Comparison between Fluoride and Nano-hydroxyapatite in Remineralizing Initial Enamel Lesion: An *in vitro* Study

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ABSTRACT

Aim: The aim of this study was to compare the effectiveness of nano-hydroxyapatite (nano-HAP) paste and fluoride varnish in remineralizing initial enamel lesion in young permanent teeth and their ability to resist secondary caries under dynamic pH cycling quantitatively and qualitatively.

Materials and methods: Initial caries-like lesions were artificially developed on 45 specimens. Specimens were divided into three groups: (1) Control (without treatment), (2) fluoride varnish (3M ESPE), and (3) nano-HAP paste (Desensibilize Nano P). The nano-HAP paste was applied twice separated by one pH cycle, and the varnish was applied only once followed by 7 days of pH cycling. All specimens were examined using DIAGNOdent[®] pen (KaVo, Germany), and a representative specimen was randomly selected from each group for qualitative evaluation using scanning electron microscope (SEM) at four stages: Baseline, after lesion formation, immediately after remineralization, and after pH cycling. Data were statistically analyzed with Statistical Package for the Social Sciences (SPSS), version 20.

Results: The degree of demineralization was significantly elevated in control group; however, no significant difference was found between fluoride varnish group and nano-HAP paste group ($p < 0.001$).

Conclusion: Nano-HAP paste showed promising long-term protective effect in terms of surface depositions and maintaining a smooth surface compared with fluoride varnish.

Clinical significance: Based on the findings of this study, nano-HAP paste might be recommended as alternative remineralizing agent with lower fluoride concentration than fluoride varnish that could be beneficial for children, pregnant females, and those who are at high risk of dental fluorosis.

Keywords: Enamel lesion, Nano-hydroxyapatite, Remineralization.

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INTRODUCTION

The modern dental practice is focusing on prevention and minimal intervention, and the replacement of lost tooth substance with biomimetic material is considered as one of the fundamentals of minimal intervention dentistry, with the goal of remineralizing an incipient lesion rather than extensive surgical replacement. Several methods were introduced to remineralize an early tooth structure loss.^{1,2}

Fluoride varnishes have been known to be effective in the prevention of incipient enamel lesion in the presence of calcium and phosphate ions as it advances the development of fluorapatite.³ However, the adoption of remineralization of initial enamel lesions within the concept of minimal intervention dentistry led to the introduction of several agents and compounds as bioactive formulation of tricalcium phosphate, bioactive glass (calcium sodium phosphosilicate), casein-based products, and, finally, nano-HAP.⁴⁻⁶

The biomimetic nano-HAP has the ability to protect the teeth with the creation of a new layer of synthetic enamel around the tooth, rather than hardening the existing layer with fluoride.⁴ Several studies on nano-HAP as a biomimetic material showed the capability of

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remineralizing initial enamel lesions under pH cycling model.⁷⁻⁹

The newly introduced nano-HAP paste is a bioactive agent that contains calcium nano-phosphate organized in a crystalline form of HA incorporated with highly concentrated sodium fluoride of 9000 ppm fluoride ion (Desensibilize Nano P, FGM, Joinville, Brazil). This paste is indicated for desensitization and/or remineralization of the enamel and is commercially available for professional use. According to the manufacturer, the maximum remineralizing effect can be observed by two professional applications of the paste, but the estimated number of sessions may vary according to the clinical judgment.

The purpose of this study was to evaluate the effect of fluoride varnish *vs* nano-HAP paste in remineralizing initial enamel caries quantitatively using quantitative laser fluorescence DIAGNOdent[®] pen (KaVo, Germany) and qualitatively using SEM. It was hypothesized that there is no difference between the effects of the two remineralizing agents on initial enamel caries.

MATERIALS AND METHODS

This controlled experimental *in vitro* study was conducted using intact human premolars extracted for orthodontic reason. Before initiating the study, the approval of the institutional review board committee was obtained. The teeth were inspected by naked eye, and any carious or malformed teeth were excluded. Teeth were thoroughly cleaned of debris and refrigerated in 0.1% thymol until being used within 1 month after extraction.¹⁰ Buccal or lingual surfaces of teeth were examined using a DIAGNOdent[®] pen. Forty-five specimens were prepared from those surfaces showing a reading less than 7, while surfaces showing reading more than 7 were excluded. The sample size was calculated using OpenEpi, version 3, open source calculator, SS mean.¹¹

Specimen Preparation

The teeth were mounted from the root portion in self-cure acrylic resin blocks showing the crown only, the selected buccal or lingual surfaces were sectioned mesiodistally in approximately 2 mm thickness from the cusp tip using a diamond water-cooled diamond saw.¹²

A polyvinyl stencil of 4 × 4 mm dimension was placed on the specimen surface. The surface was coated with a transparent acid-resistant nail varnish (REVLON, Oxford, UK). The stencil was removed after the set of the varnish, leaving a 4 × 4 mm window on the surface.¹³ In this area, the artificial caries lesion was developed, assessed using the DIAGNOdent[®] pen and scanned by SEM.

