J Dent Mater Tech 2026; 15 (3): 99-108
DOI: 10.22038/jdmt.2026.96834.2016

J Dent Mater Tech

Original Article Open Access

Comparative effects of sodium fluoride varnish with and without CPP-ACP addition on *Streptococcus mutans* counts and salivary calcium and phosphate levels in preschool children

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Abstract

Objective: This study compared the effects of 5% sodium fluoride varnish (NaF) with those of 5% NaF varnish containing casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) on *Streptococcus mutans (S. mutans)* counts in dental plaque and salivary calcium and phosphate levels in preschool children.

Methods: This randomized single-blind clinical trial included 33 caries-free children aged 3–6 years who were classified as having a moderate-to-high risk of future caries development according to the American Academy of Pediatric Dentistry (AAPD) caries risk assessment guidelines. The participants were randomly allocated (1:1) to receive either sodium fluoride (NaF) varnish alone (n = 16) or NaF varnish containing CPP-ACP (n = 17). *S. mutans* counts in dental plaque and salivary calcium and phosphate concentrations were assessed at baseline and follow-up intervals. Data were analysed using repeated-measures ANOVA at the significance level of $P < 0.05$.

Results: Plaque *S. mutans* counts changed significantly over time ($P < 0.001$), but did not differ significantly between the two groups ($P > 0.05$). Salivary calcium and phosphate concentrations showed no significant changes over time or differences between groups ($P > 0.05$).

Conclusions: The addition of CPP-ACP to NaF varnish did not provide a statistically significant additional benefit in reducing plaque *S. mutans* counts or changing salivary calcium and phosphate levels compared with NaF varnish alone.

Keywords: Calcium phosphates, Dental caries, Dental plaque, Fluorides, Saliva, *Streptococcus mutans*

Introduction

Early childhood caries (ECC) remains one of the most prevalent chronic conditions in preschool children and continues to be an important public health problem (1). Recent global estimates indicate that untreated caries in primary teeth affects more than 530 million children worldwide (2). ECC is defined as the presence of one or more decayed, missing, or filled primary teeth in children younger than 71 months (3). Among cariogenic bacteria, *Streptococcus mutans (S. mutans)* is strongly associated with the initiation and progression of dental caries because of its acidogenic characteristics (4). The accumulation of cariogenic biofilm on tooth surfaces leads to acid production and subsequent enamel demineralization (5, 6).

The application of 5% sodium fluoride (NaF) varnish remains a widely used preventive strategy for early childhood caries. Recently, interest has grown in modified varnish formulations containing bioactive agents that may enhance remineralization (7). Among these, casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) has attracted considerable attention because of its potential to promote enamel remineralization (8, 9). CPP-ACP stabilizes bioavailable calcium and phosphate ions and, in the presence of fluoride, promotes the formation of fluorapatite (10).

The reservoir of bioavailable ions facilitates the diffusion of calcium and phosphate into subsurface enamel lesions and thus enhances remineralization (11). Some studies have suggested that CPP-ACP may reduce *S. mutans* adhesion to the acquired pellicle and tooth surface by binding to bacterial cells and pellicle proteins, thereby interfering with bacterial attachment and co-aggregation (12, 13).

Previous studies have investigated the effects of fluoride varnishes containing CPP-ACP on

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Accepted: 28 August 2026. Submitted: 24 June 2026

microbiological and mineral-related outcomes in children. Patel et al. (3) compared fluoride varnish, chlorhexidine varnish, and CPP-ACP-containing fluoride varnish in children aged 6–12 years and assessed salivary *S. mutans* at baseline and at 1, 3, and 6 months. They reported significant reductions in *S. mutans* levels with both fluoride varnish and CPP-ACP-containing varnish, with no significant difference between the two fluoride varnish groups. Poureslami et al. (14) evaluated salivary and plaque mineral concentrations 60 minutes after CPP-ACP or fluoride-containing CPP-ACP application in children aged 6–9 years and found no significant differences between the materials for most outcomes.

