Remineralization of Early Caries by a Nano-Hydroxyapatite Dentifrice

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Abstract

- **Objective:** The purpose of this randomized, double-blind, crossover, *in situ* study was to evaluate the efficacy of Nano-hydroxyapatite (nHAP) dentifrices on caries remineralization and demineralization inhibition.
- **Methods:** Three demineralized enamel blocks (A,B,C) and one healthy block (D), cut from each of 30 molars, were exposed respectively to dentifrices of A) 5% nHAP, B) 10% nHAP, C) 1100 ppm fluoride, and D) 10% nHAP via an intra-oral appliance worn by 30 adults in this four-phase study lasting 28 days, per phase. Baseline and post-test mineral loss (ΔZ) and lesion depth (LD) were quantified using microradiography.
- **Results:** Pair-wise comparison (Baseline versus Test) demonstrated significant ($p < 0.001$) reductions in ΔZ and LD in A, B, and C. ANOVA showed no significant differences among the three products in percent mineral gain. No demineralization occurred in the sound enamel specimens exposed intra-orally while using 10% nHAP.
- **Conclusion:** nHAP dentifrice caused remineralization comparable to a fluoride dentifrice, and inhibited caries development, thus suggesting that an nHAP dentifrice can be an effective alternative to fluoride toothpaste.

(J Clin Dent 2011;22:000–000)

Introduction

It is now well established that dental caries in its early stage of formation (non-cavitated) can be remineralized,[1,2] and this remineralization can be facilitated by such agents as fluoride, delivered via either mouthrinse or dentifrice.[3,4] However, extensive use of fluoride mainly in the form of a dentifrice has contributed to a rising incidence of dental fluorosis, particularly in preschool children, due to chronic ingestion of these products.[5] Hence, there is a need for an alternative caries remineralizing and preventive agent as effective as fluoride, but without its harmful effects.

Recent research has emphasized the relevance and necessity of biomimetic oral health products containing nano-sized hydroxyapatite particles in modern preventive dentistry.[6-9] Synthetic nano-hydroxyapatite (nHAP) has the same chemical-physical properties as the apatite structure within enamel, making it an interesting ingredient for oral health compounds. nHAP shows strong affinity to the tooth, and can strongly adsorb on enamel surfaces.[8] With advances in nanotechnology, a number of dentifrice and mouthrinse formulations containing nano-sized biomimetic apatite particles have been developed. These products are envisaged to promote remineralization due to size-specific effects of the apatite nano-particles corresponding to the ultrastructure of the enamel.[9] However, the efficacy of nHAP-based products in promoting remineralization and inhibiting demineralization of tooth enamel has not been widely investigated. In Japan, nano-hydroxyapatite has been used in toothpastes since 1980, and was approved as an anti-caries agent in 1993. This approval was based on evidence including anti-caries field trials in Japanese schoolchildren.[11] Other available studies are mainly *in vitro* [8,9,12-14] and therefore lacking the influence of the biological variable within the oral environment. Hence, the objective of the present study was to investigate the *in situ* remineralization efficacy of dentifrices containing different concentrations of nano-sized hydroxyapatite, comparing it to that of a fluoride-containing dentifrice. This study sought to test two hypotheses. The first hypothesis was that each of the three dentifrices would promote enamel remineralization that is significantly greater than zero. The second hypothesis was that the three dentifrices differ with respect to post-treatment remineralization. Of special interest was whether the nHAP-based dentifrices promote greater enamel remineralization relative to the fluoride dentifrice.

