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THE IN SITU POTENTIAL OF SYNTHETIC NANO-HYDROXYAPATITE FOR TOOTH ENAMEL REPAIR

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Abstract.This study was designed to evaluate whether nano-hydroxyapatite toothpastes with or without fluoride would be more advantageous than a fluoride toothpaste in the repair of eroded enamel in situ. Twenty-one subjects participated in this single-blind, randomized, cross-over design study with three 7-day treatment phases. In each phase, the subjects wearing a palatal appliance containing five sterilized enamel specimens used either one of the two test regimens (1% nano-hydroxyapatite toothpaste and 2.25% nano-hydroxyapatite/1450 parts per million (ppm) fluoride toothpaste) or one control (1400 ppm fluoride toothpaste). Enamel specimens were extraorally demineralized (4 × 5 min/day) and were intraorally treated with the toothpastes (2 × 2 min/day). The nano-hydroxyapatite toothpaste groups exhibited significantly higher surface microhardness than did the standard fluoride toothpaste group ($p < 0.05$). Enamel surface hardness was increased only by nano-hydroxyapatite toothpastes after in situ treatment compared with the baseline ($p < 0.05$). Morphological analysis demonstrated an apatite-type crystal deposition on the eroded enamel surface produced by nano-hydroxyapatite toothpastes, while fluoride toothpaste failed to show any significant surface deposition. Chemical analysis showed a higher content of calcium and phosphorus in the enamel surface treated with nano-hydroxyapatite toothpastes compared with that in the control one ($p < 0.05$). It is concluded that home use of nano-hydroxyapatite containing toothpastes may have a protective effect against erosion at the enamel surface.

1 Introduction

Mature enamel is almost completely composed of minerals (>97 wt% apatite) in the form of highly elongated and oriented prisms of carbonate apatite crystals in the nanometer size range.1,2 Therefore, synthetic hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, has been generally used as a model for dental enamel due to its chemical and phase similarities.3–6 In acidic erosion, hydroxyapatite crystallites become partly or completely dissolved and demineralized by the low pH of the mouth caused by acidic beverages and foods or gastric juices.7,8 To prevent dental erosion, toothpastes containing hydroxyapatite as an active ingredient, an alternative to fluoride (F^-)-containing pastes, were introduced to help the repair of enamel.6,9

The biomimetic repair strategies for demineralized enamel or micro-sized tooth surface defects are based on nano-sized hydroxyapatite particles and are thought to be more effective than traditional use of micrometer-sized hydroxyapatite in toothpastes.5,10,11 It has been demonstrated experimentally that using nano-sized hydroxyapatite particles that mimic the size of natural dentin (20 nm) and enamel (100 nm) apatite results in an effective adsorption of the artificial materials to the natural tissue, in a process that not only repairs defects caused by erosive demineralization3,12,13 but also helps prevent further erosion formation by strengthening mechanically the enamel surface.3

It should be noted that the literature available on novel applications of nanotechnologies in toothpastes mainly relies on pure in vitro conditions.3,12,14,15 To the authors' knowledge, there is no clinical evidence yet supporting the effect of nano-hydroxyapatite (n-HAP) in commercial toothpastes on erosive enamel lesions using an in situ erosion model. Moreover, the effect of the saliva and pellicle in the oral environment must be taken into consideration as the crystallization process and the behavior of toothpastes may be affected by the physiological conditions during biomineralization.10,16–18 In this study, therefore, the authors hypothesized that toothpastes containing 1% nano-sized hydroxyapatite and 2.25% nano-sized hydroxyapatite with 1450 parts per million (ppm) fluoride would be more advantageous than a control toothpaste containing 1400 ppm fluoride to repair eroded enamel in situ. Whether n-HAP toothpaste containing fluoride provides greater protection against erosion relative to that containing n-HAP alone was also tested.

2 Material and methods

2.1 Ethical aspects and participants

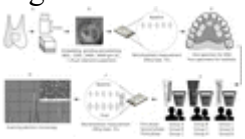
This in situ study was a one-center, single-blind, randomized, three-phase, cross-over study. The study protocol was approved by the local ethics committee in clinical research, and written informed consent was obtained from each participant at the beginning of the study.