There is limited evidence on the effectiveness of 5% NaF varnish containing CPP-ACP in preschool children. Therefore, this study compared the effects of NaF varnish with and without CPP-ACP on plaque *S. mutans* and salivary calcium and phosphate concentrations in caries-free children aged 3–6 years at moderate to high caries risk.

Materials and methods

Study design and participants

This randomized, single-blind, parallel-group clinical trial was conducted at the Department of Pediatric Dentistry, School of Dentistry, Shahid Sadoughi University of Medical Sciences, Yazd, Iran. The study protocol was approved by the ethics committee of Shahid Sadoughi University of Medical Sciences (IR.SSU.REC.1396.207) and registered in the Iranian Registry of Clinical Trials (IRCT20140601017935N7). The study was conducted in accordance with relevant ethical principles for research involving human participants. Written informed consent was obtained from the parents or legal guardians of all participating children before enrollment. Participation was voluntary, and the confidentiality of participants' information was maintained throughout the study. The study was reported in accordance with the Consolidated Standards of Reporting Trials (CONSORT) guidelines (15).

A total of 60 children aged 3–6 years were initially examined for eligibility. These children were recruited from kindergartens and preschools in Yazd, Iran, during 2018–2019 using convenience sampling. Among them, 33 caries-free children who were classified as having a moderate-to-high risk of future caries development and met the other inclusion criteria were included in the study. Table 1 summarizes the caries-risk assessment criteria established by the American Academy of Pediatric Dentistry (AAPD), which were used for participant screening (16). Risk factors, protective factors, and disease indicators were evaluated. Each child's overall caries risk (low, moderate, or high) was determined by reviewing all risk factors, protective factors, and disease indicators together, along with the examiner's clinical judgment, as recommended by the AAPD. No numerical cutoff was used to define risk level. Additional inclusion criteria included positive or definitely positive behavior (scores 3 or 4) on the Frankl Behavior Rating Scale (17).

Exclusion criteria involved systemic diseases or medical conditions affecting oral health, a history of milk allergy, professional fluoride treatment within the previous 3 months, antibiotic use within the previous month, the need for antibiotic therapy during the study period, and failure to attend follow-up visits or sample collection sessions. Written informed consent was obtained from the parents or legal guardians of all participants before enrollment.

Randomization and blinding

A total of 33 eligible children were enrolled in the study and randomly assigned to either the 5% sodium fluoride (NaF) varnish group (n = 16) or the 5% sodium fluoride varnish containing casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) group (NaF/CPP-ACP) (n = 17) at an approximately 1:1 ratio.

Allocation concealment was achieved with sequentially numbered, opaque, sealed envelopes that were opened only after eligibility confirmation and enrollment. Due to differences in varnish packaging, the operator was aware of the group allocation; however, the participants, outcome evaluator, and statistical analyst remained blinded to the assigned groups.

Baseline sample collection

Before baseline sampling, participants were instructed to avoid toothbrushing for 24 hours and to refrain from eating or drinking for at least 1 hour before sample collection. Baseline dental plaque and unstimulated whole saliva samples were collected between 9:00 and 11:00 a.m.

Dental plaque was obtained from the buccal surfaces of the maxillary right first and second primary molars using a sterile toothpick and immediately transferred into test tubes containing brain heart infusion (BHI) transport medium (Merck, Darmstadt, Germany). Plaque samples were transported to the microbiology laboratory within 2 hours of collection.

