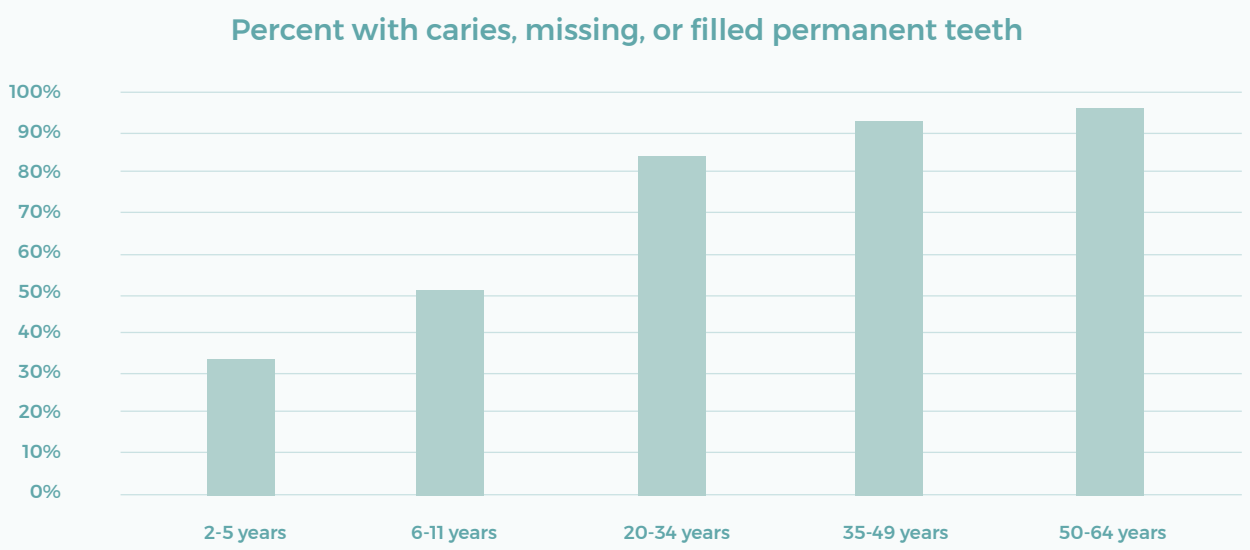
Introduction to Hydroxyapatite

Compiled by RiseWell

There has been a promising decline in dental caries over the last few decades, thanks to the hard work of dentists, who are engaging more with prophylactic treatments and providing better patient education on brush and flossing techniques. However, according to the National Institute of Dental and Craniofacial Research, 92% of adults have had cavities develop in their permanent teeth[1]. The prevalence of caries in the youngest population is just as alarming: over 50% of children aged 6-11 have had caries in their primary teeth.[2].



The unfortunate fact is, our teeth are constantly under attack. Enamel may be the hardest substance in the human body, but it struggles to remain resilient against the daily acid attacks that are largely the product of our modern lifestyle and diet. The substance that gives enamel its strength is hydroxyapatite (Ca5(PO4)3(OH)), aka HA, which forms a unique crystal structure that makes up 97% of enamel, 70% of dentin, and 60% of our bones.

To build enamel strength and help resist acid attack, toothpastes have used active ingredients like fluoride, instead of HA, to harden and fortify enamel. Microcrystalline HA was pioneered by NASA in 1970 as a remineralizing agent for astronauts returning from space with mineral loss in their teeth and bones as a result of zero gravity. It was subsequently adopted by the Japanese market, where it has become the gold standard in oral care. Countless studies comparing HA to fluoride and other remineralizing agents like

[1] https://www.nidcr.nih.gov/research/data-statistics/dental-caries/adults
[2] https://www.nidcr.nih.gov/research/data-statistics/dental-caries/children

Tri-Calcium Phosphate (CPP-ACP, aka MI Paste) have supported the use of HA.

In a recent study in Nature (included in the attached), microcrystalline HA was compared to fluoride in a one-year double-blind randomized clinical trial in children looking at the impact on caries rates. The study shows that "the impact of the daily use of a toothpaste with microcrystalline HA on enamel caries progression in the primary dentition is not inferior to a fluoride control toothpaste." In fact, the results for the HA toothpaste in the study were slightly better than the fluoride toothpaste.