Twenty-one participants were included. All volunteers were recruited among students of the school of dentistry. Volunteers were included if they were in good oral hygiene condition with adequate physiological saliva flow rates as determined by a salivary flow test (stimulated > 0.8 ml/min; unstimulated > 0.2 ml/min)19 and able to wear a palatal appliance and to comply with study procedures. The exclusion criteria were presence of systemic diseases, any medication that could be expected to interfere with saliva secretion, medical conditions with reflux, caries activity, tooth wear lesions, periodontal disease and use of orthodontic appliances. The participants were extensively trained for all procedures and received written instructions and a schedule of procedures.

2.2 Specimen preparation

Three hundred and fifteen enamel blocks (5 × 4 × 3 mm) were prepared from the buccal and/or palatal surfaces of freshly extracted non-erupted human third molars, which were stored in a 0.1% thymol solution at room temperature until use. Specimens were cut at the middle of the crown surface using a cylindrical diamond bur with a high-speed turbine. After preparation, specimens were embedded in acrylic resin block and the enamel surfaces were ground flat with water-cooled 800-, 1200-, 2400- and 4000-grit silicon carbide paper and polished with a 1.25 μm diamond suspension using a grinding and polishing device (LaboPol-5, Struers, Denmark), resulting in 3.0 mm (±0.1 mm) thickness. Specimens were viewed under a stereomicroscope (S4E, Leica Microsystems, Germany) at ×40 magnification to ensure that they were free of cracks or other defects. Specimens were then cleansed ultrasonically in deionized water for 10 min to remove any residues of the polishing procedure and stored in deionized water. Baseline microhardness measurements were taken before in situ treatment. Sterilization of the enamel specimens was accomplished by a gas method using ethylene oxide (Amsco Eagle 3017, Steris, Mentor, OH, USA). The specimens were then randomly allocated to the volunteers and the respective treatment phases. The study protocol is summarized in

Figure 1.



2.3 Intraoral appliances

Removable acrylic palatal appliances were fabricated for each subject and were retained by stainless steel circumferential clasps in the molar region. Five cavities were cut into the acrylic base; four cavities were made in the posterior region (two on each side), and one cavity was made in the anterior region of the appliance. Four specimens were positioned in the posterior cavities, mounted with a flowable resin composite (i-Flow, i-dental, Siauliai, Lithuania) and light-cured. One specimen for scanning electron microscopy (SEM) analysis was placed in the anterior cavity of the appliance with the flowable resin composite without light-curing, allowing the specimen to be removed first and preventing further debris buildup created by bur during removal.

2.4 Study procedure

The study procedure is given in a flowchart (Figure 2). The study had cross-over design consisting of three treatment phases of 7 days each with a washout period of 7 days between each phase. At the beginning of each treatment, subjects had five blocks of non-demineralized permanent human enamel placed into the base of their palatal appliances. In the first phase of the experiment, using

a computer-generated randomization list, the subjects received the following treatments: a toothpaste containing 1% n-HAP with 1450 ppm fluoride as sodium fluoride (NaF) (ApaCare, Cumdente, Tübingen, Germany), a toothpaste containing 2.25% n-HAP without fluoride (PrevDent, PrevDent International BV, Ilpendam, the Netherlands) and a standard toothpaste containing 1400 ppm fluoride as sodium fluoride (control; Parodontax Fluoride, GlaxoSmithKline, sold in Turkey). The compositions of the toothpastes are described in Table 1. The toothpastes were crossed over in the second and third phases. The tubes of toothpastes were covered with white paper and coded to ensure blinding of the subjects. The covers were not broken until the completion of the statistical analysis. Seven days prior to and throughout the entire experiment including the washout periods, the subjects brushed their teeth two times a day (before the experiment and before bed) with a fluoride-free toothpaste (Parodontax Original, GlaxoSmithKline, sold in Turkey) and standard soft-bristle toothbrush (Colgate, Ultra Compact Slim Soft, Colgate-Palmolive Company, USA) that were distributed. They were also instructed to refrain from using any other oral hygiene products (e.g. non-study toothbrushes, toothpaste, floss and mouthwashes) for the duration of the study.

