Effect of Two Remineralizing Agents on Initial Caries-like Lesions in Young Permanent Teeth: An in Vitro Study

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ABSTRACT

Aim: To compare the effect of nano-hydroxyapatite (9000 ppm F) and casein phosphopeptide-amorphous calcium phosphate fluoride (900 ppm F) pastes on initial enamel carious lesions of young permanent teeth.

Materials and methods: Sixty extracted young premolars with a standardized window on enamel were immersed in a demineralizing solution for 48 hours to produce subsurface enamel lesions. They were divided into three groups according to remineralizing agents (n = 20) group I: nano-hydroxyapatite paste; group II: casein phosphopeptide-amorphous calcium phosphate fluoride paste; and group III: control (without an agent). The enamel surface microhardness (SMH) was measured at baseline, after the incipient enamel lesion, and after treatment. Additional twenty young premolars were selected and prepared as mentioned above for surface morphology evaluation by scanning electron microscope (SEM).

Results: No significant difference was found in mean surface microhardness in teeth treated with nano-hydroxyapatite paste and those treated with casein phosphopeptide-amorphous calcium phosphate fluoride paste p = 0.26. SEM showed improvement in surface defects of demineralized enamel in the two test groups.

Conclusion: Nano-hydroxyapatite and casein phosphopeptide-amorphous calcium phosphate fluoride pastes were effective in rehardening the initial enamel caries lesions in young permanent teeth.

Clinical significance: The best strategy for caries management is to focus on the methods of improving the remineralization process with the aid of the remineralizing agents. The current study compared the remineralizing effect of two remineralizing agents. Within the limitations of the study, both remineralizing agents were effective for remineralization of early caries-like lesions.

Keywords: Casein phosphopeptide-amorphous calcium phosphate fluoride, Initial caries lesion, Nano-hydroxyapatite, Young permanent teeth.


Source of support: Nil

Conflict of interest: None

INTRODUCTION

Dental caries is considered a significant problem across the world. When the pH drops and the disturbance of the equilibrium between the tooth surface and the surrounding plaque acid occurs, the result is a loss of minerals from the tooth surface. Fluoride is considered an effective method for controlling and reducing enamel demineralization in both the primary and permanent dentitions by the formation of a calcium fluoride (CaF2)-like layer on the demineralized surface. Fluoride varnish has been extensively used to treat and prevent incipient enamel caries lesions due to its high fluoride concentration and adhesion capacity to tooth enamel. However, dental fluorosis occurs as a result of excessive fluoride ingestion during tooth formation. New tooth re-mineralization technologies have been developed including compounds with the additional or synergistic effects of fluoride to enhance the remineralization process and improve the mechanical properties of the demineralized surface, such as casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) and nano-hydroxyapatite (Nano-HAP). Casein phosphopeptide-amorphous calcium phosphate is a bioactive agent with a base of milk products. Casein...
phosphate (CPP) stabilizes amorphous calcium phosphate (ACP) and localizes it in dental plaque. This complex acts as a reservoir for storing bioavailable calcium and phosphate and maintains the solution supersaturated, hence facilitating remineralization. A number of reports have proved the efficacy of the CPP-ACP technology in inhibiting demineralization and enhancing remineralization of enamel and dentin in vivo and in vitro. There are two types of phosphopeptide-based dental products: CPP-ACP in paste or mousse form and casein phosphopeptide-amorphous calcium phosphate fluoride (CPP-ACPF) product. The availability of calcium, phosphate and fluoride in one product is the main advantage of CPP-ACPF. However, De Carvalho et al. stated that CPP-ACPF did not show any additional protection on artificial early enamel caries development on permanent teeth.

As hydroxyapatite considered being the main mineral component found in enamel and comprising more than 60% of tooth dentin by weight, thus the ideal method of increasing remineralization is through using it for reconstruction of the depleted tissues. The nano-hydroxyapatite paste is one of the most biocompatible and bioactive agents. The nanocrystals of phosphate are smaller than 100 nm, improving the bioactivity of the agent due to the increase in the superficial area of hydroxyapatite nanoparticles. Thus the innovation of incorporating the Ca and PO4 ions, as nano-HA crystals, with 9,000 ppm of fluoride may improves the surface hardness of the demineralized enamel and promotes remineralization.

