Original Article

Comparative Evaluation of Remineralization Potential of Casein Phosphopeptide- Amorphous Calcium Fluoride Phosphate and Novamin on Artificially Demineralized Human Enamel: An *In vitro* Study

Abstract

Aim: This study aimed to quantitatively compare the remineralization potential of casein phosphopeptide-amorphous calcium fluoride phosphate (CPP-ACFP) and Novamin on artificially induced enamel subsurface lesions using scanning electron microscope-energy dispersive X-ray (SEM-EDX). **Materials and Methods:** Enamel specimens were prepared from seventy caries-free premolars and were kept for demineralization for 4 days. Following demineralization, enamel specimens were assigned to three groups: (1) Control group, (2) Group with CPP-ACFP-containing toothpaste, and (3) Group with Novamin-containing toothpaste. Further, both the experimental groups (CPP-ACFP group and Novamin) were then divided into three subgroups based on the duration of re-mineralization as follows: (a) 7 days, (b) 14 days, and (c) 21 days. Enamel specimens of experimental groups were then subjected to SEM-EDX to evaluate mineral content after treating with CPP-ACFP and Novamin. **Results:** Both the experimental groups showed very high significant differences between Ca/P ratios of remineralized samples. **Conclusion:** Even though both CPP-ACFP and Novamin showed remineralization potential, remineralization was found to be higher in the samples treated with CPP-ACFP.

Keywords: *Casein phosphopeptide-amorphous calcium fluoride phosphate, demineralization, Novamin, remineralization, scanning electron microscope-energy dispersive X-ray*

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Introduction

The remineralization potential of damaged tooth surfaces is appreciable, especially in children. As it is well documented that white spot lesions are the earliest macroscopic evidence of enamel caries, the enamel surface layer stays intact during subsurface demineralization, but without any intervention, it will eventually collapse into cavitation.[1,2] Standard procedures for protection of these teeth are fissure sealing and topical fluoride application. So far, none of these procedures are completely efficient. Therefore, attempts have been made to find an effective anticariogenic and remineralizing agent to have ions directly delivered to when and where they are needed most. This leads to the development of aided remineralization.[3]

A number of remineralization techniques have been tried out, among which the use of milk and milk products appeared to have a protective effect against the development of dental caries. It has been suggested that the anticariogenic properties of milk are due to the presence of casein, calcium, and phosphate, which are responsible for resistance to acid dissolution. Casein phosphopeptide-amorphous calcium fluoride phosphate (CPP-ACFP) contains nanocomplexes of milk protein.[4,5] The fluoride ion incorporated into the ACP phase was stabilized by the CPP to produce a novel ACFP which was observed to be responsible for anti-cariogenic effect. When CPP-ACFP is applied to the oral environment, the sticky CPP part of the CPP-ACFP complex binds readily to the enamel, biofilm, and the soft tissues, delivering the calcium phosphate ions, thereby maintaining a supersaturated state of essential minerals. Fluoride ions help in remineralization by forming fluorapatite in the presence of calcium and phosphate ions over the enamel surface.[6]

Novamin, a bioactive glass of highly biocompatible materials, was originally developed as bone regenerative material.[7]

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In the aqueous environment of the tooth, sodium ions from the Novamin particles rapidly exchange with hydrogen cations (in the form of H_3O^+) to release calcium and phosphate (PO_4^{3-}) ions. There will be a localized transient increase in pH during the initial exposure of the material due to the release of sodium. This increase in pH helps to precipitate the extra calcium and phosphate ions provided by the Novamin particles to form a precipitated calcium phosphate layer. As these reactions continue, this layer crystallizes into hydroxycarbonate apatite (HCA) which is chemically and structurally equivalent to naturally occurring biological apatite.[7-9]

With this background, this study was undertaken to evaluate the ability of topically applied CPP-ACFP and Novamin in bringing about remineralization changes on enamel surface that has been exposed to an artificial caries challenge in a simulated oral environment.

Materials and Methods

Seventy caries-free premolars extracted for orthodontic reasons without any visible caries, hypoplastic lesions, and white spots on any surface of the tooth were collected and stored in 0.1% thymol solution until processing.