The subjects wore the appliance 8 h/day (from 08:00 to 17:00) continuously, except for 1 h for lunch. While the appliances were in the mouth, the participants were instructed not to eat and were allowed to drink only one cup of coffee or black tea. Appliances and contained specimens were stored in plastic containers with small amount of distilled water at room temperature when removed from the mouth or overnight. Prevention of bacterial plaque accumulation was achieved by soaking the appliances with contained specimens, in 0.2% chlorhexidine digluconate solution for 3 min before and after use in the mouth.

Volunteers started wearing the appliance 1.5 h prior to performing the protocol tested to allow salivary pellicle formation. The enamel specimens were demineralized extraorally with a fresh cola-based soft drink (Coca-Cola, Coca-Cola Türkiye, pH 2.7) four times a day at 1 h intervals (10:30, 11:30, 13:30 and 14:30) for 5 min. After demineralization, the appliances were rinsed with tap water and reinserted into the mouth. The soft drink used for each erosive procedure was discarded. Before the first (09:30) and after the last demineralization (15:30) of each day, participants used the test products intraorally. They placed a pea-sized amount of toothpaste on the head of the manual toothbrush (Colgate, Ultra Compact Slim Soft, Colgate-Palmolive Company). A toothpaste slurry was produced by placing the toothbrush on the buccal surfaces of the participant's own upper molars, and then, the head was moved in a vertical position (90° angle) next to the specimens in the appliance. The specimens were brushed for 2 min with circular motion. Afterward, the slurry was spit out and the oral cavity was rinsed with tap water. Then, the appliance was removed from the oral cavity, rinsed under tap water and reinserted into mouth. The same procedures were repeated in the cross-over phase. All demineralization and remineralization procedures were carried out under supervision. At the completion of each treatment phase, the enamel specimens were stored in distilled water for further surface microhardness measurement.

2.5 Surface microhardness measurement

Enamel surface microhardness was determined before in situ treatment (baseline) and after in situ treatment (after 7 days). At each time point, the Vickers hardness number (VHN) was determined by making five indentations located about 100 µm apart from each other with a diamond pyramid Vickers indenter (Lake Bluff, IL, USA) under a load of 200 g for 10 s. For each specimen, the

average value of five indentation scores was calculated. For the hardness measurements at the baseline, the line of five indentations was positioned at the centre of the specimen. After in situ treatment, the line of five indentations was placed at 100 µm directly below the baseline indentations. All measurements were performed by the same examiner.

2.6 Scanning electron microscopy

SEM was used for analyzing the enamel surface morphology of the specimens. Twenty-one specimens from each toothpaste group (one for each participant) were evaluated at the end of the experimental procedures. The specimens were dried in a vacuum desiccator for 24 h, mounted on stubs with carbon conductive tape and observed under a scanning electron microscope (Vega II, Tescan, Cambridge, UK) operating at 20 kV using backscatter detection mode. No sputter coating was performed. SEM microphotographs of the surfaces of each specimen at ×200, ×500, ×1000 and ×2500 magnifications were obtained. Thereafter, the same set of specimens was prepared for energy-dispersive spectroscopy (EDX).

2.7 Energy-dispersive X-ray spectroscopy

Chemical characterization of the specimen surface was performed through SEM coupled with EDX to determine the levels of calcium (Ca), phosphorus (PO_4) and fluoride (F). At the end of the in situ treatment, the specimens were placed in a vacuum desiccator for 24 h, mounted on stubs using carbon conductive tape, sputter-coated with gold and examined by SEM/EDX (Zeiss, Leo 1430 VP, Carl Zeiss, Oberkochen, Germany). EDX spectra were collected during sample surface scanning using an accelerating voltage of 20 kV at a working distance of about 26 mm. Final results were normalized to 100% and presented as a relative ratio of element mass (weight %).