Materials and Methods

Participant Recruitment

The study was approved (approval #HSC20090352H) by the Institutional Review Board of The University of Texas Health Science Center at San Antonio (UTHSCSA). Thirty healthy adults (12 males, 18 females) with a mean (SD) age of 37.8 (7.9), from different ethnic origins and socioeconomic status participated in this study. The subjects were identified with code numbers. After providing informed written consent, subjects underwent a complete intra-oral examination and completed a medical history questionnaire. The inclusion criteria were: age 21–40 years, having at least 22 teeth with a past history of dental caries but no clinically active caries, periodontal disease, or other oral pathology, and having a mandibular first molar with a sound, unrestored buccal surface. Other inclusion criteria were normal salivary function with unstimulated and stimulated salivary flow rates $\geq$ 0.2 ml/min and $\geq$ 0.7 ml/min, respectively, measured according to the Sreebny and Valdini procedure,[15] and not taking any antibiotics or medications which could affect saliva flow rate. The sample size was based on a power analysis, and the sample size calculations were performed using nQuery Advisor software (Statistical Solutions, Cork, Ireland). The size was based on previous results obtained in this group.[16]

In that study, the mean pre-treatment % ΔZ was equal to 28.5, with a standard deviation equal to 31.2. For our null hypothesis, that each of the three dentifrices promotes enamel remineralization that is significantly greater than zero, the proposed sample size of n = 30 would have power greater than 0.95 with a 0.05 one-sided significance level to detect a difference between a null hypothesis mean of zero and a sample mean % ΔZ equal to or greater than 10%. The primary end-point of the present study was disease treatment, *i.e.* remineralization of early caries lesions.

Artificial Enamel Lesion Preparation

Freshly extracted human third molars, following prescribed treatment, in various clinics of the dental school of UTHSCSA, were collected and stored in 0.1% thymol solution at 4˚C prior to use. Thirty teeth without caries, cracks, or enamel malformations were selected and cleaned with pumice. The teeth were then painted with two coats of acid-resistant nail varnish, except for an exposed window (9 mm length × 2 mm width) on the buccal surface of the tooth. Caries-like lesions were created in the exposed windows by seven days' immersion in an acidified gel (0.1 M sodium hydroxide, 0.1 M lactic acid, and 6% w/v hydroxyethyl cellulose, pH 4.5) at 37ºC.[17] Following lesion production, the nail varnish was carefully removed with acetone, and three lesion-bearing tooth blocks (~ 3 mm length × 2 mm width × 1.5 mm thick), to be used for the remineralization test, were cut from each window. In addition, one sound tooth block of similar size was cut from the lingual surface of same tooth to be used for the demineralization inhibition test. One tooth section (control) of approximately 100 µm thick was cut from each experimental block for the measurement of baseline parameters of the lesion, and for selection of the suitable lesions for the study. The sections were processed and microradiographed, and the images were visualized with transverse microradiography (TMR) analysis software version 3.0.0.11 (Inspektor Research Systems, Amsterdam, Netherlands) as described in previous publications.[17] At this point, the images were used only for selection of the suitable lesions for the study. Only the controls that showed caries-like lesions with subsurface lesions and pseudo-intact surface layer, which display a fairly uniform width throughout their length, were selected for the remineralization process, and their test blocks were used for construction of the *in situ* appliance.

Intra-Oral Appliances

Each tooth block was covered with polyester gauze (Bard Peripheral Vascular, Inc., Tempe, AZ, USA) and mounted within an intra-oral appliance, a customized orthodontic bracket (Figure 1). The appliance consisted of an orthodontic molar pad with retentive mesh backing, which had a stainless steel band welded to it so the band closely enclosed each test enamel block. The enamel specimen was retained within the bracket using fluoride-free intermediate restorative material. In order to control the plaque thickness and thus have a more natural plaque on the enamel surface, the specimens were mounted slightly recessed below the edges of the band. Each tooth successfully completing the fabrication process produced four *in situ* appliances; three with carious surface and one with sound surface. The appliances were sterilized with gamma irradiation.[18]

