Original Article

Enamel Remineralization System: Front Line in Caries Management

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Abstract

Background: The initial carious lesions are the professed "white spot" lesions, which indicatethat there is a subsurface area with most of the mineral loss underneath a relatively intact enamel surface. With a clear understanding regarding the demineralization-remineralization theory of caries initiation, importace in present-day dentistry is on remineralization of carious lesion. Thus implying that remineralization could occur in clinically detectable incipient lesions. Therefore, the aim of the present study was to compare and evaluate the effectiveness of the remineralization potential of Nanohydroxyapatite and Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP) toothpastes on enamel. **Materials and Methods:** Freshly extracted caries-free intact human primary molar were used for this study. Forty-eight enamel blocks (4 mm × 4 mm) were prepared from the smooth surfaces. All the specimens were immersed in the demineralizing solution for a period of 6 days. These specimens were randomly divided into two groups of 24 each. In Group-I specimen were treated with Nano-hydroxyapatite (n-HAP) toothpaste and in Group II specimen by CPP-ACP mousse. Specimens were manually brushed, twice daily for 3 min and were stored in the saliva solution. After 2 weeks, 12 samples of each groups were sent to the laboratory for evaluation of enamel surface morphologies, using scanning electron microscope (SEM) and for evaluation of Calcium and Phosphate mineral component, SEM energy dispersive X-ray (SEM- EDX). **Results:** SEM: n-HAP Toothpaste Group demonstrated more remineralized structure than CPP-ACP Group. The surface of the teeth treated with n-HAP displayed nucleation of apatitic crystals. SEM-EDX: Mean Ca (4.084 ± 1.603) and *P* (2.787 ± 1.699) was higher in n-HAP Toothpaste Group than CPP-ACP Group. This was statistically significant difference. **Conclusion:** Based on the data obtained from the present study, it was concluded that although both CPP-ACP and n-HAP demonstrated remineralization potential, the samples treated with n-HAP illustrated statistically significant higher remineralization.

Keywords: Calcium, Casein Phosphopeptide-Amorphous Calcium Phosphate, Demineralization, Nano-hydroxyapatite, Phosphate, Primary Teeth, Remineralization, Scanning electron microscope, Scanning electron microscope - energy dispersive X-ray.

Introduction

The first clinical sign of dental caries is the appearance of a white spot lesion (WSL) on the tooth surface that can be considered the initial stage of enamel demineralization.[1] If the demineralization process continues, the initial lesion may lead to cavity formation. Thus, enhancing remineralization of early carious lesions is a valuable, non-invasive treatment for maintaining healthy dentition. Among initial strategies, the use of fluorides has shown certain degree of effectiveness in managing WSLs.[2]

Fluoride increases the remineralization of the outer enamel and decreases the demineralization of the inner enamel, resulting in sufficient mineral gain. However, some authors have suggested that using high concentrations of fluorides causes remineralization especially in the superficial part of WSL. This superficial layer might impede calcium and phosphate, inhibiting deeper remineralization and restraining the cosmetic improvement of the WSLs. Therefore, there is a need to investigate other anti-carious agents that could have synergistic effect with fluoride or be an alternative to enhance remineralization of dental caries.[3]

For almost a decade, Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP) imitative from cow's milk has been reported to reduce the demineralization process and improve the remineralization process of the tooth structure.[4]

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In-depth examination have shown that a particular part of casien protein, CPP, is responsible for tooth-protective activity. CPP plays an important role as ACP carrier, thus localizing the highly soluble calcium phosphate phase at the tooth surface. This localization maintains high concentration gradients of calcium and phosphate ions in the subsurface enamel, thus enabling remineralization.[5]