All the teeth were trimmed to 4 mm × 4 mm × 1 mm-sized enamel specimens from the buccal surfaces of tooth using a low-speed diamond disc. About 2 mm × 2 mm section from each sound enamel specimen was taken and is then evaluated for mineral content (% weight) using scanning electron microscope-energy dispersive X-ray analysis (SEM-EDX).

Lesion formation

4 mm × 2 mm section of the enamel specimens was coated with acid-resistant nail varnish on all the surfaces except the buccal surface and was subsequently immersed in demineralizing solution (20 ml) containing 2.2 mM calcium chloride, 2.2 mM sodium hypophosphate, and 20 mM acetic acid with pH adjusted to 4.4 using 50% sodium hydroxide for 4 days at 37°C to produce artificial carious lesions.

After demineralization, sectioning was done for 4 mm × 2 mm enamel samples which was then divided into two specimens, where 2 mm × 2 mm demineralized enamel specimens were taken for remineralization process and the remaining 2 mm × 2 mm demineralized enamel specimens were evaluated for any loss of mineral content (calcium/phosphorous ratios) using SEM-EDX on the 5th day after the removal of acid-resistant nail varnish carefully using acetone.

Test groups

The remaining 2 mm × 2 mm demineralized enamel specimens were coated with acid-resistant nail varnish on all the sides except the buccal surface and were randomly assigned to three groups:

1. Group I: Control group (ten specimens)
2. Group II: CPP-ACFP-containing toothpaste (thirty specimens)
3. Group III: Novamin-containing toothpaste (thirty specimens).

Experimental groups, namely CPP-ACFP group and Novamin group, were then divided into three subgroups with ten specimens in each group based on the duration of remineralization.

- Subgroup A – 7 days
- Subgroup B – 14 days
- Subgroup C – 21 days.

Enamel specimens of Group I (control group) were incubated in the artificial saliva containing 0.213 g/L calcium chloride, 0.738 g/L sodium hypophosphate, 0.381 g/L sodium chloride, and 1.114 g/L potassium chloride with pH adjusted to 7 using 85% acetic acid at 37°C for 21 days, but received no treatment with remineralizing paste. Whereas enamel specimens of Group II and Group III (experimental groups) were treated with CPP-ACFP- and Novamin-containing toothpaste using a stainless steel spatula for 7 days (subgroup A), 14 days (subgroup B), and 21 days (subgroup C) twice daily for 3 min followed by incubation in artificial saliva at 37°C.

The dentifrices were cleaned under copious water spray using a 20 ml disposable syringe to eliminate the possible leftovers. Artificial saliva was changed every 24 h just before the immersion of freshly treated samples.

At the end of the 21st day, the acid-resistant nail varnish on the enamel specimens was carefully removed using acetone, and SEM-EDX were done to measure the mineral content (% weight) for specimens in Group I after the demineralization process and for the specimens in Group II and Group III SEM-EDX was done to assess the remineralization process of the tooth enamel after 7, 14 and 21 days respectively.

Statistical analysis

Collected data were entered into Microsoft SpreadSheet of Microsoft Windows 2007 (Microsoft Office, USA) and descriptive statistics were calculated using Statistical Package for the Social Sciences (SPSS version 19) software (IBM, USA).

Results

The data pertaining to Ca/P ratio in sound, demineralized, and remineralized specimens were collected. Observations and results are divided into two parts: (1) Statistical analysis for Ca/P ratios obtained from EDX and (2) SEM microphotographs.

Mean and SD of seventy sound and demineralized samples taken on the 5th day after demineralization were 2.07 (0.07) and 1.53 (0.05), respectively.

The difference in Ca/P ratio recorded after 7th, 14th, and 21st days of remineralization within the two experimental groups, CPP-ACFP and Novamin, is tabulated in Table 1.

The remineralization potential between the study groups, namely Control, CPP-ACFP, and Novamin, which was recorded after 21 days was analyzed, and the pair-wise comparison is tabulated in Tables 2 and 3.

The difference in Ca/P ratio in the remineralized specimens between Novamin and CPP-ACFP and the remineralization potential of control after 21 days of remineralization are tabulated in Tables 4 and 5, respectively.

Scanning electron microscope morphological characters/scanning electron microscope evaluation

The typical SEM images of the enamel surfaces in the different groups showed that the sound enamel [Figure 1a] had an orderly rod appearance. The enamel crystals were homogenously arranged with a clear outline.