2.8 Statistical analysis

Power analysis was performed with the G*Power software package (G*Power version 3.0.10, Franz Faul, Universität Kiel, Germany). It was determined that 18 subjects were required for the study, with $\alpha = 0.05$ type I and $\beta = 0.05$ type II error rates for 95% power in $d = 0.62$ impact width. To cover possible data loss, three substitute subjects were added, and the decision was made to include a total of 21 subjects.

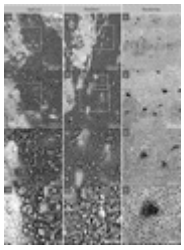
Statistical analysis was performed using the SPSS version 11.5 software (SPSS Inc., Chicago, IL, USA). The significance level for all tests was set at $p = 0.05$. The intra-examiner reliability was assessed by calculating the intraclass correlation coefficient (ICC) with 95% confidence interval. The repeated measurements performed by the same examiner were used to calculate the intra-examiner ICC. All measurements were tested for their fit to normal distribution (Kolmogorov–Smirnov test) and homogeneity of variances (Levene test). As normality assumptions were not met by all groups, a Kruskal–Wallis non-parametric test followed by Bonferroni/Dunn's post hoc test was carried out to compare the microhardness values of different treatment groups at each time point of measurement. Furthermore, a Wilcoxon signed-rank test was performed to analyze the results obtained on surface microhardness measurement over time in the same treatment group. Differences in the weight ratios of elements (calcium, phosphorus (P) and fluorine (F)) among the treatment groups were tested by one-way analysis of variance (Anova) and Tukey's honestly significant difference (HSD) post hoc test to assign groups of significance.

3 Results

A total of 21 volunteers (10 female and 11 male) with a mean age of 23.43 ± 1.43 years participated in this study. They had an average of unstimulated and stimulated salivary flow rates of 0.59 ± 0.11 and 1.78 ± 0.27 ml/min, respectively. All volunteers completed the treatment according to the study protocol, no specimens were lost and the number of specimens removed after 7-day in situ treatment and providing valid data for statistical analysis was 315.

toothpastes (ApaCare and PrevDent) demonstrated statistically significant hardness values of the enamel as indicated by an increase in VHN ($p < 0.05$), while the surface microhardness of the enamel blocks after intraoral application of the standard fluoride toothpaste (Parodontax) was significantly lower compared with the respective baseline microhardness ($p < 0.05$).

Figure 3 shows SEM images of enamel surfaces treated using different treatment products. At 7 days, there was an accentuated demineralization of the entire surface, being more pronounced on the interprismatic portion. The enamel surface clearly exhibited a porous and etching-like appearance. The enamel specimens treated with n-HAP toothpastes presented deposition of n-HAP globules on the demineralized enamel surfaces. The porous interprismatic and prismatic enamel structures were mostly hidden by a thick, uniform apatitic layer with n-HAP crystallites in contrast to the specimens treated by sodium fluoride, which exhibited evidence of uncovered porous surfaces. The enamel specimens after treatment with Parodontax toothpaste showed mainly demineralized enamel and only a few small deposits on the surface.



The results of EDX analysis are presented in Table 3. The enamel surfaces treated using n-HAP toothpastes showed a higher content of calcium and phosphorus than the enamel surfaces treated using standard fluoride toothpaste ($p < 0.05$). Comparison between the n-HAP toothpastes showed that the presence of calcium was higher for the PrevDent group ($p < 0.05$), while both groups presented a similar content of phosphorus. Unlike PrevDent, higher quantities of fluoride appeared in the enamel of ApaCare and Parodontax groups ($p < 0.05$).

percent, normalized to the total. Comparisons of mean values among the three treatment groups using one-way Anova followed by Tukey's HSD post hoc comparison test. *p*-values underlined with a full line are smaller than 0.05 and thus indicate significant difference; *p*-values underlined with a dashed line represent a nearly statistically significant difference