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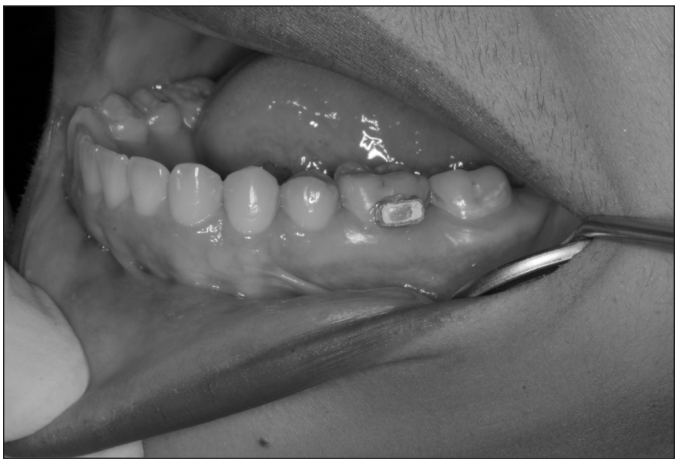

Figure 1: *Clinical photograph of the enamel specimen housed in the customized orthodontic bracket (San Antonio* in situ *model).*

Study Procedure

This was a double-blind study in which the subjects were randomly exposed to each of the following four distinct phases in a crossover design: A) 5% nHAP dentifrice (Apagard® nHAP toothpaste, Sangi Co., Japan; B) 10% nHAP dentifrice (Apagard® nHAP toothpaste, Sangi Co., Japan), or C) 1100 ppm NaF fluoride dentifrice (Crest® Cavity Protection, Procter & Gamble, Cincinnati, OH, USA) while subjects wore appliances with a carious tooth block; and D) 10% nHAP dentifrice (Apagard® nHAP toothpaste, Sangi Co., Japan) with subjects wearing appliances with a sound tooth block. Subjects were randomly exposed to the products in such a manner that while some subjects were using product A, others were using B, C, or D. Each phase lasted for 28 days, and was preceded by a seven-day washout period during which the subjects used their next test dentifrice without wearing the appliance. This was to eliminate the residual effect of previously used product.

The four *in situ* appliances originating from the same tooth were assigned to one subject. Following this assignment, the first of the four assigned appliances was bonded, in accordance with current principles of orthodontic practice, on the buccal surface of the chosen lower molar tooth. The appliance was fitted by a qualified dentist, who was different from the laboratory assistant that processed and analyzed the samples to produce the final data. The dentifrices were specially prepared and coded by the manufacturing company, and the codes were not released until data collection and analysis were completed. This procedure permitted blinding of the clinician, the laboratory assistant, and the study coordinator who provided instructions to the subjects. The subjects were provided with their appropriate dentifrice and a toothbrush. Subjects received oral and written instruction to brush their teeth with the product three times daily for five minutes on each occasion, preferably morning, immediately after lunch, and the last thing before bed. Subjects were also requested to refrain from the use of any other oral hygiene products for the duration of the trial, and maintain their normal dietary habits. These measures were to ensure uniformity in the use of the oral hygiene product that might otherwise unduly influence the de-/remineralization cycle during the study periods. Subjects were also requested to record in the provided diary the number and

time of tooth brushing each day, and return the remaining toothpaste after each study phase. The weight of toothpaste was measured before and after the study phase. These measures were applied to monitor compliance and adherence. After each 28-day period, the appliance was detached, and after the washout period the next appliance was cemented in place on the same tooth as the first appliance. This procedure was repeated until the four phases were completed by each subject.