Nano-hydroxyapatite (n-HAP) is considered one of the most biocompatible and bioactive materials and has gained wide acceptance in medicine and dentistry in recent years.[6] Despite the fact that earlier attempts to use hydroxyapatites clinically did not succeed, synthesis of nano-scaled Zinc Carbonate Hydroxyapatite ($ZnCO_3$/n-HAp) yielded a significant progress, and showed significant affinity to the enamel surface.[7] Nano-sized particles have resemblance to the apatite crystals of tooth enamel in morphology and crystal structure. Recently, a few reports have revealed that n-HAP has some potential to repair dental enamel, but no information is available for established dentine lesions. To date, it can be concise that for remineralization of subsurface lesions by n-HAP containing products, different formulations have been developed, and early data have recommended remineralizing properties.[8]

However, evidence is still incomplete to substantiate claims by manufacturers so far. Owing to its chemical and structural resemblance with enamel minerals, the application of n-HAP to biomimetic repair the damaged enamel directly has received great awareness in today's dental research. Nano-sized particles have similarity to the apatite crystal of tooth enamel in morphology, crystal structure, and crystallinity.

The biomimetic hydroxyapatite function is to protect the teeth with the formation of a new layer of synthetic enamel around the tooth, before hardening the existing layer with fluorine, that chemically changes into calcium halophosphate [Ca (PO) F]. Therefore, the aim of the present study was to evaluate thus effectiveness and remineralization potential of n-HAP toothpaste on enamel.[9]

Materials and Methods

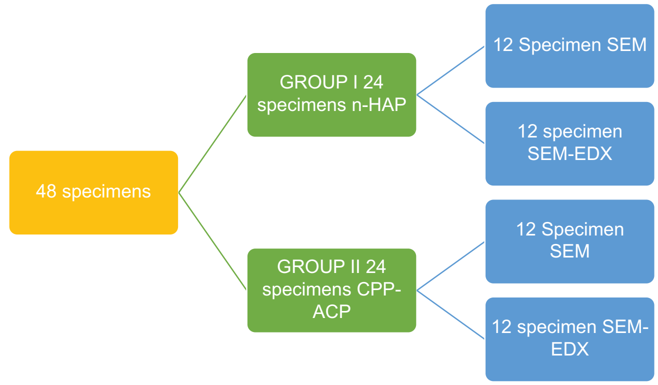
Current research was an *in vitro* study performed with approval of the Institutional Ethics Committee.

Sample size and selection

Forty-eight freshly extracted primary molars were collected from the Dept. of Pediatric and preventive dentistry and stored in 1% Thymol.

I. Study design
II. Preparation of specimen.

The crowns of primary molar teeth were separated from the roots by diamond disc, under continuous water cooling. Forty-eight enamel specimens of size 4 mm × 4 mm were prepared from the smooth surfaces of teeth using carborundum disc with slow speed handpiece under water cooling. Sound primary teeth were included in the study.



Demineralizing solution

KH_2PO_4 (potassium dihydrogen phosphate)-2.2mm/L, $Ca(No^3)^2$ (calcium Nitrate)-2.2 mm/L, Acetic acid–50 mm/L, NaF (sodium fluoride)-0.1ppm pH 4.5 is adjusted.

Procedure

All the 48 specimens were immersed in demineralizing solution for a period of 7 days at a constant temperature of 37° C. The solution was changed daily in the course of this experiment. The specimen was rinsed with deionized water using cotton swabs. All demineralized specimen were randomly divided into two groups. Twenty-four specimens of each group were stored in saliva solution in separate container. In Group-I specimen were treated with n-HAP toothpaste and in Group II specimen was treated by CPP-ACP mousse. n-HAP toothpaste and CPP-ACP containing paste were applied for 3 min to respective group specimens twice a day for 2 weeks using a soft toothbrush (Meridol, GABA, Lorrach Germany) and with minimum pressure. After brushing specimens were stored in the saliva solution without rinsing. The saliva solution was changed daily over the duration of 2 weeks. After 2 weeks, twelve samples of each group were evaluated for enamel surface morphologies in the corresponding treatments using SEM. The specimen was treated using a standard protocol as requied for scanning electron microscope (SEM). And, twelve samples of each group were evaluated for Ca and P mineral component using SEM- Energy Dispersive X-ray (EDX). For this the specimen were mounted on aluminum stubs using double-sided adhesive tape; the stubs were then placed in the vacuum chamber of the SEM-EDX. Data were analyzed using SPSS version 23. Frequencies, Descriptive statistics, paired *t*-test was done for intergroup comparison.