HA has one key advantage over fluoride—it is completely bioavailable and has zero systemic side effects. Hydroxyapatite products carry no "Poison Control" warning label, and the ingredient itself is completely safe to eat. Fluoride, in contrast, can lead to fluorosis and systemic side effects if swallowed, which is why products containing fluoride must contain a warning label. The safety of HA when swallowed makes it particularly well suited for uses in toothpastes for vulnerable populations, including children, the elderly, or patients undergoing medical treatments like chemotherapy.

The preeminent book Toothpastes by C. van Loveren summarizes much of the research on hydroxyapatite in oral care, pointing out that it has "been commercially available in Japan since the 1980s, and was approved as an anticaries agent in 1993 based on randomized anticaries field trials in Japanese school children."

The book also lays out the mechanism of action by which it is thought that hydroxyapatite works. Hydroxyapatite "functions by directly filling up micropores on demineralized tooth surfaces. When it penetrates the enamel pores, it also acts as a template in the remineralization process by continuously attracting large amounts of calcium and phosphate ions from the remineralization solution to the enamel tissue, thus promoting crystal integrity and growth."

Attached, and summarized below, are some of the clinical studies on hydroxyapatite. In addition to our selected studies, we would encourage you to use the power of Google to find studies of your own—HA is well researched remineralizing agent that is only beginning to make headway in the United States with our products. RiseWell is a science-driven company, and we believe you will come to the same conclusion we did on HA. If not, we would still love to hear from you, as we would love to know how we can help and serve your patients as we grow as a company.

Thank you for taking the time to read this and for your interest in RiseWell.

[1] https://www.nidcr.nih.gov/research/data-statistics/dental-caries/adults
[2] https://www.nidcr.nih.gov/research/data-statistics/dental-caries/children

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Nano-hydroxyapatite and its applications in preventive, restorative and regenerative dentistry: a review of literature

Erlind Pepla, MD
Lait Kostantinos Besherat, MD
Gaspare Palaia, DDS
Gianluca Tenore, DDS
Guido Migliau, DDS

Department of Oral and Maxillo-Facial Sciences, "Sapienza" University of Rome, Italy

Corresponding author:
Erlind Pepla
Department of Oral and Maxillo-Facial Sciences
"Sapienza" University of Rome
Viale Regina Elena 278/a
00198 Rome, Italy
E-mail: kledion2004@libero.it

Summary

This study aims to critically summarize the literature about nano-hydroxyapatite. The purpose of this work is to analyze the benefits of using nano-hydroxyapatite in dentistry, especially for its preventive, restorative and regenerative applications. We also provide an overview of new dental materi-als, still experimental, which contain the nano-hy-droxyapatite in its nano-crystalline form. Hydroxya-patite is one of the most studied biomaterials in the medical field for its proven biocompatibility and for being the main constituent of the mineral part of bone and teeth. In terms of restorative and preven-tive dentistry, nano-hydroxyapatite has significant remineralizing effects on initial enamel lesions, cer-tainly superior to conventional fluoride, and good results on the sensitivity of the teeth. The nano-HA has also been used as an additive material, in order to improve already existing and widely used dental materials, in the restorative field (experimental ad-dition to conventional glass ionomer cements, that has led to significant improvements in their me-chanical properties). Because of its unique proper-ties, such as the ability to chemically bond to bone, to not induce toxicity or inflammation and to stimu-late bone growth through a direct action on os-teoblasts, nano-HA has been widely used in peri-odontology and in oral and maxillofacial surgery. Its use in oral implantology, however, is a widely used practice established for years, as this sub-stance has excellent osteoinductive capacity and improves bone-to-implant integration.

Key words: nano-HA, preventive, restorative, regenerative.

Introduction

The purpose of this work is to analyze what, up to now, is reported in the literature about the advantages of nano-hydroxyapatite in dentistry, especially in preventive and restorative dentistry, up to its use in oral surgery, such as implantology and periodontal regeneration. We also attempt to provide a broad overview of the new materials that are being born from experimental research, with particular attention to the materials commonly used in restorative dentistry, such as composite resins.