Post-Treatment Processing

After intra-oral exposure, three tooth sections, approximately 100 µm thick, were cut from each block. Although the pre-test control sections had been microradiographed and visualized for selection of the appropriate lesions for the study, they were microradiographed again, together with the post-test sections, and then analyzed together for quantification of the lesion parameters of mineral loss (ΔZ) and lesion depth (LD), using the TMR analysis software to eliminate variation due to different processing conditions. Quantification of ΔZ and LD yielded the following information: 1) Pre-test ($\Delta Z_{pre\text{-}test}$ and $LD_{pre\text{-}test}$) and 2) post-test ($\Delta Z_{post\text{-}test}$ and $LD_{post\text{-}test}$) parameters of the lesions, and their pre-test and post-test microradiographic images. The mean value of ΔZ and LD of the three sections from each post-test block was used for statistical analysis. Relative change in mineral loss before and after intra-oral exposure was calculated for each specimen as: $(\Delta Z_{pre\text{-}test} - \Delta Z_{post\text{-}test}) / \Delta Z_{pre\text{-}test}$ and expressed as a percentage. Relative change in lesion depth was calculated similarly.

Statistical Analysis

After assumptions of normality and homogeneity of variances had been verified by normal probability plots, ANOVA was applied to compare the percentage change in mineral loss and lesion depth between the three treatment groups, while intra-group comparisons were performed using the paired Student's t-test (STATA version 10.0, StataCorp LP, College Stations, TX, USA) at a significance level of 0.05. Correlation between the saliva flow rate (stimulated and unstimulated) and the lesion parameters (mineral gain and lesion depth reduction) was tested using Pearson's correlation coefficient.

Results

The unstimulated and stimulated saliva flow rates of the subjects ranged from 0.2 to 1.6 ml/min and 0.8 to 3.6 ml/min, respectively. The Pearson's correlation coefficient showed that the mineral gain and lesion depth reduction were poorly correlated with saliva flow rate (stimulated and unstimulated). The only significant correlation was between the stimulated flow rate and mineral gain in 5% nHAP dentifrice (n = 30; Pearson's $r = 0.4616$, $p < 0.01$). The mean DMFT of the participants was 1.12.

ANOVA analysis verified there were no statistically significant differences among the treatment groups (A, B, and C) with respect to mineral loss ($p = 0.5$) and lesion depth ($p = 0.8$) at baseline. The paired t-test demonstrated all dentifrice formulations to significantly ($p < 0.001$) promote mineral gain in the enamel subsurface lesions (Table I). However, ANOVA indicated no significant differences among the three treatments with respect to percent mineral gain (Table II). Post-remineralization images of the lesions demonstrated a reduction in lesion depth and clear evidence of remineralization of the subsurface lesions (Figure 2) in all groups. In group D, which examined the ability of the 10% nHAP dentifrice formulation to inhibit demineralization of sound tooth surface, there was no evidence of demineralization in any of the tooth blocks following intra-oral exposure.

Table II
Mean Percentage in Mineral Gain and Reduction in Lesion Depth in the Three Treatment Groups

	% Change					
Response Variable	Group A (n = 30)	Group B (n = 30)	Group C (n = 30)	df	F	p-value†
ΔZ	32.9 (17.1)	28.5 (11.5)	30.3 (16.3)	28	0.92	0.40
LD	14.1 (11.0)	10.1 (9.4)	10.2 (15.6)	28	0.72	0.48

†ANOVA.

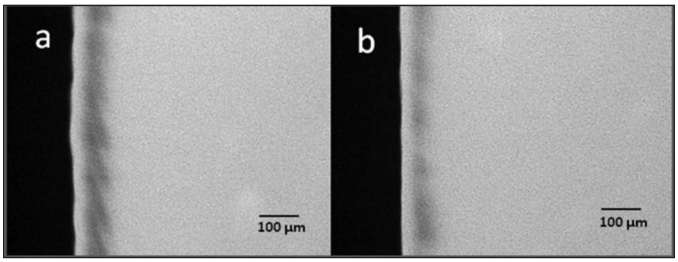

Figure 2: *Representative microradiographic images of enamel subsurface lesions: (a) before (control); and (b) after* in situ *remineralization by use of 5% nHAP dentifrice formulation. The scale bar indicates 100 µm.*

Table I
Mean Values of Mineral Loss (Vol % µm) and Lesion Depth (µm) in the Three Study Groups Before and After Treatment and Their Differences (n = 30)