De Carvalho et al. evaluated the remineralization effects of nano-HA paste on artificial early enamel lesions of permanent teeth. They concluded that nano-HA paste was effective in remineralizing the initial enamel caries lesions. Moreover, several studies have shown that nano-HA had the potential to remineralize artificial carious lesions when added to toothpaste and mouthwashes.

Limited researches compared the effect of nano-HA paste and CPP-ACP on initial enamel carious lesions. That is why this study was designed to compare the remineralizing capacity of nano-HA and CPP-ACP pastes on initial enamel carious lesions in young permanent teeth. The null hypothesis tested was that there was no difference between the effects of the two remineralizing agents on enamel microhardness.

MATERIALS AND METHODS

The Scientific Research Ethical Committee, Faculty of Dentistry, Alexandria University, Egypt, approved this study. Calculation of sample size was done using SPSS software, version 21.0. Armonk, NY: IBM Corp. Significance level was set to 0.05, and maximum accepted was up to 20 percent, with a minimum power of 80 percent.

The sample consisted of 80 young premolars freshly extracted due to orthodontic purposes, 60 for microhardness evaluation and 20 for scanning evaluation. Teeth were collected from the outpatient clinic of the Faculty of Dentistry, Alexandria University and private Dental Orthodontic Clinics in Alexandria Governorate. Teeth free of caries, cracks, and developmental defects were included in the study.

Materials

- Calcium nano phosphate paste (Desensibilize Nano P, FGM Produtos Odontológicos, Joinville, Brazil) which is Calcium nanophosphate organized in a crystalline form of hydroxyapatite, potassium nitrate, water, surfactant, tensioactive, flavor, 9000 ppm sodium fluoride.
- Casein phosphopeptide-amorphous calcium phosphate fluoride (GC MI Paste Plus, GC America Inc., Alsip, Ill., USA), which is a water-based, sugar-free cream containing Recaldent (milk derived with lactose content less than 0.01 percent) with fluoride.
- The Demineralizing solution consisted of 2.2 mM calcium chloride, 2.2 mM potassium dihydrogen phosphate, 0.05 M acetic acid, and 1 M potassium hydroxide (KOH) to maintain a pH of 4.4.
- Artificial saliva was prepared by mixing 500 ml distilled water, 20 g potassium chloride, 0.843 g sodium chloride, 0.051 g magnesium chloride, carboxymethyl cellulose, 20 ml tricalcium phosphate, and 0.05 M sodium hydroxide to maintain a pH of 6.8.
- The artificial saliva was renewed daily through the whole study period.
- Distilled water.

Methods

All teeth were thoroughly cleaned using fluoride free pumice and were stored in saline solution until required for use. Roots were removed at the cement-enamel junction (CEJ) with a water-cooled diamond saw of a precision sectioning machine. Specimens were embedded in acrylic resin with their buccal surface facing upwards. The buccal enamel of all the specimens was grinded using silicon carbide papers (grades 600 to 1200) underwater irrigation and polished to produce a flat surface. Every tooth specimen was coated with acid-proof nail varnish, exposing only a small window in the cervical third of the buccal surface of enamel (4 × 4 mm) which were exposed to the demineralizing solution to produce caries-like lesions.

The null hypothesis tested was that there was no difference between the effects of the two remineralizing agents on enamel microhardness.
lesion. The exposed window was standardized in all the specimens. Before any cariogenic challenge, all specimens were assessed for enamel surface microhardness (SMH) using digital display vickers micro-hardness tester with a Vickers diamond indenter and a 20X objective lens. A load of 50 g was applied to the surface of the specimens for 10 seconds (initial baseline assessment). Five indentations were equally placed over a circle of 1 mm diameter at the cervical third of the specimens. The diagonal length of the indentations were measured by a built in scaled microscope and Vickers values were converted into micro-hardness values. The surface Micro-hardness was obtained using the following equation:

\[ HV = \frac{1.854 P}{d^2} \]

where HV is Vickers hardness in Kef/mm², P is the load in Kef and d is the length of the diagonals in mm. Baseline evaluation was done, so that the effect of Nano-HAP paste and the CPP-ACPF paste, on the enamel specimens, could be verified.