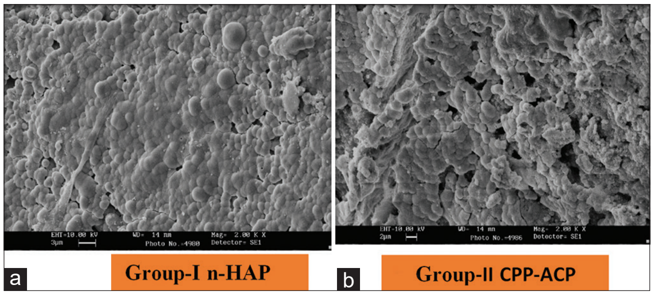
Results

SEM

The samples were then analyzed using a SEM (Carl Zeiss EVO 40, Germany) for surface morphology (Bruker X Flash Detector 3010, U.S.A.).

Group I and II demonstrated either amorphous crystals or particles scattered on the surface or lines of remineralization.

More crystals and fewer voids were observed in n-HAP Toothpaste Group than CPP-ACP Group. n-HAP Toothpaste Group was showing greater remineralized structure than CPP-ACP Group.



((a) SEM image showing effect of n-HAP after remineralization revealing smoothening of the enamel surface. (b) SEM image showing effect of CPP-ACP after 15 days revealing smoothening of the enamel surface.

The surfaces of the teeth treated with n-HAP displayed nucleation of apatitic crystals. The porous interprismatic and prismatic enamel structures were completely hidden by a thick, uniform apatitic layer with typical fastened needle-like HA crystallites in contrast to the specimens treated by CPP-ACP which exhibited evidence of uncovered porous surfaces. The surfaces of the teeth treated with n-HAP displayed nucleation of apatitic crystals in the pores created by demineralization.

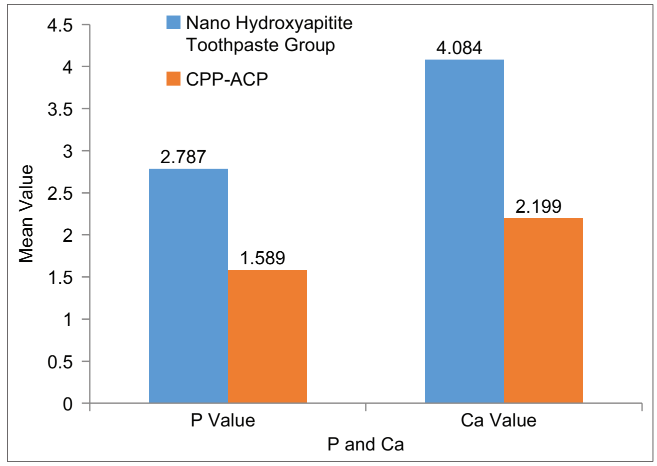
A superior surface texture of enamel was noted with n-HAP compared to CPP-ACP. This could be qualified to the resemblance of the nano-sized particles to the apatitic crystals of tooth enamel in morphology, crystal structure, and crystallinity.

SEM-EDX

Mean P was high in n-HAP Toothpaste Group (2.787 ± 1.699) than CPP-ACP Group (4.084 ± 1.603) and Ca was high in n-HAP Toothpaste Group (4.084 ± 1.603) than CPP-ACP Group (2.199 ± 1.865). Statistically, significant difference was present in value between Both Groups.

P and Ca are directly related with remineralization so n-HAP Toothpaste Group demonstrated greater remineralization than CPP-ACP Group in this study.