Treatment Groups	Before Treatment) (Baseline)	After Treatment ΔZ [Mean (SD)]	Difference (95% CI)	p-value†
A (5% nHAP)	1294.5 (417.6)	860.3 (350.6)	434.2 (327.6 to 540.7)	< 0.001
B (10% nHAP)	1386.5 (351.3)	997.9 (319.2)	388.6 (324.8 to 452.4)	< 0.001
C (1100ppm) NaF)	1267.9 (420.7)	863.8 (271.7)	404.1 (298.2 to 507.3)	< 0.001
	LD [Mean (SD)]	LD[Mean (SD)]		
A (5% nHAP)	73.8 (13.9)	62.7 (11.8)	11.1 (7.2 to 14.9)	< 0.001
B (10% nHAP)	74.8 (13.1)	66.8 (11.7)	8.0 (5.1 to 10.9)	< 0.001
C (1100 ppm) NaF)	72.5 (15.1)	64.6 (15.0)	7.9 (3.6 to 12.2)	< 0.001

†Student's t-test.

Discussion

In the present study, the San Antonio *in situ* model was used to test the efficacy of nHAP and fluoride dentifrice formulations in the remineralization of incipient caries-like lesions produced in human enamel. Upon recommendation by the UTHSCSA IRB, this study did not include a fluoride-free dentifrice (*i.e.*, negative control) due to possible adverse dental health concerns; however, each subject served as his/her own control. The results revealed no significant differences in the caries remineralizing efficacy of the two nHAP dentifrice formulations (5% and 10%) and the commercially available sodium fluoride dentifrice. A significant improvement in mineral content of enamel lesions was observed for each of the three treatment modalities after intra-oral exposure, with no significant difference in remineralization efficacy between the fluoride and nHAP (5% and 10%) treatment groups, although the results were slightly in favor of the 5% nHAP. These findings were consistent with those observed in a previous *in vitro* study[12] that examined the effect of varying nHAP concentrations (1, 5, 10, 15%) and exposure time (3, 6, 9, 12 days) on remineralization of early caries under *in vitro* pH-cycling conditions. In this study, lesions were found to be re-hardened significantly compared to baseline; however, a dose-response was observed up to 10% concentration and 9 days of cycling in 5, 10 and 15% concentrations, after which the effect plateaued with no significant difference in remineralization with increase dose or cycling time. This plateauing effect was thought to be related to a certain level of unavoidable aggregation of the particles at higher concentrations. The influence on particle aggregation of the *in vivo* biological conditions, such as saliva and the longer exposure period (28 days) applicable in our present *in situ* study, has not been investigated since this is the first study comparing the two concentrations *in vivo*. Furthermore, contrary to the results of our present study, they reported significantly greater remineralization with 1000 ppm NaF compared to nHAP treatments. These differences may be attributed to the different experimental designs and conditions, intra-oral exposure as opposed to *in vitro* studies, different treatment regimens, and/or analytical techniques. Being an *in situ* study, the caries lesions in the present study were exposed to the biological variables within the oral cavity, which are not encountered *in vitro*. The influence of these variables on the remineralization efficacy of nHAP may differ with varying concentrations of nHAP and other factors, such as the intra-oral clearance of the product. The dietary differences among subjects, a possible confounding factor, may be responsible for the high standard deviations in the result. In a previous *in vitro* pH cycling study that compared 10% nHAP dentifrice and 950 ppm fluoride dentifrice using a dentifrice with neither nHAP nor fluoride as the negative control, the dentifrice that contained 10% nHAP showed similar remineralization efficacy with the dentifrice that contained 950 ppm NaF, while the caries-like lesions subjected to the dentifrice with neither fluoride nor nHAP exhibited further demineralization.[13] A similar pH cycling study with a dentifrice containing 10% nHAP with or without additional fluoride, showed similar remineralization efficacy when compared with the dentifrices that contained only fluoride.[14] Combining fluoride and nHAP did not show any synergistic effect. Another clinical study reported the application of synthetic hydroxyapatite on enamel hypoplasia in children and adolescents[19] to promote remineralization process in decalcified areas on enamel surface.