**Demineralization Phase**

All teeth included in the study were immersed in a demineralizing solution (10 mL for each specimen) for 48 hours to produce subsurface enamel lesions (white spot lesion without cavitation). Specimens were rinsed with distilled water and stored in artificial saliva. The demineralizing solution was renewed after 24 hours to prevent depletion or saturation of solution and accumulation of enamel dissolution agents. After initial caries formation, post-lesion microhardness test was conducted with the same static load and time applied for obtaining the baseline measurements (Second assessment).

**Remineralization Phase**

For quantitative evaluation, the 60 teeth were divided randomly into three groups according to remineralizing agents (n = 20): Group I: Nano-hydroxyapatite paste group, specimens of this group were treated with nano-HA once daily for 5 minutes for 7 days. Nano-HA paste was applied with a microbrush with friction for 10 seconds. After this, the paste was kept in contact with the enamel for five minutes and removed with distilled water then specimens were stored in artificial saliva.

Group II: casein phosphopeptide-amorphous calcium phosphate and fluoride paste group, specimens of this group were treated with CPP-ACPF once daily for 5 minutes for 7 days. The paste was applied with a cotton tip and maintained in contact with enamel for five minutes and removed with distilled water then specimens were stored in artificial saliva.

Group III: control group, specimens of this group were left untreated and stored in artificial saliva.

The post-treatment microhardness test was conducted with the same static load and time applied for baseline and post-lesion measurements (Final assessment). All data were transferred to SPSS 21.0 software (IBM, Armonk, N.Y., USA), and analyses were performed. The Kruskal–Wallis test was used for comparing two or more independent not-normally distributed samples of equal or different sample sizes. The Friedman test was used for comparing two or more dependent not-normally distributed samples. Post-hoc pair-wise comparison when Kruskal-Wallis test or Friedman test was significant was carried out using Mann-Whitney tests. A five percent level of significance was adopted.

For qualitative evaluation, scanning electron microscope (SEM) was used. Additional twenty young permanent teeth were selected and prepared as mentioned before. They were labeled and divided into four groups (A–D) (five for each group). Group A: To assess the sound enamel surface, teeth were labeled from 1' to 5'; Group B: To assess the demineralized enamel surface, teeth were labeled from 6' to 10'; Group C: To assess the demineralized enamel surface after treatment with nano-HA paste, teeth were labeled from 11' to 15' and Group D: To assess the demineralized enamel surface after treatment with CPP-ACPF, teeth were labeled from 16' to 20'. All specimens were dehydrated through ascending grades of ethyl alcohol 50%, 70%, 90% and absolute alcohol. Specimens were vacuumed and gold sputter coated with gold-palladium layer before examination then they were examined by SEM JSM-5300 at operating magnification 3500 at 20 KV to study the surface of enamel. Photomicrographs were taken to achieve comparison between the groups.

**RESULTS**

Results of the present study showed that the SMH values of the sound enamel (baseline) were not significantly different among the three groups (p = 0.577) (Table 1). After immersion in the demineralizing solution for 48 hours, there was no statistically significant difference among the three groups (p = 0.740) (Table 2). Post treatment, there were significant differences among groups (p = 0.028). By pair-wise comparison using Mann-Whitney test, there was no statistically significant difference in surface microhardness between nano-HA group and CPP-ACPF group (p = 0.261). The nano-HA group showed the highest mean microhardness values (201.46 ± 19.3), followed by CPP-ACPF group (195.6 ± 19.6), and control group (181.4 ± 26.6) (Table 3).