A total of 45 specimens were individually separated in plastic test tubes and randomly assigned to three groups

of 15 specimens each: in Group I (control), there was no treatment; Group II was treated by Clinpro[™] white fluoride varnish containing 5% sodium fluoride (22,600 ppm fluoride ion) and innovative tricalcium phosphate ingredient, ethyl alcohol, n-hexane, thickener, and sweetened with xylitol (3M ESPE, USA), while Group III was treated with nano-HAP paste (Desensibilize Nano P, Brazil) containing calcium nanophosphate organized in crystalline form of HA, potassium nitrate, water, surfactant, tensoactive, flavor, and 9000 ppm sodium fluoride.

Lesion Formation

Each specimen was placed in continuously stirring incubator at 37°C and immersed in 8 mL of demineralizing solution for 4 days (96 hours). The solution was prepared using the formula 2.2 mM CaCl₂, 2.2 mM KH₂PO₄, and 0.05 M acetic acid. KOH (1 M) was used to adjust pH to 4.4.¹⁴

The solutions were changed every day to avoid aggregation of demineralization products and pH change. After 96 hours, every specimen was washed thoroughly using deionized water and placed back in their test tubes immersed in deionized water until it was either measured by the DIAGNOdent pen or SEM.

REMINERALIZATION

Both fluoride varnish and nano-HAP paste were applied using 1 cm³ BD[™] U-100 insulin syringe (BD, Franklin Lakes, New Jersey, USA) to standardize the amount of agent applied on the lesion surface. A quantity of 0.02 cm³ of both agents was sufficient to cover the lesion surface of the specimens.⁶

In Group II, fluoride varnish was applied and air-dried for 30 seconds. The specimens were stored in the remineralizing solution. After 4 hours, the varnish was removed with a scalpel blade gently to avoid touching the lesion surface. The surface of every specimen was washed using a syringe containing the remineralization solution: 1.5 mM CaCl₂, 0.9 mM NaH₂PO₄, and 0.15 M KCl at pH of 7.¹⁴

In Group III, according to the manufacturer's instructions, nano-HAP paste was applied on the specimen followed by friction of 10 seconds using a microbrush, then it was left in contact with the lesion surface for 4 minutes and removed with deionized water. This was done once a day for 2 days separated by one pH cycle.

pH Cycling

Standard pH-cycling regimen was used to mimic the pH dynamics of the oral environment. It included 3 hours of demineralization and 21 hours of remineralization for 7 consecutive days.

Table 1: DIAGNOdent® pen values interpretation

Value	Inference
0–13	Healthy tooth structure
14–20	Enamel caries
21–29	Deep enamel caries
30+	Dentin caries

DIAGNOdent® Pen Analysis

The examination of the specimens was done using DIAGNOdent® pen to quantify the white lesions objectively. The instrument was calibrated against its own ceramic disk provided by the manufacturer before every measurement session. The instrument probe B was used in this study as it is indicated for smooth surface caries' detection.

The specimens were placed horizontally with the lesion facing upward, which allowed the standardization of the measurement by holding the tip 90° to the lesion surface.

Measurements were interpreted according to the manufacturer of the DIAGNOdent® pen (Table 1). However, for research purpose and for inclusion phase, values measuring <9 were interpreted as shown in Table 2.¹² Measurements were done at four stages: Baseline, after lesion formation, immediately after remineralization phase (posttreatment), and after 7 days of pH cycling.

Scanning Electron Microscope Evaluation

Scanning electron microscope analysis was carried out to qualitatively analyze the surface morphology of the specimens at the baseline, after demineralization, post-treatment, and after pH cycling. The randomly assigned specimens were air-dried and sputter-coated with gold under vacuum using Cressington 108 auto-sputter coater (Cressington Scientific Instruments Ltd., UK) and then examined under SEM at ×1,000 magnification (Seron, Korea).

Statistical Analysis

Statistical analyses were performed using SPSS (Windows, version 20, Chicago, Illinois, USA). The alpha error was set at 0.05. Kolmogorov–Smirnov tests were carried out to assess the normal distribution of continuous variables. Nonparametric tests were used for statistical comparisons because variables were not normally distributed.