In contrast, the demineralized enamel [Figure 1b] was disorganized with increased surface porosity and clear destruction of the enamel surface, resulting in significant depressions and irregularities.

Group II (CPP-ACFP) after 7 days of remineralization revealed only a few deposits [Figure 2a]. At the end of the 14th day, the interprismatic substances were evident and amorphous crystals were arranged on the surface [Figure 2b].

After the 21st day of remineralization, the microphotographs of Group II (CPP-ACFP) showed discernable enamel rods and prismatic substance. The areas of calcified deposits were found to be more evident [Figure 2c].

Group III (Novamin) revealed mainly demineralized enamel after 7 days of remineralization, with only a few deposits, which appears to be less homogeneous than Group II [Figure 3a]. At the end of the 14th day, remineralization was displayed along the prismatic borders [Figure 3b].

The configuration of enamel topography after 21 days of remineralization of Group III (Novamin) showed areas of mineralized deposits which are discernable and seen profusely scattered along the porous defects. The interprismatic substances are evident and more thickened areas of remineralization are seen along the prismatic borders [Figure 3c].

Discussion

This study has quantitatively compared the remineralization potential of CPP-ACFP and Novamin on artificially induced enamel subsurface lesions using SEM-EDX.

A very limited number of studies had evaluated and compared the remineralization potential of CPP-ACFP and Novamin quantitatively using an *in vitro* method, and SEM-EDX was used to record the structural changes

Table 1: Comparison of remineralized enamel specimens for 7, 14, and 21 days within casein phosphopeptide-amorphous calcium fluoride phosphate and Novamin

	Remineralization	*n*	Mean	SD	*F*	*P*
Group II (CPP-ACFP)	7th day	10	1.7110	0.01912	98.437	0.001
	14th day	10	1.7930	0.02791		
	21st day	10	1.9130	0.04473		
Group III (Novamin)	7th day	10	1.6240	0.06979	12.074	0.001
	14th day	10	1.6650	0.05874		
	21st day	10	1.7480	0.03994		

*One-way ANOVA. CPP: Casein phosphopeptide; ACFP: Amorphous calcium fluoride phosphate; SD: Standard deviation

Table 2: Comparison between study groups (control, casein phosphopeptide-amorphous calcium fluoride phosphate, and Novamin) for 21 days of remineralization

	Groups	*n*	Mean	SD	*F*	*P*
Remineralization	Group I (control)	10	1.5550	0.04170	180.523	0.001
	Group II (CPP-ACFP)	10	1.9130	0.04473		
	Group III (Novamin)	10	1.7480	0.03994		

*One-way ANOVA. CPP: Casein phosphopeptide; ACFP: Amorphous calcium fluoride phosphate; SD: Standard deviation

Table 3: Intragroup comparison for 21 days

Pair wise comparison	Mean of 21 days remineralization
Group I (control) versus Group II (CPP-ACFP)	Highly significant
Group I (control) versus Group III (Novamin)	Highly significant
Group II (CPP-ACFP) versus Group III (Novamin)	Highly significant

**Post hoc* analysis - Tukey's tests. CPP: Casein phosphopeptide; ACFP: Amorphous calcium fluoride phosphate

Table 4: Comparison between experimental groups (Group II-casein phosphopeptide-amorphous calcium fluoride phosphate and Group III-Novamin)

	Groups	*n*	Mean	SD	*t**	*P*
7th day	Group III (Novamin)	10	1.6240	0.0697	−3.802	0.001
	Group II (CPP-ACFP)	10	1.7110	0.0191		
14th day	Group III (Novamin)	10	1.665	0.0587	−6.224	0.001
	Group II (CPP-ACFP)	10	1.793	0.0279		
21st day	Group III (Novamin)	10	1.748	0.0399	−8.700	0.001
	Group II (CPP-ACFP)	10	1.913	0.0447		

*Unpaired *t*-test. CPP: Casein phosphopeptide; ACFP: Amorphous calcium fluoride phosphate; SD: Standard deviation

Table 5: Comparison for 21 days remineralization for Group I (control) with the sound and demineralized enamel

Group I (control)	Mean	SD	*F*	*P*
Sound	2.0783	0.07231	1394.31	0.001
Demineralized	1.5386	0.05284		
Remineralized	1.5550	0.04170		