Unstimulated whole saliva (minimum volume, 1 mL) was collected by spitting into sterile containers for 5

Table 1. Table 1. AAPD caries-risk assessment criteria used for participant screening

Category	AAPD caries-risk criterion	AAPD risk category
Risk factors: social/behavioral/medical	Parent/caregiver has lifetime poverty or low health literacy	High risk
	Child has frequent exposure (>3 times/day) to between-meal sugar-containing snacks or beverages	High risk
	Child is a recent immigrant	High risk
	Child has special health care needs	High risk*
	Child frequently uses a bottle or nonspill cup containing natural or added sugars between meals and/or at bedtime	High risk
Risk factors: clinical	Visible plaque on teeth	High risk
	Dental enamel defects	High risk
Protective factors	Optimally fluoridated drinking water or fluoride supplements	Protective
	Daily toothbrushing with fluoridated toothpaste	Protective
	Topical fluoride from a health professional	Protective
	Dental home/regular dental care	Protective
Disease indicators	Noncavitated (incipient/white-spot) caries lesions	High risk
	Visible caries lesions	High risk
	Recent restorations or missing teeth due to caries	High risk
Additional criteria for children aged ≥6 years	Medication associated with reduced salivary flow	High risk
	Low salivary flow	High risk
	Intraoral appliance	High risk
	Defective restorations	High risk

The risk classification for children with special health care needs may vary depending on the specific medical condition and clinical circumstances.

minutes. Saliva samples were transported to the laboratory on the day of collection.

Following baseline assessment and sample collection, oral hygiene instructions and dietary recommendations were provided to all participants and their parents. Parents were encouraged to maintain these practices throughout the study period.

Varnish application

After baseline sampling, the tooth surfaces were cleaned and isolated with cotton rolls, and a saliva ejector was placed. Participants received either NaF varnish (V-varnish™ Premium, Vericom Co., Ltd., Chuncheon, Korea), containing 5% sodium fluoride, or NaF/CPP-ACP varnish (MI Varnish™, GC Corporation, Itabashi-ku, Tokyo, Japan), containing 5% sodium fluoride and 2% RECALDENT™ (CPP-ACP). The varnishes were applied to the tooth surfaces, and dental floss was used to facilitate varnish contact with the proximal surfaces in areas with tight interproximal contacts. Following application, participants were instructed to avoid eating, drinking, and rinsing for 1 hour and to refrain from toothbrushing until the following morning. All varnish applications were performed by the same trained operator, who also provided standardized instructions before and after each application.

Follow-up sampling procedures

Saliva sampling was repeated 1 hour after varnish application, and dental plaque sampling was repeated the following morning. Both plaque and saliva samples were collected 1 month after varnish application again. The same sampling procedures were used at all subsequent time points.

Figure 1 illustrates the study design, participant flow, group allocation, sample collection schedule, exclusions, and subsequent microbiological and biochemical analyses.

Dental plaque microbiological analysis

Dental plaque samples were thoroughly mixed for 30 seconds and then diluted tenfold (from 10^{-1} to 10^{-4}) in sterile phosphate-buffered saline (PBS, pH 7.2).

One milliliter of each dilution was separately inoculated onto freshly prepared mitis salivarius agar supplemented with 300 units of bacitracin and 20% sucrose (15). The plates were incubated at 37 °C in an atmosphere containing 5% CO_2 for 48 hours.

Following incubation, *S. mutans* colonies were identified based on their characteristic frosted-glass appearance on Mitis Salivarius agar. Species identification was confirmed by the presence of Gram-