Graph A: show mean SEM-EDX result of, Comparison between n-HAP and CPP-ACP groups for, calcium and phosphate levels



Discussion

In a primary tooth, progress of enamel caries into underlying dentine is rapid. For the carious process to proceed, the pH and the ionic activities of Ca and P in plaque fluid are critical as they determine the stability of the tooth structure under cariogenic attack. Topical agents prevent and inhibit caries progression by inhibition of demineralization and/or enhancement of remineralization, thus creating a more caries-resistant surface due to the remineralized crystals.[10]

The early enamel lesion is characterized by four distinct histopathologic zones. Two zones of demineralization are present: (i) the translucent zone (1% pore volume) along the advancing front of the lesion; and (ii) the body of the lesion (>5–25% pore volume) representing the majority of the lesion and situated approximately 15–30 μm beneath the overlying intact enamel surface.

Two zones of remineralization are also present: (i) the dark zone (2–4% pore volume) situated near the advancing front just superficial to the translucent zone; and (ii) the surface zone (1–<5% pore volume) forming the intact surface overlying the lesion.[11]

The initial development of the lesion is due to the dissolution of HAP from the enamel prisms forming the enamel surface. The initial dissolution results in the loss of a small amount of mineral within the enamel and would have a similar appearance to the translucent zone (negative birefringence in Quinoline).[12]

Scientific literature proposes that clinical management of tooth demineralisation should accentuate on early detection and prevention, before a restorative approach is applied. Decades of research has steered the advancement of technologies that can promote enamel remineralisation or down scale demineralisation thereby reinforcing and aiding oral health.[13]

CPP-ACP is the acronym for a complex of Casein Phosphopeptides And Amorphous Calcium Phosphate. It has been proposed that the anticariogenic mechanism of CPP-ACP is due to localization of ACP at the tooth surface which then buffers the free Ca and P ion activities, which helps to sustain a state of super saturation with respect to enamel, thereby depressing demineralization and promoting remineralization.[14]

HAP is one of the most biocompatible and bioactive materials and is widely applied to coat artificial joints and tooth roots. Nano-sized particles have similarity to the apatite crystal of tooth enamel in morphology, crystal structure and crystallinity.[15]

Huang *et al*., explained how there is increased potential of n-HAP to directly fill up defects and micropores on demineralized teeth. If n-HAP penetrates the enamel pores, n-HAP will act as a pattern in the precipitation process and will continuously attract a large amount of $Ca^{2+}$and PO_3^{-4} from the remineralization solution to the enamel surface to fill the unoccupied positions of the enamel calcium crystals. This will encourage crystal integrity and growth.[16]

Vyavhare *et al*., compared remineralisation potential of 1%, 5%, 10%, 15% n-HAP under SEM and found 10% and 15% n-HAP was found to have similar remineralizing effectiveness as that of 1000 ppm fluoride. They considered it as an effective alternative to 1000 ppm fluoride for promoting remineralization on daily basis.[17]

Singh, *et al*., compared four pastes for the remineralizing efficiency, and found- Aclaim and Apagard n-HAP pastes to be superior to Clinpro Tooth Crème and Colgate Total. In addition, that Aclaim and Apagard on daily application would provide maximum protection against enamel demineralization in orthodontic patients, by the configuration of new n-HAp layer which is insensitive to demineralization.[6] In the present research, the tooth sections were subjected to a chemical caries model for the production of artificial caries lesion and pH cycling for testing the efficacy of remineralizing agents, which were consecutively exposed to demineralizing and remineralizing solutions with intermediary treatments with the agents. These methods have improved understanding of the mechanism of demineralization and remineralization. Further, they provide information about the effects of caries preventive agents on de/remineralization dynamics at the surface of the teeth.

Demineralization begins at the atomic level on the crystal surface inside the enamel or dentin and can continue but for halted with the endpoint being cavitation. The initial stages of the carious lesion are characterized by a partial dissolution of the tissue, leaving a 2–50-µm thick mineralized surface layer and a subsurface lesion with a mineral loss of 30–50% extending into enamel and dentin. In a clinical examination, the lesion will appear chalky white and softened. In practice, the goal is to stop the process at the WSL stage, when intervention can still be nonsurgical.[18] If the lesion advances, the outer enamel layer can eventually become cavitated. At this point, the lesion is not reversible and requires operative interference. The demineralization of teeth was carried out as per the method described by Damle *et al*. In this method, a buffer solution system was used, and the lesions were created over 6 days at 37°C.[19]