Friedman tests followed by Wilcoxon tests were performed to compare the degree of remineralization and demineralization over time in each treatment group. Kruskal–Wallis tests followed by Mann–Whitney tests were performed to compare the three groups.

Table 2: DIAGNOdent® pen <9 values interpretation

Moment value	Inference
3–7	Normal
7–9	Demineralization
>9	White spot lesion

Graph 1: Mean degree of demineralization for the three groups at different quantitative assessment stages

RESULTS

DIAGNOdent® Pen

At baseline, the mean values were not significantly different between the three groups ($p = 0.297$) (Graph 1). Moreover, there was no significant difference between the three groups after lesion formation ($p = 0.816$). After treatment, there was no significant difference between the fluoride varnish group and the nano-HAP paste group ($p = 0.834$). After pH cycling, a significant difference was found between the three groups ($p < 0.001$), where the degree of demineralization was significantly elevated in control group ($p < 0.001$); however, no significant difference was found between fluoride varnish group and nano-HAP paste group ($p = 0.171$; Mann–Whitney test).

Scanning Electron Microscope

The SEM observation at ×1,000 magnification showed smooth and intact surface at the baseline (Fig. 1). In Group I, after lesion formation, there was an increase in the surface porosity (Fig. 2A). After pH cycling, a marked increase in the porosities was evident (Fig. 2B). In Group II, SEM showed an evidence of reduction in the surface porosity (remineralization) (Figs 3A and B). However, after pH cycling, certain areas of porosity reappeared (Fig. 3C). In Group III, SEM showed a decrease in the porosity with deposition of nano-HAP crystals (Figs 4A and B). While, after pH cycling, nano-HAP crystals still could be observed with a smoother surface than that of fluoride group (Fig. 4C).

Fig. 1: Normal enamel at $\times 1,000$ magnification

DISCUSSION

Remineralization of noncavitated caries lesions noninvasively is one of the goals of modern noninvasive dentistry.¹ Nano-sized HA provides high levels of biomimetic properties by its structure, composition, and physiochemical properties, which, in turn, could represent the key element for biomineralization of dental enamel. In recent years, the addition of nano-HAP to toothpastes and

mouthwashes has shown some potentiality in remineralizing artificial demineralized lesions.⁹ The present study aims to compare the effect of a newly introduced chairside professional topical paste containing nano-HAP crystals with fluoride to fluoride varnish in remineralizing initial enamel lesion.

The sample size was calculated by comparing the fluoride varnish and nano-HAP paste Knoop microhardness (KNH) mean values (183.7 ± 29.0 , 150.7 ± 30.5 respectively), which represent one of two studies done on desensitized nano-P paste.¹⁵ Minimal sample size of 12 in each group was needed to detect a difference with a power of 80% at 95% confidence level. Moreover, 10% was added to the sample size to cover the SEM samples which were lost through the treatment protocol.

In this study, two applications of nano-P paste were applied to simulate two professional applications, whereby the maximum remineralizing effect could be observed according to the manufacturer instructions. The fluoride varnish was applied once, similar to other *in vitro* studies.¹⁶⁻¹⁸ The specimens were left for 4 hours in the remineralizing solution before the removal of the fluoride varnish to stimulate clinical application protocol

Figs 2A and B: Group I: Control group (A) after lesion formation; and (B) after pH cycling

Figs 3A to C: Group II (fluoride varnish): (A) After lesion formation; (B) immediately after treatment with the arrows marking the evidence of reduction in the surface porosity; and (C) after pH cycling, certain areas of porosity reappeared

Figs 4A to C: Group III (Nano-HAP paste): (A) After lesion formation; (B) immediately after treatment with the arrows marking deposition of nano-HAP crystals; and (C) after 7 days of pH cycling, the nano-HAP crystals still could be observed

Table 3: Mean values and standard deviation of quantitative assessment of the degree of demineralization at different stages

	Baseline	After lesion formation	Immediately after treatment	After pH cycling
Control group (n = 13)	2.85 ± 0.689 ^a	15.54 ± 1.506 ^a	–	18.62 ± 2.063 ^b
Fluoride varnish group (n = 12)	2.58 ± 0.669 ^a	15.25 ± 2.379 ^a	11.17 ± 2.290	13.08 ± 2.678 ^a
Nano-HAP group (n = 12)	3.00 ± 0.953 ^a	14.83 ± 2.250 ^a	10.67 ± 2.535	11.50 ± 2.067 ^a
Significance	0.297	0.816	0.834	<0.001