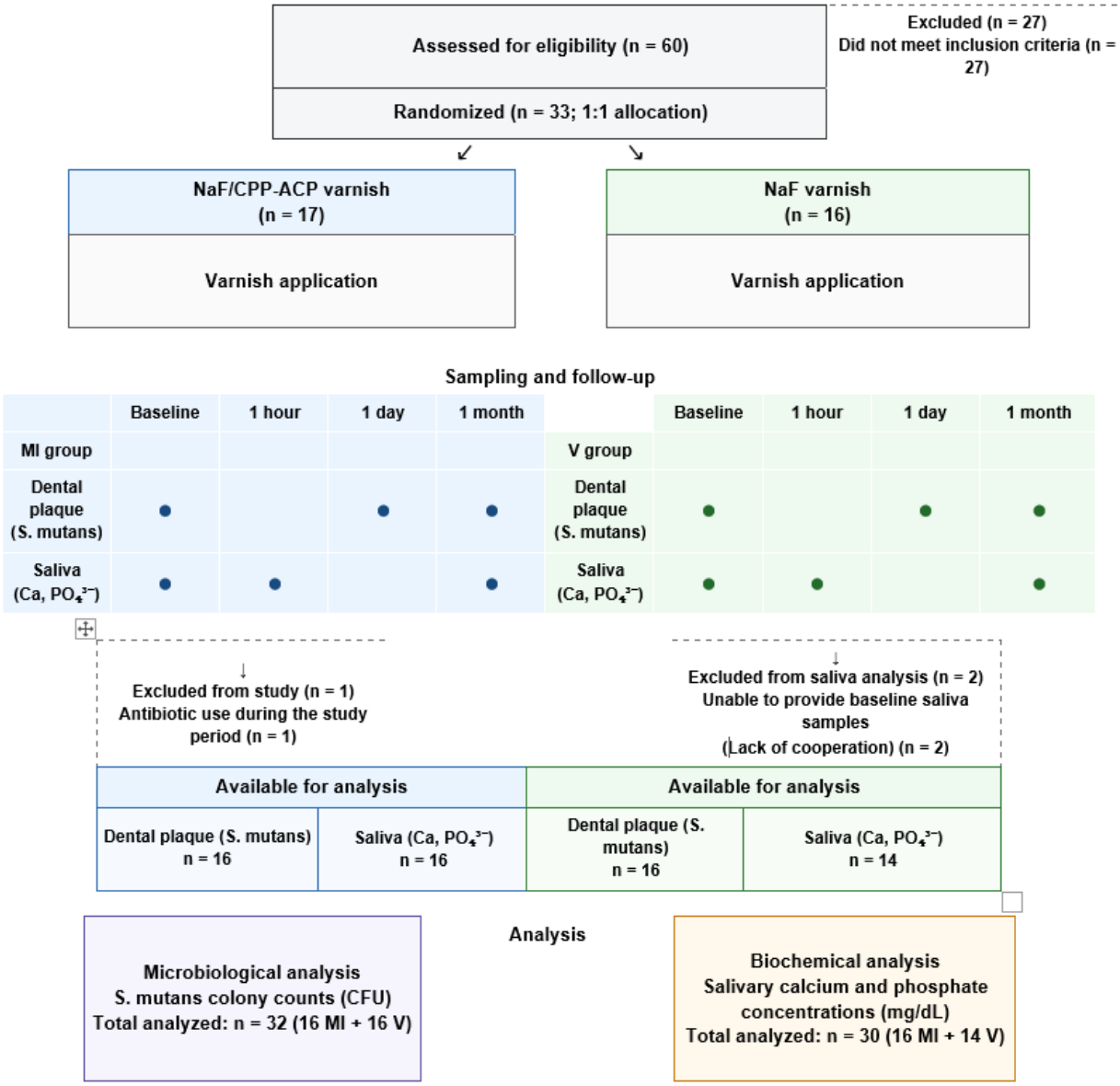

Figure 1. Flow diagram illustrating participant enrollment, allocation, study procedures, sample collection schedule, exclusions, microbiological assessment, and salivary biochemical analyses (NaF: Sodium fluoride varnish; NaF/CPP-ACP: Sodium fluoride varnish containing casein phosphopeptide-amorphous calcium phosphate; Ca: Calcium; $PO4^{3-}$: Phosphate)

positive cocci under microscopic examination and negative catalase and bile esculin tests (15). Colony enumeration was performed by a single investigator using a colony counter. The number of colonies was multiplied by the dilution factor, and the results were expressed as colony-forming units (CFU/mL).