4 Discussion

For simulating intraoral erosion as closely as possible, it is desirable to assess the erosive process by using a pH-cycling regime.11,16 In the present study, a cyclic demineralization and remineralization model in situ was used to evaluate the effect of n-HAP and fluoride toothpastes on erosive enamel lesions. The effects of different treatment procedures on the prevention of acidic erosion were compared prior to and after the enamel was demineralized.21 The demineralization process comprised intermittent extraoral immersion of specimens in cola-based drink (pH 2.7) for 5 min. After exposing the enamel specimens to the oral environment for 1.5 h, the specimens were intraorally exposed to the saliva/toothpaste mixture for 2 min and were intraorally brushed within this time.

Two in vitro studies compared the remineralization effect of fluoride-containing toothpaste with that of nano-carbonate hydroxyapatite containing toothpaste on enamel demineralized by 37% phosphoric acid.12,15 Electron microscopic analysis demonstrated that in the prismatic and interprismatic enamel, uncovered areas appeared after treatment with fluoridated toothpaste in contrast to the completely hidden areas after treatment with carbonated hydroxyapatite

toothpaste.12,15 The authors concluded that the remineralization process produced by carbonate–hydroxyapatite nanocrystals was a result of new mineral apatitic deposition on the demineralized enamel surface.15 The findings of the study were consistent with those observed in previous in vitro studies (Figure 3).

The remineralization effect of early caries lesions increased with increasing n-HAP concentrations up to 10% and stabilized after 6 days of pH cycling.25 That is why a concentration of 10% n-HAP is considered to be optimal for remineralization of early enamel caries lesions.25,26 In another study monitoring the crystallization process of nanoparticles in toothpastes under physiological conditions, the time required for the crystallization process was observed to be shorter with a higher concentration of nanoparticles due to the formation of larger crystals and amount of nucleation centers in the same study.17 In the present study, the effectiveness of the products on enamel erosion was measured for 7 days and at the end of the treatment period, surface microhardness significantly increased compared with the baseline for n-HAP toothpastes, suggesting an improved mechanical strength, and no significant difference was noted between 1 and 2.25% n-HAP concentrations. The findings of this study regarding microhardness are in agreement with other in vitro studies.9,27

In a recently published study investigating the interaction mechanisms between chemically pure synthetic hydroxyapatite powder and bovine tooth enamel, each sample was pretreated with phosphoric acid and exposed to dispersions containing different n-HAP concentrations for 1 min, simulating the application procedure of a commercial mouth rinse.28 The results showed a significant dose–response relationship, and the size of the total area covered by particles increased as the concentration of hydroxyapatite increased (from 1 to 5 and 10 wt%).28 In an in situ study investigating the protective properties of different commercially available prophylactic products against erosive enamel demineralization, no continuous layer formation was observed with the use of mouth-rinsing solution containing less than 1% hydroxyapatite microclusters after 30 min.29 In the present study, neither 1 or 2.25% concentration of n-HAP in the toothpaste was able to cover the enamel surface entirely at the end of the 7 days. Further evaluation is required whether material precipitation on eroded enamel is increased by a higher n-HAP concentration, repetitive applications and a longer application/treatment time.

Microparticles and nanoparticles of hydroxyapatite are thought to refill the defects on dental hard tissue under erosive attack and to smoothen the affected surface.29,30 In vitro data, based on repair of initial enamel erosion, indicated that n-HAP particles strongly adsorb to the etched enamel surface and attachment of these nanoparticles to the enamel surface was stronger compared with that of conventional hydroxyapatite and nano-amorphous calcium phosphate.3,13 Additionally, the protective layer composed of hydroxyapatite particles formed on the tooth surface might protect the tooth from acid attacks.30 These effects have been confirmed in the present in situ trial not only because the specimens treated with n-HAP toothpastes demonstrated greater surface hardness after 7 days of treatment compared with the baseline, but also because an in-situ-formed persistent n-HAP coating on the enamel surface could be visualized by SEM. The obtained results demonstrated that the interaction between toothpaste, water and saliva results in n-HAP crystallization. It is also important to note that the layer of hydroxyapatite nanoparticles was not removed on brushing, suggesting that hydroxyapatite nanocrystals can chemically bind to the surface of natural enamel apatite.13,15 It has been shown that hydroxyapatite particles also attach to the surface of eroded enamel by forming solid interfaces between crystallites of hydroxyapatite particles and enamel.28