It has been observed that the relative value of critical pH is significantly higher for children than for adults in both resting and stimulated saliva. In addition, children exhibit a greater thermodynamic driving force for demineralization at low oral pH, and a lower force for remineralization at normal oral pH. This has led to an increased risk of demineralization in the child population. This pH cycling model has been utilized successfully to assess the anti caries potential of dentifrice formulations since it simulates *in-vivo* high caries risk situations and also measures the net result of the inhibition of demineralization and the enhancement of remineralisation.[19] The protocol adopted in the current study, the dynamic cycles of demineralization and remineralization was simulated by sequentially immersing enamel specimens in acidic (demineralizing) and supersaturated (remineralizing) solutions. Dentifrice use was simulated by topical application of the agents twice per day during the de- and remineralization stages. The demineralizing solution uses an acid buffer of pH 4.4, while the remineralization solution contains Ca and P at a known degree of saturation at pH 7.0. This solution approximates the mineral ion composition and supersaturation of saliva.[13]

The pH cycling protocol to simulate demineralization, adopted for this study was based on the model described by Featherstone.,[20] and Ten Cate and Duijsters.[21]

The efficiency of n-HAP paste under dynamic pH cycling may be referred to the explicit filling and adherence of globular deposits of n-HAP crystals in the enamel porosities which was observed by the SEM evaluation. n-HAP crystals might have attracted a larger amount of Ca2+ and PO4 3−from the remineralizing agent which acted as a reservoir during pH cycling.[22]

Several techniques have been in use for the assessment of enamel remineralization. This can be achieved both quantitatively by mineral content and hardness profiles or SEM-EDX and SEM.[22]

In the current study, the Ca/P ratio decreased to a considerable level in both groups after demineralization. Although both the groups showed, post-application a significant increase in Ca/P ratio ($P < 0.05$), it was found that the recovery of mineral content was higher in the HA group than in the CPP-ACP group and the difference was statistically significant ($P < 0.05$). Similar results were observed by Swarup and Rao,[23] studied the specimen treated with synthetic n-HAP exhibited surface Ca/P ratio close to that of the biologic enamel, indicating that apatite coating deposition on the demineralized enamel surface As on remineralization by n-HAP crystal expressed that the enamel slabs brushed with the material showed increase in Ca/P ratio.[22]

SEM analysis allowed the investigation of the morphology of remineralized enamel and the features observed after remineralization procedures induced by *in vitro* application of biomimetic carbonated HAP nanocrystals. Remineralization could be estimated in terms of Ca and P content of enamel also.

In the current study, the particles of n-HAP had the typical rod-like morphology and their lengths were hundreds of nanometres. The result of n-HAP was conducive with the lattice structure of hydroxyapatite and exhibit excellent crystallinity. However, there is no clear diffraction pattern for ACP, which indicates that its main composition is amorphous phase. Numerous needle-like apatite crystallites were oriented to form the organized structure on the enamel surface. Similar crystalline structures were observed in, smooth surface morphology formation in studies performed by Swarup and Rao,[23] and Roveri *et al*.[22]

In this current research, the SEM analysis of the surface after remineralization induced by n-HAP paste showed cluster depositions of n-HAP crystals post-treatment, which was also noticed after pH cycling, this indicates that the adherence of the crystals was stable. These observations were in accordance with de Carvalho *et al*.,[24] who found similar cluster deposition using the same n-HAP paste. In addition, similar results were observed by Vyavhare *et al*.,[17] who studied the effect of adding synthetically processed n-HAP to toothpaste in remineralizing initial enamel lesion. Issa Daas *et al* showed a decrease in the porosity with deposition of n-HAP crystals similar to our study.

In the current study the CPP-ACP group, immediately post-treatment SEM showed a reduction in the surface porosity with areas of smooth enamel surface; however, an evidence of uncovered porous surfaces was observed after pH cycling. This result was not in accordance with the study of de Carvalho *et al*.,[24] who reported the presence of a homogeneous protective layer after pH cycling. The difference in morphology in corresponding treatment perhaps was due to different mechanisms for promoting remineralization.