*One-way ANOVA. SD: Standard deviation

of the enamel surface and to determine the calcium and phosphorous content of sound, demineralized, and remineralized enamel, which was similar to that utilized by Hegde and Moany in 2012.[10]

Remineralization is a natural repair process for noncavitated lesions. CPP-ACP is a known source of calcium and phosphate ions, which is likely to inhibit demineralization and enhance remineralization or possibly both. In addition to it, CPP-ACFP has fluoride. Elsayad *et al*. in 2009 reported that addition of fluoride to CPP-ACP could give a synergistic effect on enamel remineralization of early carious lesion.[11] Studies have also shown that remineralization of small lesions with low-dose fluoride therapy is more efficient. CPP-ACFP releases fluoride as well as calcium and phosphate ions, thereby providing all the three ions which are required for the formation of acid-resistant fluorapatite. In a study conducted by Patil *et al*. in 2013, it was showed that CPP-ACP + fluoride is more effective than CPP-ACP alone.[12]

Novamin is a component made of bioactive glass particulates, which has been tested for its effectiveness in remineralizing hard tooth structure occluding dentinal tubules.[13] It provides silica, calcium, phosphorous, and sodium ions when it comes in contact with the aqueous media binding the tooth structure, thereby initiating the remineralization process. The particle reaction continues and deposition of calcium and phosphate complex takes place which crystallizes to calcium hydroxyl apatite, also known as Hydroxycarbonate apatite.[7,8]

An oral environment with low salivary clearance was simulated by lowering the concentrations of calcium and phosphate in the demineralization solution which was done in accordance with the in vitro pH-cycling study done by Ogata *et al.* in 2010 where the *in vitro* pH-cycling was done for suitable remineralization environment to validate the effect of CPP-ACFP and Novamin.[14]

Artificial early caries-like lesions of enamel show all of the principal histological features of natural caries and have been successfully used to study the demineralization of enamel *in vitro* as discussed by Soumya *et al*. in 2011.[15]

Mean Ca/P ratio of both the experiment groups (CPP-ACFP and Novamin) were found to be increased after 7, 14, and 21 days compared to the demineralized enamel specimens, but were found to be less than the sound enamel even after

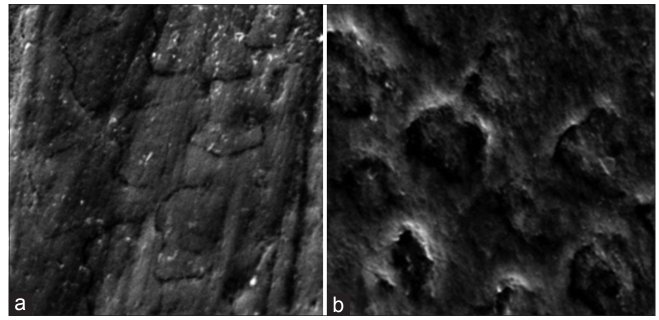

Figure 1: (a and b) Sound enamel and demineralized enamel

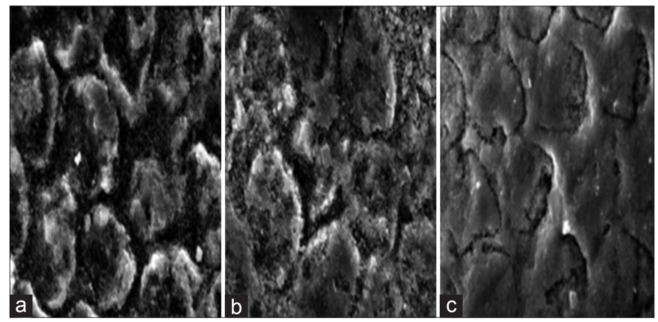

Figure 2: (a-c) Remineralization at the end of 7, 14, and 21 days for Group II (Casein phosphopeptide-amorphous calcium fluoride phosphate)