Incorporation of nanodimensional apatite in toothpastes has been suggested due to its well-proven potential and ability to repair caries lesions by biomimetic coating.12,14,31 However, information about repair and prevention potential of hydroxyapatite in oral health-care products for enamel erosive lesions is extremely scarce. One study showed that a 10% n-HAP solution could protect enamel against erosion from 5 min of in vitro soft drink exposure.32 The inhibitory effect

in situ.33 The formation of a biomimetic coating by the use of a toothpaste containing zinc (Zn)–carbonate hydroxyapatite nanostructured microcrystals on the hypersensitive cervical areas of the teeth and also on erosive enamel surfaces was demonstrated in an ex–in vivo study34 and in vitro experiments,23,24,27 respectively. In agreement with these findings, hydroxyapatite nanoparticles from toothpastes with or without fluoride clearly improved the microhardness of demineralized enamel specimens in the present in situ study, which could be attributed to the crystalline structure formed by nanoparticles on the enamel surface.

Sodium fluoride served as a control, since this compound is the most common active ingredient in toothpastes available on the market. Recent studies indicated that the evidence for the protective effect of sodium and amine fluoride-based products at low and medium concentrations commonly used in commercially available toothpastes and rinses was not strong as that of other protective agents.35–38 Because calcium fluoride (CaF_2)-like precipitates seem to be short-lived under the action of low pH of acids, forming a continuous surface coating cannot be expected when using neutral fluoride toothpastes, as mentioned in the studies.35,37 This was confirmed by the present study in situ that ApaCare and PrevDent toothpastes were shown to be superior to toothpaste containing sodium fluoride (1400 ppm fluoride) in terms of protective effect against erosion for enamel. Thus, the authors do not reject the null hypothesis.

The aforementioned results were also confirmed by EDX used for analyzing the elemental distribution of sample surfaces in different groups after in situ treatment. The remineralization and repair effect of the toothpaste containing various remineralization systems has been determined by analysis of either the calcium/phosphorus molar ratio or elemental distribution in the enamel surface, demonstrating incorporation of different elements into the enamel structure.12,34,39 Comparison of the samples treated with either n-HAP or fluoride toothpastes showed a higher calcium and phosphate content for both n-HAP toothpastes, which likely suggests the formation of surface deposits and a protective layer on the enamel surface in the present study. The results also demonstrated that the PrevDent treatment group exhibited a significantly higher calcium content of the enamel surface than did the ApaCare treatment group after in situ treatment. This finding may be related to the higher n-HAP concentration in PrevDent (2.25%) compared with that in ApaCare (1%).

In this study, measurement of surface microhardness showed that a similar protective effect against erosion was achieved after exposure to either n-HAP toothpaste with fluoride or nano n-HAP toothpaste without fluoride for demineralized enamel specimens. The results of this study could not support the finding that combination of n-HAP and fluoride enhanced the effectiveness of both n-HAP and fluoride.26,40 Further experiments under optimized conditions are needed to show whether synergistic action between n-HAP and fluoride is available.

5 Conclusions

Under the conditions of this in situ study, it was demonstrated that toothpastes containing n-HAP (1% n-HAP/1450 ppm fluoride as sodium fluoride and 2.25% n-HAP) may have a protective effect against erosion at the surface of enamel as measured by microhardness, elemental analysis and scanning electron microscopy. However, fluoridated toothpaste (1400 ppm fluoride as sodium fluoride) presented no beneficial effect on enamel rehardening or protective layer formation on the enamel surface. This study indicates that the use of n-HAP-containing toothpaste would be a promising preventive strategy based on self-applied product for the management of dental erosion.

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