In the current study, EDX results indicated that there was a decrease in the wt% of both Ca and P following the demineralization of samples. Furthermore, after the treatment for15 days, there was a significant increase of the elements. The results of this study indicated that n-HAP toothpaste was significantly more effective in increasing the mineral content (Ca and P) as compared to CPP-ACP over longer treatment duration. This difference in the results can be accredited to the fact that n- HAP toothpaste acts differently than CPP-ACP paste.

Mean C was high in n-HAP Toothpaste Group (4.084 ± 1.603) than CPP-ACP Group (2.199 ± 1.865). Statistically, significant difference was observed in Ca value between both Groups.

Treatment with both CPP-ACP and n-HAP resulted in a slight increase in thickness of the surface enamel. n-HAP group had a better increase in enamel thickness, and the difference was statistically significant ($P > 0.05$).

Evidences from the studies by Kumar *et al*.,[25] Petersen,[26] have also shown with SEM EDX, that lesions can be re-hardened by deposition of hydroxyapatite that is initially deposited near the surface layer of the enamel and this was found to be significant.

In the current *in vitro* research n-HAP group illustrated more remineralized structure than the CPP-ACP Group. The surface of the teeth treated with n-HAP displayed nucleation of apatitic crystals. The porous interprismatic and prismatic enamel structures were completely hidden by a thick, uniform apatitic layer with typical fastened needle-like HA crystallites in contrast to the specimens treated by CPP-ACP which exhibited evidence of uncovered porous surfaces.

It is necessary to note that remineralization *in vitro* may be quite unpredictable when compared to changes occurring in the oral cavity *in vivo*. One of the limitations of the study is the absence of the control group, which would have provided a more accurate wt % of the changes in the Ca, P elements.

Clinical significance: Based on the findings of this study, n-HAP paste might be recommended as alternative remineralizing agent that could be beneficial for children.[27,28] A number of studies have established CPP-ACP to have anticariogenic activity in laboratory, animal, and human *in situ* experiments. n-HAP is a material with high tissue biocompatibility with structure similar to enamel.

The enamel of primary teeth is less mineralized, exhibits a greater diffusion coefficient, and therefore, more vulnerable to acid dissolution compared to the enamel of permanent teeth. At present, less data are available in the literature on remineralization efficacy in primary teeth.[16] Thus, the present study was conducted to evaluate the remineralization potential of n-HAP and CPP-ACP.

Current *in vitro* study concluded that n-HAP has the potential to remineralize initial enamel lesions. A concentration of 10% n-HAP may be optimal for remineralization of early enamel caries. Given the small number of experiments and limitations of the *in-vitro* studies comparing the re-mineralizing potential of n-HAP toothpaste on enamel, the effectiveness of this toothpaste seems to be obvious in improving the initial enamel carious lesions. Various literature reports have shown the value of DIAGNOdent as a trustworthy non-invasive cariesdetecting device. This device could prove contributory in the assessment of the efficacy of n-HAP in a clinical scenario, in a natural oral environment.[16]

Conclusion

Based on the data obtained from the present study, it was concluded that even though both CPP-ACP and n- HAP showed remineralization potential, remineralization was found to be higher in the samples treated with n-HAP.

Within the boundaries of our *in vitro* study, we conclude the following:

1. Both the agents n-HAp and CPP-ACP, were effective in causing remineralization of enamel
2. n-HAP was more effective as compared to CPP-ACP in increasing the Calcium and Phosphate content of enamel, and this effect was more evident over a longer treatment period
3. Hardness of the tooth structure improved following the application of n-HAP than CPP-ACP.

n-HAP paste showed potential long-term protective effect in terms of surface depositions and maintaining a smooth surface.

Hence, in the present study, n-HAP showed enhanced remineralization and therefore can be expected to be effective in high-risk children who have not developed good oral hygiene habits.

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