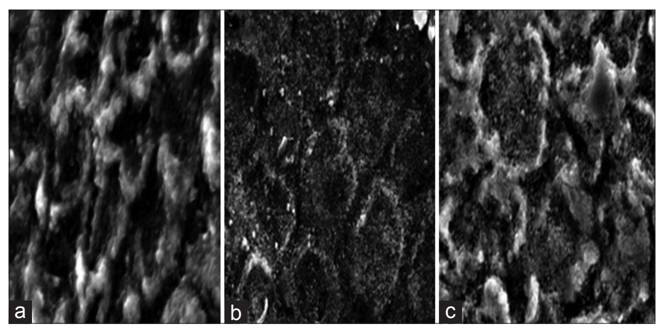

Figure 3: (a-c) Remineralization at the end of 7, 14, and 21 days for Group III (Novamin)

21 days of remineralization. These results were similar to the study done by Hegde and Moany in 2012.[10]

Remineralization enhanced in enamel specimens where CPP-ACFP (mean Ca/P ratio = 1.91) was applied than that of Novamin (mean Ca/P ratio = 1.74) and artificial saliva (mean Ca/P ratio = 1.55) at the end of 21 days. The findings of the present study were similar to the study conducted by Soares *et al*. in 2011.[16] The mineral formed in the subsurface lesions was in consistent with hydroxyapatite and fluorapatite for remineralization with CPP-ACFP.[16]

Calcium sodium phosphosilicate (Novamin) is delivered in the form of a solid bioactive glass that must first dissolve before it can be active. CPP-ACFP, on the other hand, is in a dissolved active, noncrystalline form which accounts for its superior remineralizing capacity.[17] With the use of low fluoride concentration as is present in CPP-ACFP (0.2% or

900 ppm of NaF), there is a complex localization of free calcium phosphate and fluoride ion activities and is thus an excellent local slow delivery system to treat the white spot lesion.[18]

Enamel remineralization by the bioactive glass-containing toothpaste occurred by a different mechanism, namely the incorporation of different elements into the enamel structure. The surface chemical reaction results in the formation of a HCA layer which is chemically and structurally equivalent to biological apatite.[3,19]

Remineralization of both the groups (CPP-ACFP and Novamin) was found to be maximum for the samples kept till 21 days when compared to those of 7 days and 14 days. With these findings, in this study, it is found that remineralization increases with exposure duration and dose level. These results were similar to the study conducted by Patil *et al*. in 2013[12] on remineralization mechanism of CPP-ACFP and similar to the study conducted by Hegde and Moany in 2012 who demonstrated that remineralization of subsurface enamel using CPP-ACP was dose dependent which increases the time of exposure and duration of the study.[10]

SHY-NM is a "fluoride-free" toothpaste containing nanometric bioactive glass (Novamin). Fluoride has been shown to have substantial positive effects on preventing tooth decay. However, there are also negative effects to excessive ingestion of fluoride. Evidence is mounting that every day there is ingestion of fluoride through normal blood, beverage, and water intake supplies, most or all of the fluoride necessary for good oral health. For this reason, SHY-NM is not supplemented with fluoride.[17]

CPP-ACFP showed a better remineralization effect than CPP-ACP. This may be attributed to a combination of CPP-ACP and fluoride ions at the enamel surface, presumably as CPP-ACFP nanocomplexes.[6,20]

In the present study, Group I (artificial saliva) showed a very minimal remineralization of enamel surface at the end of 21 days, which is similar to the study conducted by Rirattanapong *et al*. in 2011.[21] Although saliva has some remineralization potential, it cannot increase the levels of calcium and phosphate release.[22]

Although the present study could not completely simulate the complex oral environment, the study results still demonstrated the remineralization effectiveness of the CPP-ACFP and Novamin on the artificial demineralized human enamel.

Shortcoming of the present study is the period of remineralization used in the study was 21 days, which could not remineralize artificial caries completely, thus the period of application for complete remineralization cannot be described for remineralizing agents used in the study.

Conclusion

Based on the data obtained from the present study, it was concluded that even though both CPP-ACFP and Novamin showed remineralization potential, remineralization was found to be higher in the samples treated with CPP-ACFP. Hence, in the present study, CPP-ACFP showed enhanced remineralization and therefore can be expected to be effective in high-risk children who have not developed good oral hygiene habits. Children usually have the habit of swallowing the paste during daily tooth brushing. This could be counted as one of the advantages of Novamin over fluoride-containing pastes, making it recommendable and safe to be prescribed as a toothpaste of choice for young children.

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Conflicts of interest

There are no conflicts of interest.

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