Using the Friedman test, in the nano-HA group, the mean value of the post treatment phase (201.46 ± 19.93) was significantly higher than of post-lesion phase.
Table 1: Descriptive statistics of the enamel surface microhardness of three groups at baseline

<table>
<thead>
<tr>
<th></th>
<th>Nano-hydroxyapatite paste group</th>
<th>Casein phosphopeptide-amorphous calcium phosphate paste group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± Std. deviation</td>
<td>204.75 ± 19.4</td>
<td>207.16 ± 18.28</td>
<td>202.37 ± 25.39</td>
</tr>
<tr>
<td>Median</td>
<td>200.000</td>
<td>205.500</td>
<td>195.500</td>
</tr>
<tr>
<td>Inter-quartile range</td>
<td>191.00-214.60</td>
<td>192.195–212.000</td>
<td>180.000–227.825</td>
</tr>
<tr>
<td>KS test of normality</td>
<td>D = 0.147</td>
<td>D = 0.196</td>
<td>D = 0.191</td>
</tr>
<tr>
<td></td>
<td>p = 0.200 NS</td>
<td>p = 0.043*</td>
<td>p = 0.054 NS</td>
</tr>
<tr>
<td>Independent-samples Kruskal-Wallis test</td>
<td>p = 0.577 NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

KS: Kolmogorov–Smirnov
*: Statistically significant (p < 0.05)
NS: Statistically not significant (p > 0.05)

Table 2: Descriptive statistics of the enamel surface microhardness after initial caries formation by demineralizing solution

<table>
<thead>
<tr>
<th></th>
<th>Nano-hydroxyapatite paste group</th>
<th>Casein phosphopeptide-amorphous calcium phosphate paste group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± Std. deviation</td>
<td>184.03 ± 22.83</td>
<td>187.04 ± 21.39</td>
<td>183.48 ± 26.97</td>
</tr>
<tr>
<td>Median</td>
<td>182.3000</td>
<td>187.700</td>
<td>176.3500</td>
</tr>
<tr>
<td>Inter-quartile range</td>
<td>155.00-195.675</td>
<td>171.025–195.300</td>
<td>162.00–211.325</td>
</tr>
<tr>
<td>KS test of normality</td>
<td>D = 0.109</td>
<td>D = 0.179</td>
<td>D = 0.208</td>
</tr>
<tr>
<td></td>
<td>p = 0.200 NS</td>
<td>p = 0.093*</td>
<td>p = 0.023*</td>
</tr>
<tr>
<td>Independent-Samples Kruskal-Wallis Test</td>
<td>p = 0.740 NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

KS: Kolmogorov–Smirnov
*: Statistically significant (p < 0.05)
NS: Statistically not significant (p > 0.05)

Table 3: Descriptive statistics of the enamel surface microhardness after treatment.

<table>
<thead>
<tr>
<th></th>
<th>Nano-hydroxyapatite paste group</th>
<th>Casein phosphopeptide-amorphous calcium phosphate paste group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± Std. deviation</td>
<td>201.46 ± 19.93</td>
<td>195.61 ± 19.61</td>
<td>181.42 ± 26.62</td>
</tr>
<tr>
<td>Median</td>
<td>199.000</td>
<td>193.800</td>
<td>174.500</td>
</tr>
<tr>
<td>Inter-quartile range</td>
<td>187.475-211.200</td>
<td>181.175–203.300</td>
<td>150.700–208.575</td>
</tr>
<tr>
<td>KS test of normality</td>
<td>D = 0.149</td>
<td>D = 0.195</td>
<td>D = 0.210</td>
</tr>
<tr>
<td></td>
<td>p = 0.200 NS</td>
<td>p = 0.045*</td>
<td>p = 0.021*</td>
</tr>
<tr>
<td>Pair-wise comparison</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compared with control</td>
<td>p = 0.018*</td>
<td>p = 0.045*</td>
<td></td>
</tr>
<tr>
<td>Compared with nano-hydroxyapatite paste group</td>
<td>p = 0.261 NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

KS: Kolmogorov–Smirnov
*: Statistically significant (p < 0.05)
NS: Statistically not significant (p > 0.05)

Pair-wise comparison was done using Mann–Whitney test

(184.03 ± 22.83). Although post-treatment value was still significantly lower when compared to the baseline phase (204.75 ± 19.4) (p = 0.001). On the same line, the mean value of post-treatment phase of CPP-ACPF (195.61 ± 19.61) was significantly higher than post-lesion phase (187.04 ± 21.39). However, they were still significantly lower than those of the baseline phase (207.16 ± 18.28)(p = 0.001). For the control group, there was a statistically significant difference between the three phases. The mean value of post-treatment phase was (181.42 ± 26.62) while the mean values of post-lesion and baseline phases were (183.48 ± 26.97, 202.37 ± 25.39) respectively (p = 0.001) (Graph 1).