Salivary laboratory analysis

Saliva samples were transferred into 5-mL tubes and centrifuged at 2,000 rpm for 5 minutes using a laboratory centrifuge (Behdad, Tehran, Iran).

Salivary calcium and phosphate concentrations were measured using an automated chemistry analyzer (BT3000; Biotecnica Instruments S.p.A., Italy). Calcium concentration was determined using the Arsenazo III colorimetric method at 630 nm, whereas phosphate concentration was determined using the molybdate-UV photometric method at 340 nm (16, 17). Results were expressed as mg/dL. Because of the high phosphate concentration, saliva samples were diluted 1:1 with normal saline before phosphate analysis.

Sample size calculation

Based on Lakade et al. (18), a sample size of 14 participants per group was calculated with a 95% confidence level (α = 0.05), 90% power (β = 0.10), and a minimum detectable difference of 0.20. Although a total of 28 participants were required, 33 were enrolled to account for potential dropout.

Statistical analysis

Data were analyzed using IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA). The normality of data distribution was assessed using the Shapiro–Wilk test. Salivary calcium and phosphate concentrations showed normal distributions (P > 0.05), whereas plaque *S. mutans* counts did not (P < 0.05). Therefore, plaque S. *mutans* counts were logarithmically transformed before statistical analysis, resulting in an approximately normal distribution (P > 0.05).

Repeated-measures analysis of variance (ANOVA) was performed to assess changes over time in plaque *S. mutans* counts and salivary calcium and phosphate concentrations within and between groups, with time as the within-subject factor and treatment group as the between-subject factor. The effects of time, group, and the time × group interaction were evaluated for each outcome variable. Statistical significance was set at P < 0.05.

Results

A total of 33 children aged 3–6 years were enrolled and randomly allocated to the NaF varnish group (n = 16; mean age 4.6 ± 0.9 years; 56% female) and the NaF/CPP-ACP varnish group (n = 17; mean age 4.9 ± 1.05 years; 59% female). No significant between-group differences were observed in age (P = 0.231) or sex distribution (P = 0.663) between the groups.

During the study period, one participant in the NaF/CPP-ACP varnish group was excluded due to antibiotic use. In addition, two participants in the NaF varnish group were excluded from the salivary analyses because of non-cooperation during saliva collection. Consequently, plaque samples from 32 participants were included in the microbiological analysis, while saliva samples from 30 participants were available for salivary calcium and phosphate measurements.

Plaque Streptococcus mutans counts.

Table 2 presents the mean ± SD of log-transformed plaque *S. mutans* counts at baseline, 1 day, and 1 month after varnish application. Repeated-measures ANOVA of the log-transformed counts showed a significant effect of time (*P* < 0.001). Pairwise comparisons showed significant differences among all three time points (all *P* < 0.001), with *S. mutans* counts decreasing after varnish application, followed by a partial increase at 1 month. However, *S. mutans* counts at 1 month remained significantly below the baseline levels. Neither the overall group effect (*P* = 0.522) nor the time × group interaction (*P* = 0.089) was statistically significant. The changes in plaque *S. mutans* counts over time in the study groups are illustrated in Figure 2.

Salivary calcium concentration

Table 3 presents the mean salivary calcium concentrations at baseline, 1 hour, and 1 month after

Table 2. Log-transformed dental plaque *Streptococcus mutans* counts in the study groups at baseline, 1 day, and 1 month after varnish application

Study group	Baseline	1 day	1 month
	Mean ± SD	**Mean ± SD**	**Mean ± SD**
NaF varnish (n = 16)	3.39 ± 0.76^{A}	2.35 ± 0.66^{c}	2.89 ± 0.69^{B}
NaF/CPP-ACP varnish (n = 16)	3.60 ± 0.76^{A}	1.99 ± 1.09^{c}	2.57 ± 0.90^{c}
The effect of time	P < 0.001		
The effect of group	P = 0.522		
The time × group interaction	P = 0. 089		

Different superscript uppercase letters indicate statistically significant differences between time points within each group.