^{a,b}Mean of the multiple comparison tests. The same letter means no significant difference. The letter “a” means the smallest, etc.

where the patients are asked not to eat, brush, or floss for a minimal of 4 hours to prevent removal of the material from the tooth surface.¹⁹

In this study, the DIAGNOdent[®] pen was used as a noninvasive method that could give a quantitative assessment for the degree of demineralization. Healthy tooth structure displays little or no fluorescence, resulting in very low readings. However, when a demineralized area is present, the fluorescence will increase, which is indicated by the increase in the audible sound. Higher pitched sounds and higher number of sounds indicate more demineralization.¹² The efficacy and the reproducibility of the DIAGNOdent[®] have been investigated by several studies.^{19,20} Moreover, DIAGNOdent[®] is found to be capable of diagnosing dental caries that were neither visible clinically nor radiographically.^{21,22}

The results of this study showed that there was no significant difference between the three groups in the degree of demineralization at baseline (Table 3). Furthermore, there was no significant difference between the three groups after lesion formation. This indicates that standardized uniform artificial enamel lesions were obtained using the demineralization solution which made it possible to establish objective comparison among groups.

Immediately after treatment, both nano-P paste and fluoride varnish showed a significant decrease in demineralization with no significant difference between them. This showed that nano-HAP paste had a similar effect to fluoride varnish despite the fact that nano-HAP paste was left in contact with the specimen surface for a total of 8 minutes only and contained a lower fluoride

concentration. Meanwhile, the fluoride varnish was left in contact with the specimen surface for 4 hours with higher fluoride concentration. This can be attributed to nano-HAP's high affinity and attachment onto enamel surface as suggested by several studies.^{23–25} The bioactive and biocompatible properties increased the potential of the nano-HAP to directly fill up defects and micropores on the demineralized enamel.

After pH cycling, there was a significant difference between the three groups, and the degree of demineralization was significantly elevated in control group (Table 3). However, there was no significant difference between the nano-P paste and fluoride varnish groups. Medeiros et al¹⁵ investigated the performance of a single application of nano-P paste and fluoride varnish in the prevention of enamel erosion. Similar to our results, they found no significant difference among nano-P paste and fluoride varnish groups as assessed by surface KNH. Nevertheless, they observed that nano-P paste showed the formation of a protective layer on enamel surface by atomic force microscopy, which, to some extent, simulate the cluster deposition of nano-HAP crystals found in our study using SEM. The efficiency of nano-P paste under dynamic pH cycling may be referred to the explicit filling and adherence of globular deposits of nano-HAP crystals in the enamel porosities which was observed by the SEM evaluation as shown in Figure 4. Nano-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.⁹

On the contrary, de Carvalho et al⁶ reported that nano-HAP group showed significantly higher difference

in surface hardness recovery after using the same pH cycling method as this study than fluoride varnish. This may be attributed to the number of applications of the nano-P paste, where it was applied for a total of seven times, while the fluoride varnish was applied only once. Taking into consideration the difference in the assessment method as they used KNH, it can be deduced that increasing the times of application of the nano-P paste could enhance the effect of remineralization.

Scanning electron microscope analysis of the surface after remineralization induced by nano-HAP paste showed cluster depositions of nano-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,⁶ who found similar cluster deposition using the same nano-HAP paste. In addition, similar results were observed by Swarup and Rao²⁶ and Vyavhare et al,⁹ who studied the effect of adding synthetically processed nano-HAP to toothpaste in remineralizing initial enamel lesion.

In the fluoride varnish group, immediately posttreatment 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,⁶ who reported the presence of a homogeneous protective layer after pH cycling. This might be due to the variation of contact hours between the fluoride varnish and the tooth structure where the fluoride was applied for 4 hours in this study, while in de Carvalho et al⁶ study, it was applied for 24 hours, thus 4 hours might have been not enough to affect the surface morphology, where the effect of fluoride varnishes depends on the prolong contact of a thin layer which acts as a slow-release reservoir of fluoride.²⁷

CONCLUSION

Within the limitation of this study, we can conclude that nano-HAP paste had similar potential in remineralizing initial enamel lesion as fluoride varnish. Moreover, under dynamic pH cycling conditions, nano-HAP paste showed promising results in surface depositions and maintaining a smooth surface over a longer duration.

CLINICAL SIGNIFICANCE

Based on the findings of this study, nano-HAP paste might be recommended as alternative remineralizing agent with lower fluoride concentration than fluoride varnish that could be beneficial for children, pregnant females, and those who are at high risk of dental fluorosis. Further *in vivo* investigations to assess the clinical performance of nano-HAP paste should be done.

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