.6 ± 15.6	−23.6	+7.40	ΔΔZ: $p < 0.01$ ΔLD: ns ($p = 0.82$) ΔSZmax: $p = 0.01$

ΔΔZ: Alteration in integrated mineral loss (vol%·µm), ΔLD: Alteration in lesion depth (µm), ΔSZmax: Alteration in surface zone maximum mineral density (%), ns: Not significant.
FV: fluoride varnish, DW: deionized water.

DISCUSSION
The current research aimed to evaluate the remineralization efficacy of Casein Phosphopeptide-Amorphous Calcium Fluoride Phosphate (CPP-ACFP) on enamel caries lesions created using the Methylcellulose model, utilizing transverse microradiography (TMR) for quantitative assessment.
All specimens used in this study were subjected to the Methylcellulose (MeC) lesion formation protocol, chosen for its ability to simulate early enamel caries with a relatively intact surface layer and controlled subsurface porosity[22]. This standardized model provided consistent baseline lesion characteristics across all treatment groups, enhancing the reliability of interventional comparisons[23]. The uniformity of lesion morphology and mineral distribution within this model allowed for a focused evaluation of remineralization dynamics without the confounding effects of structural heterogeneity[24].

For determining lesion depth and mineral density, TMR is regarded as the gold standard 2D approach. It is a dependable and extremely sensitive destructive method for measuring the thickness and level of surface layer mineralization, in addition to the quantity of mineral gain or loss from dental substrate [16].

In our study, CPP-ACFP achieved greater increases in surface zone mineral density (ΔSZmax) compared to fluoride varnish, confirming its effectiveness in enhancing enamel remineralization. This was agreed with the findings of Tuloglu et al. [12], who found that fluoride containing CPP-ACP varnish offered the greatest resistance against acid attack of the dental enamel when compared to fluoride varnish, due to higher mineral gain at the enamel surface. Additionally, this was in line with Varma et al. [9], who found that CPP–ACFP enhanced the remineralization artificial enamel lesions.

By preserving their amorphous state (ACP), CPP stabilizes Ca and P, increasing the amount of minerals in the enamel surface [8]. Furthermore, it promotes the development of calcium phosphate ions, andthese ions operate more strongly in the depth of the enamel, causing remineralization in the lesion's body [25].

Additionally, it was proposed that the calcium and phosphorus produced by CPP-ACP can create nanocomplexes in the biofilm next to the original caries lesion, strengthening the enamel's defenses against further acid attacks[26]. Moreover, CPP promotes the remineralization of intimal enamel lesions in along with boosting the integration of fluoride throughout the biofilm [8]. Consequently, the CPP-ACP complex serves as a calcium and phosphate reservoir, raising the amount of these free ions and assisting in the preservation of a supersaturated condition with regard to tooth enamel [27]. It has a stronger remineralizing capability when the CPP-ACP coupled with fluoride forms a stable amorphous phase of fluorinated calcium phosphate [28]. According to some research, the combination of fluoride within the CPP-ACP has better remineralization benefits [29–31].

Also, our findings align with those from Ma et al.[7], who conducted a systematic review and meta-analysis showing that CPP-ACFP significantly improves enamel surface microhardness and fluorescence recovery across clinical and in vitro studies.

Saliva cannot raise calcium and phosphate release levels on its own, despite having some remineralization capacity.Calcium and phosphate ions have to initially pierce the enamel's outer layer in order for mineral deposition to take place inside the lesion's body.This clarifies the reason why the CPP-supported metastable in nature calcium phosphate remedies are like effective remineralizing remedies: they may sustain the substantial concentration gradient through the lesion by producing greater calcium and phosphate ions, involving $CaHPO_4$, which are able to use the acid produced throughout enamel lesion remineralization[2].

However, these findings disagreed with those of de Souza et al. [32], indicating that ACP and F could undergo a chemical interaction that renders the two inorganic constituents ineffective and/or decreases the amount of soluble fluoride available.

Fluoride remains the gold standard in caries prevention due to its ability to facilitate fluorapatite formation and inhibit enamel demineralization. However, its effectiveness is limited by the availability of salivary calcium and phosphate ions[3]. In contrast, CPP-ACFP delivers bioavailable minerals directly to the tooth surface, enhancing remineralization independent of salivary composition [7].According to Pithon et al. [33], fluoride varnish without CPP-ACP was less successful than fluoride varnish with additional CPP-ACP, in decreasing the depth of caries lesions.

On the other hand, our results disagreed with those of Bandekar et al. [13], who concluded that fluoride varnish exhibits a stronger capacity for remineralization of initial enamel caries lesions compared to CPP-ACFP.Also, Lata et al. [34] stated that, when compared to fluoride varnish alone, a mixture of fluoride and CPP-ACP did not offer any additional remineralization capability at the surface level. Initial enamel carious lesion cannot be remineralized at the subsurface level using fluoride varnish, CPP-ACP, or a combination of the two.

In comparison to the deionized water group (negative control), the fluoride varnish group (positive control) in the present research showed a significant increase in surface zone mineral density after remineralization.It was reported that the fluoride in the oral environment increases the enamel surfaces' resilience to acid by forming fluorapatite and preventing hydroxyapatite from dissolving [35]. The capacity of fluoride to produce calcium fluoride has also been linked to the preventative impact of topical fluoride administration [36].

Even though calcium fluoride leaches readily and gradually when exposed to acid, it keeps minerals from dissolving from enamel by forming a physical barrier on the surface of the enamel [12]. It may additionally serve as a reservoir for fluoride for further reactions, which helps preserve high fluoride concentrations in the oral environment and promote enamel remineralization [37].The present findings are consistent with an investigation by Santos et al. [6] as they discovered that all of the fluoride groups had significantly lower lesion depth values than a control group that didn’t obtain fluoride treatment.

The limitations of this study were in vitro design, which cannot replicate the dynamic conditions of the oral environment, such as salivary flow, microbial activity, and patient variability.The short duration of intervention and remineralization phases limits understanding of long-term effectiveness and stability of mineral deposition. The assessment relies solely on TMR, which does not account for potential clinical factors like esthetic

improvements. Future in vivo studies with longer durations and multiple lesion models as well as other remineralizing agents are recommended.

CONCLUSIONS

Within the limitations of the presentresearch, CPP-ACFP demonstrated significant potential in promoting enamel remineralization, particularly in increasing surface zone mineral density. While its effect on lesion depth was not statistically distinct from fluoride varnish. These findings support the incorporation of CPP-ACFP into caries management protocols, especially for early enamel lesions with defined porosity.

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