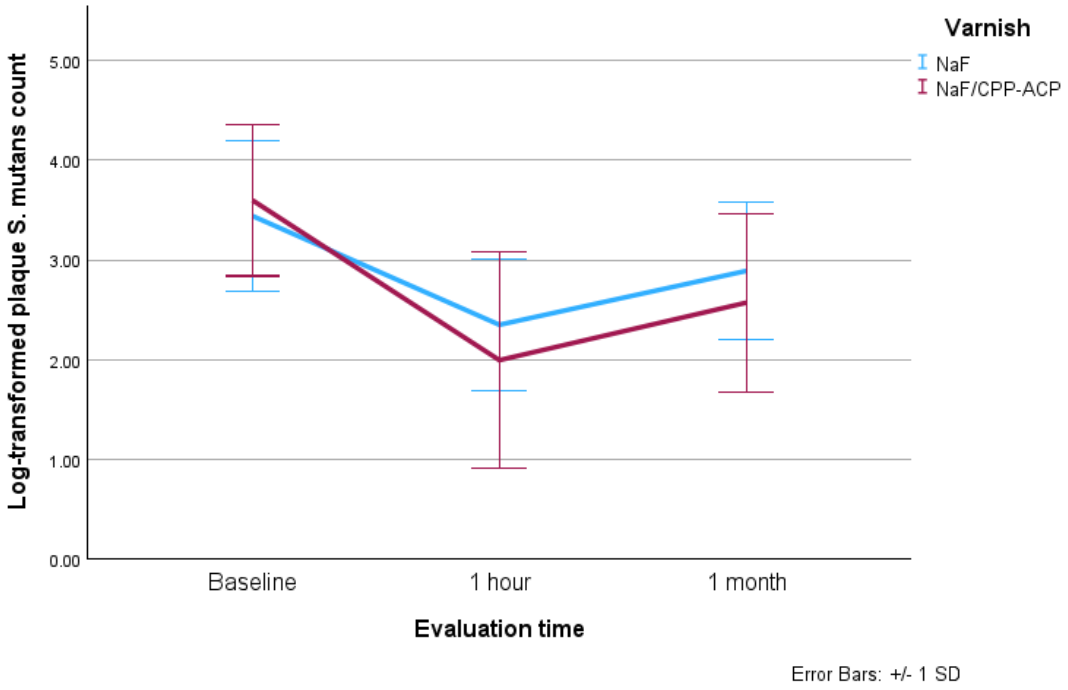

Figure 2. Changes over time in mean log-transformed dental plaque Streptococcus mutans counts in the NaF and NaF/CPP-ACP varnish groups at baseline, 1 day, and 1 month after varnish application. Error bars represent ±1 standard deviation (SD).

varnish application. Repeated-measures ANOVA showed no significant effects of time (P = 0.054), group (P = 0.483), or time × group interaction (P = 0.123) on salivary calcium concentrations.

Salivary phosphate concentration

Table 4 presents the mean salivary phosphate concentrations at baseline, 1 hour, and 1 month after varnish application. Repeated-measures ANOVA revealed no significant effects of time (P = 0.716), group (P = 0.390), or time × group interaction (P = 0.652) on salivary phosphate concentrations.

Discussion

The present randomized clinical trial investigated the effects of NaF/CPP-ACP and conventional NaF varnishes on plaque *S. mutans* counts and salivary calcium and phosphate concentrations in caries-free preschool

Table 3. Salivary calcium concentrations (mg/dL) in the study groups at baseline and 1 day and 1 month after varnish application

Study group	Baseline	1 hour	1 month
	Mean ± SD	Mean ± SD	Mean ± SD
NaF varnish (n = 14)	2.64 ± 0.78	2.64 ± 0.71	2.37 ± 0.65
NaF/CPP-ACP varnish (n = 16)	2.94 ± 0.95	2.99 ± 0.94	2.88 ± 0.98
The effect of time	P = 0.054		
The effect of group	P = 0.483		
The time × group interaction	P = 0. 123		

Different superscript uppercase letters indicate statistically significant differences between time points within each group.