Regarding SEM evaluation, the normal enamel surface (group A) appeared smooth, uniform with minimal irregularities. After demineralization (group B) the surface topography of enamel revealed deepened craters and pits simulating type I etching pattern. This appearance is characterized by preferential loss of enamel
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Graph 1: The change in enamel surface micro-hardness in the three phases of nano-HA, CPP-ACPF and control groups.

Effect of Remineralizing Agents on Initial Caries-like Lesions rods and persistence of interrod enamel (Figs 1A and B).

Following the application of remineralizing agents, group C (nano-HA), the enamel surface showed relative restoration of the normal smooth enamel with few areas showing limited scratching and minor depressions. In group D (CPP-ACPF), the surface revealed obvious improvement in surface texture. However, surface pittings, concavities with interspersed elevated zones were also detected (Fig. 1C and D).

DISCUSSION

Based on the results of the present study, the null hypothesis was accepted. As compared to the control group, both nano-HA and CPP-ACPF pastes can increase the surface hardness of the demineralized enamel.

Our results showed that the demineralized enamel surfaces in all groups revealed an overall significant decrease in microhardness, indicating loss of minerals, as all teeth in all groups were subjected to the same demineralizing solution, for the same period of time in order to achieve standardization. Microhardness analysis has been used as a method to assess loss and reincorporation of minerals to dental tissue as the reduction in the numerical hardness value presents a linear relation to mineral loss.\textsuperscript{34}

Graph 1: The change in enamel surface micro-hardness in the three phases of nano-HA, CPP-ACPF and control groups.

Figs 1A and B: (A) Scanning electron micrograph showing normal, uniformly-smooth enamel surface; (B) Demineralized enamel showing marked surface irregularities. Most of the demineralized areas simulating type I etch pattern with demineralized rod core (arrowhead) and elevated Interrod enamel (arrow). (Org. Mag. X 3.500)

Figs 1C and D: (C) Scanning electron micrograph of demineralized enamel treated with nano-HAP showing normal topography of enamel surface; smooth calcific deposits masks all the irregular signs of demineralization; (D) Demineralized enamel treated with CPP-ACPF showing relatively smooth enamel surface with few concave areas of variable depths (arrows). (Org. Mag. X 3.500)
After application of the remineralizing agents, there was a statistical significant difference in enamel SMH in each group when compared with the data obtained after demineralization. Both test groups showed a significant increase in the microhardness. Changes in the SMH of the CPP-ACPF group were in agreement with the results of Shetty et al.\textsuperscript{35}

Furthermore, Talaat and Mahmoud\textsuperscript{36} evaluated the effect of acid on enamel subsurface lesions that were previously treated with CPP-ACPF and found that CPP-ACPF was able to remineralize the enamel subsurface lesions, due to the first demineralization, and to protect them against further acid attack.

Regarding nano-HA group, the changes in microhardness were supported by the work of Huang et al.\textsuperscript{37} and Haghgoo et al.\textsuperscript{38} who evaluated its effect for remineralization of incipient carious lesions and they found that nano-HA greatly enhanced remineralization and increased tooth microhardness.