The caries remineralizing action of nHAP employed in a dentifrice formulation may be explained by the potential of hydroxyapatite nano-crystals to precipitate on the lesion surface, believed to be facilitated by its strong surface bioactivity coupled with its chemical and physical similarity with natural enamel.[9] In a recent investigation of the behavior of hydroxyapatite nano-crystals in the remineralization of demineralized enamel,[8] nHAP was found to induce a consistent enamel caries remineralization by forming a homogeneous apatite layer on the demineralized surfaces of enamel after treatment for only 10 minutes. This layer is composed of synthetic hydroxyapatite nano-crystals which chemically bond to natural enamel crystals. Interestingly, the hardness and elastic modulus of the restored enamel is similar to those of natural ones, which is of paramount importance in nHAP applications in enamel reconstruction[9]. Biomimetic apatite deposition on affected sites of enamel does not only cover and protect the enamel structure, but also provides the minerals needed for restoring the demineralized areas. Progressive transfer of hydroxyapatite nano-crystals from the new apatite coating to the lesion maintains high concentration gradients of calcium and phosphate ions in the subsurface enamel, thereby facilitating remineralization.

In the caries model used in the present study, polyester gauze, which remained intact throughout the 28-day exposure period, was used to encourage plaque accumulation on the enamel surface. In a previous study, this model has been used to develop early caries on a gauze-covered enamel surface within 14 days of intra-oral exposure. The caries-inhibitory effects of an nHAP-containing dentifrice observed in the present study can also be explained by nHAP's role in direct mineral enrichment of dental plaque. Deposition of nHAP in plaque causes a higher saturation state of plaque with respect to dental enamel, thus preventing acid dissolution of the enamel. In addition, confocal laser scanning microscopy has confirmed the potential of hydroxyapatite nano-particles in protecting the prism-prism sheath interface where enamel dissolution frequently initiates during a cariogenic challenge.[9] Hence, the layer of hydroxyapatite nano-particles can inhibit mineral loss and lesion progress in the enamel.

The San Antonio *in situ* model used in the present study was designed to combat the compliance problem encountered with removable intra-oral appliances.[19] This model is a fixed intra-oral appliance, a customized orthodontic bracket which also provides more convenience to subjects. Bonding the appliance to the buccal surface of the mandibular first molar offers the advantage of nearness to the major salivary gland and also minimizes the possibility of irritation to the surrounding mucosa. The gauze-covered model reproduces the condition that exists in caries-prone sites, such as interproximal areas and pits and fissures where ion diffusion tends to be restricted.[19] The presence of gauze enhances the accumulation of plaque and thus poses a greater acidic challenge to the enamel surface. Therefore, less net remineralization is anticipated for the gauze-covered enamel compared to the gauze-free enamel.[20]

Conclusion

Dentifrices containing nHAP have the potential for the same remineralizing capacity as a fluoride dentifrice and may be employed as an effective alternative to fluoride-containing dentifrices. Based on the findings here, nHAP-containing dentifrices may be recommended for children or those who are concerned about dental fluorosis. Since the remineralizing efficacy of topical fluorides is strictly dependent on the availability of calcium and phosphate ions, nHAP dentifrices are strongly recommended to xerostomic patients with diminished amounts of saliva. Nanohydroxyapatite may have the potential to occlude dentin tubules and, as such, may be useful for the treatment of dentin hypersensitivity, and should be investigated in the future.

Acknowledgments: We express our gratitude to Sangi Co., LTD., Japan, who provided the nano-hydroxyapatite toothpastes, which has been marketed since 1980 in Japan. None of the authors reported any disclosures.

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