Table 4. Salivary phosphate concentrations (mg/dL) in the study groups at baseline and 1 day and 1 month after varnish application

Study group	Baseline	1 hour	1 month
	Mean ± SD	Mean ± SD	Mean ± SD
NaF varnish (n = 14)	17.64 ± 3.66	17.56 ± 3.74	17.42 ± 3.95
NaF/CPP-ACP varnish (n = 16)	18.30 ± 5.77	18.37 ± 5.85	18.33 ± 5.63
The effect of time	P = 0.716		
The effect of group	P = 0.390		
The time × group interaction	P = 0. 652		

Different superscript uppercase letters indicate statistically significant differences between time points within each group.

children with moderate-to-high caries risk. Unlike previous studies that primarily focused on a single outcome, the present study simultaneously assessed microbiological and salivary mineral responses over time.

Regarding the microbiological findings, dental plaque *S. mutans* counts showed a significant change over time. Pairwise comparisons demonstrated a significant reduction in *S. mutans* counts after varnish application, followed by a partial increase at 1 month; however, the counts remained significantly lower than baseline values. Repeated-measures ANOVA showed no significant main effect of group or time × group interaction, indicating that the pattern of changes over the study period was comparable between the NaF/CPP-ACP varnish and NaF varnish groups. The present findings are consistent with previous studies reporting reductions in *S. mutans* counts following fluoride varnish application in children (19-21).

Fluoride may suppress cariogenic bacterial activity by interfering with bacterial metabolism and acid production (22). The outcomes of this study are in agreement with the findings of Deepti et al; (20) who reported a significant reduction in plaque *S. mutans* counts 24 hours after fluoride varnish application. Similarly, Narayan et al. (21) observed significant reductions in plaque *S. mutans* counts at 30, 60, and 90 days following fluoride varnish application.

No statistically significant additional reduction in plaque *S. mutans* counts was observed with the NaF/CPP-ACP varnish compared with the conventional NaF varnish. Similar findings have been reported in previous studies evaluating fluoride varnishes containing additional calcium-phosphate components (7, 23). Patel et al. (23) reported no significant difference between the CPP-ACP-containing varnish and the conventional fluoride varnish at the 1-month follow-up, although lower *S. mutans* counts were observed in the CPP-ACP group at the 3- and 6-month evaluations. Similarly, a randomized clinical trial in preschool children found that NaF varnishes containing CPP-ACP or tricalcium phosphate had antibacterial effects against *S. mutans* comparable to those of conventional NaF varnish (7).

The absence of an additional reduction in plaque *S. mutans* counts associated with CPP-ACP supplementation in the present study may be partly explained by the primary mechanism of action of CPP-ACP. CPP-ACP mainly functions by stabilizing bioavailable calcium and phosphate ions and enhancing remineralization rather than exerting a direct antimicrobial effect (24). It can maintain supersaturation with respect to tooth mineral at the tooth surface, while its reported effects on bacterial adhesion and biofilm ecology appear to be more indirect (12, 24). In the presence of fluoride, CPP-ACP may also facilitate the formation of stabilized amorphous calcium fluoride phosphate complexes and thereby enhance remineralization (25). Although no significant between-group differences were observed, the relatively small sample size may have limited the ability of the study to detect potential differences between the varnish groups.

Salivary calcium concentrations changed over time, but no significant difference was observed between the time points and the two varnish groups. In contrast, Poureslami et al. (14) observed significant increases in salivary calcium following the use of CPP-ACP and CPP-ACPF pastes in children. Kakatkar et al. (26) reported increased salivary calcium after the use of CPP-ACP-

containing chewing gum. These differences may be related to variations in product formulation and mode of delivery. Unlike pastes or chewing gum, varnish is applied directly to the tooth surface and provides sustained local ion release; therefore, changes in whole-saliva calcium concentrations may not necessarily reflect calcium availability at the tooth surface (27).