Moreover, by comparing the two test groups post-treatment, nano-HA group showed better mean microhardness value compared to CPP-ACPF group with a statistically insignificant difference. This result was supported by the result of De Carvalho et al.\textsuperscript{39} However, this insignificant result concerning the comparison between the two groups was inconsistent with another study conducted by De Carvalho et al.\textsuperscript{22} They stated that after the cariogenic challenge, the nano-HA group showed significantly higher microhardness values while the CPP-ACPF group showed no increase in surface microhardness. This result could be due to the fact that De Carvalho et al used a different study design. Additionally in the present study, although the post-treatment microhardness values of both test groups were near to those of baseline values, there was a statistically significant difference. This revealed that nano-HA and CPP-ACPF pastes were used for prevention rather than treatment. Concerning the quantification of mineral loss, further studies would benefit from combining microhardness evaluation with techniques that enable to measure the lesion depth.

Scanning electron microscope findings were consistent with the changes in the microhardness at the different stages of the in vitro study. The demineralized specimens showed a deeply eroded, pitted enamel surface with evident ultrastructural details of enamel represented by rod and interrod elements. This finding could be explained by the differential removal of hydroxyapatite crystals occurring at the central core of the enamel rod with persistence of the peripheral interrod regions upon acid dissolution.

After application of the different remineralizing agents, nano-HA and CPP-ACPF, the normal enamel surface smoothness was relatively restored showing a decrease in irregularities and pitting, denoting remineralization. However, enamel remineralization following nano-HA application showed a marked improvement when compared to specimens treated by CPP-ACPF. The mechanism of action of nano-HA is based on its bioactive and biocompatible properties. Incipient caries provides a porous surface and a larger surface area which allows a greater penetration of nano-HA crystals to react with enamel minerals. These crystals directly fill up defects and micropores on demineralized teeth, and act as a template continuously attracting Ca and PO\textsubscript{4} which in turn promotes crystal integrity and growth.\textsuperscript{11} This finding is in an agreement with Huang et al.\textsuperscript{37} who evaluated the effect of nano-HA concentration on remineralization of initial enamel lesion by using SEM and found that its particles were regularly deposited on the cellular structure of the demineralized enamel.

Regarding CPP-ACPF group, the smooth enamel surface was interrupted by few enamel cracks and craters. This finding may be interpreted by the fact that the CPP-ACFP nano-complex acts as reservoir of Ca and PO\textsubscript{4} and helps the dental plaque to maintain a state of ion supersaturation, thus depressing demineralization and enhancing remineralization.\textsuperscript{40}

This result is supported by Jayarajan et al.\textsuperscript{40} who found a greater amount of mineral deposits on the demineralized enamel surface following the application of CPP-ACPF.

A possible limitation of the present study was that the high concentration of the fluoride in the two test groups pose a safety concern when used by children below the age of 6 years as could give rise to enamel fluorosis if the child swallows it. Another limitation was the absence of positive control which was required so that the effect of nano-HA and CPP-ACPF on enamel microhardness can be compared to that of fluoride varnish to see whether the two remineralizing agents are better/worse than the conventional varnish or not.

It is imperative to note that remineralization in vitro may be quite variable when compared to changes occurring in the oral cavity in vivo. Therefore, direct extrapolations to clinical situations must be executed discreetly.

Based on the results of the present study, despite the limitations, it is important to clarify that nano-HA showed the largest increase in microhardness values. This may be due to the application method of this paste (10 seconds of friction) and the calcium nano phosphate crystals may have penetrated more deeply into the defects of the carious...
enamel, forming a “reservoir-like” deposit of Ca and Po$_4$. The reservoir-like deposit could make these ions available during a subsequent cariogenic challenge and help maintain a state of super-saturation with enamel minerals. Furthermore, the fluoride concentration in the nano-HA paste is 10 times higher (9000 ppm) than that of the CPP-ACP paste (900 ppm). This higher concentration may affect the remineralization process.

CONCLUSION

From the present study, it can be concluded that:

Nano-hydroxyapatite paste and Casein phosphopeptide-amorphous calcium phosphate Fluoride paste were more effective in rehardening the initial enamel caries lesions caused by demineralization solution than no treatment in young permanent teeth.

RECOMMENDATIONS

Clinicians, especially paediatric health professionals and patients, should be aware of prevention and early intervention of caries by using the remineralizing agents.

Further studies should be conducted to evaluate the effect of brushing and attrition on nano-HA crystals that fill up the micropores on demineralized teeth.

REFERENCES