Salivary phosphate concentrations did not differ significantly across evaluation time points or between study groups. A small numerical decrease was observed over time in the NaF varnish group, whereas a small increase occurred in the CPP-ACP-containing fluoride varnish group, although the variations were not significant. In contrast to the findings of the present study, Poureslami et al. (14) reported that CPP-ACP and CPP-ACPF pastes significantly increased salivary phosphate concentrations compared with baseline in children with early childhood caries. Evidence summarized in systematic reviews also indicates substantial heterogeneity in the effects of calcium-phosphate-based agents, reflecting differences in product formulation, application protocols, study populations, follow-up periods, and measurement methods (28).

The absence of significant changes in salivary phosphate concentrations over time or between the study groups may be related to the relatively low CPP-ACP content of the varnish, the limited follow-up period, and the use of unstimulated whole saliva, which may have reduced the ability to detect treatment-related changes (28). Similar to calcium, mineral release may have occurred primarily at the tooth surface or within dental plaque rather than resulting in measurable changes in whole-saliva phosphate concentrations (29).

This study has several limitations. The relatively small sample size and short follow-up period may have limited the ability to detect modest between-group differences and evaluate the long-term persistence of the observed effects. In addition, salivary mineral measurements obtained shortly after varnish application may reflect transient changes related to immediate ion release rather than sustained effects of the intervention. Finally, operator blinding was not feasible because of differences in product presentation, which may have introduced a risk of performance bias. Future studies with larger sample sizes and longer follow-up periods are needed to evaluate the long-term effects of different varnish formulations on caries development.

Conclusion

Within the limitations of the present study, the following conclusions can be drawn:

1. Both the CPP-ACP-containing fluoride varnish and the conventional sodium fluoride varnish significantly reduced dental plaque *S. mutans* counts in caries-free preschool children at moderate-to-high caries risk, with no significant difference between the two varnishes. Although *S. mutans* counts partially increased at 1 month, they remained significantly lower than baseline levels.
2. Salivary calcium and phosphate concentrations did not significantly change over time, and no significant differences were observed between the NaF and NaF/CPP-ACP varnish groups.
3. The NaF/CPP-ACP varnish did not demonstrate a statistically significant advantage over the conventional NaF varnish in reducing dental plaque *S. mutans* counts or enhancing salivary calcium and phosphate levels.

Acknowledgements

The authors gratefully acknowledge the staff of the Department of Pediatric Dentistry and the laboratory personnel at Shahid Sadoughi University of Medical Sciences for their assistance with the clinical and laboratory procedures of this study.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

Z.B. contributed to the conception and design of the study. S.S. contributed to data collection, implementation of the clinical procedures, and statistical analysis and interpretation of the results. H.Z. contributed to the laboratory analyses and interpretation of the laboratory data. A.F. contributed to data collection, manuscript preparation, and critical revision. All authors read and approved the final version of the manuscript.

Ethical considerations

The study protocol was approved by the ethics committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran (IR.SSU.REC.1396.207) and registered in the Iranian Registry of Clinical Trials (IRCT20140601017935N7). The study was conducted in

accordance with relevant ethical principles for research involving human participants. Written informed consent was obtained from the parents or legal guardians of all participating children before enrollment. Participation was voluntary, and the confidentiality of participants' information was maintained throughout the study. The study was reported in accordance with the Consolidated Standards of Reporting Trials (CONSORT) guidelines.

Funding

This study was supported by Shahid Sadoughi University of Medical Sciences. No specific external research grant was received for this work, and the funding body had no role in the study design, data collection, statistical analysis, interpretation of the results, or preparation of the manuscript.

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