Tooth enamel is the most mineralized tissue of human body. Its composition is 96 wt.% inorganic material and 4 wt.% organic material and water. In dentin, the inorganic material represents 70 wt.%. This inorganic material is mainly composed by a calcium phosphate related to the hexagonal hydroxyapatite, whose chemical formula is $Ca_{10}(PO_4)_6{\cdot}2(OH)$. X-ray energy dispersive spectroscopy (EDS) analysis of enamel and dentin also indicated the presence in small quantities of other elements such as Na, Cl and Mg.

Hydroxyapatite (HA) is the main component of enamel, which gives an appearance of bright white and eliminates the diffuse reflectivity of light by closing the small pores of the enamel surface. Hydroxyapatite has long been among the most studied biomaterials in the medical field for both its proven biocompatibility and for being the main constituent of the mineral part of bone and teeth. Hydroxyapatite is also an important source of calcium and phosphate, very important for the remineralization of demineralized enamel areas. The inorganic component of all the mineralized tissues of the human body is, in fact, made up of a large prevalence of calcium phosphatesalts. Other inorganic materials such as calcium carbonates and sulphates are present in smaller quantities too; in particular hydroxyapatite represents 60-70% and 90% in weight of bone and enamel respectively. The recently developed interest for nanotechnology in many fields, is producing interesting and imminent applications in dentistry for nano-hydroxyapatite, which presents crystals ranging in size between 50 and 1000 nm. The nano-hydroxyapatite has a strong ability to bond with proteins, as well as with fragments of plaque and bacteria, when contained in toothpastes. This ability is due to the size of nanoparticles, which considerably increase the surface area to which proteins can bind. Besides, nano-

hydroxyapatite also acts as filler because it repairs small holes and depressions on enamel surface, a function enhanced by the small size of the particles that compose it. The Japanese company Sangi Co. Ltd was the first to take an interest in hydroxyapatite, after purchasing the rights ifrom NASA (U.S. National Aeronautics and Space Authority) in 1970. The astronauts, in fact, lost minerals from the teeth and bones in the absence of gravity, and NASA proposed a synthetic hydroxyapatite as a repairing material. The Sangi Co. Ltd had the idea in 1978 to launch toothpaste that could repair the tooth enamel, which contains for the first time nano-hydroxyapatite (Apadent). In 2006, the first toothpaste containing synthetic hydroxyapatite biomimetic as an alternative to fluoride for the remineralization and repair of tooth enamel appeared in Europe. The biomimetic hydroxyapatite function is to protect the teeth with the creation of a new layer of synthetic enamel around the tooth, rather than hardening the existing layer with fluorine, that chemically changes into calcium halophosphate [$Ca_5(PO_4)_3F$].
In its granular form, hydroxyapatite is currently used in clinical dental practice to reconstruct periodontal bone defects, to the fill bone defects after cystectomy, after apicoectomy, after the loss of dental implants and to increase of the thickness of atrophic alveolar ridges. Shaped blocks of hydroxyapatite are especially used in maxillofacial surgery (bone defects after trauma, osteotomies and reductive stabilization, reconstruction of facial skeleton, replacement of parts of orbital and maxillary bone). Blocks, as well as granular powder, can also be used in pre-prosthetic surgery to increase the thickness of the alveolar ridge.
Studies on biocompatibility have shown that hydroxyapatite chemically binds to bone and induces no phenomena of toxicity nor inflammatory, local or systemic. Some researches show that the hydroxyapatite, unlike tricalcium phosphate, doesn't undergo resorption. Other authors have instead found resorption of hydroxyapatite. Thanks to its chemical and crystallographic affinity with inorganic components that constitute the bone, hydroxyapatite is able to establish chemical bonds and to ensure a more rapid integration of titanium implants to bone and surrounding tissues. Numerous studies have highlighted the role of hydroxyapatite in facilitating the process of osteointegration with or without other polymeric space.

Analysis of the scientific literature (in restora-tive dentistry)

The use of nano-hydroxyapatite as a material that could improve the properties of materials currently used in restorative dentistry has been studied. Moshaverina et al. in 2008 (1) have focused on the addition of N-vinylpyrrolidone containing acids, nano-hydroxyapatite and fluorapatite to conventional glass ionomer cements (GIC). These cements have unique properties such as biocompatibility, anticariogenic action (due to the release of fluorides) and adhesion to many dental structures. In this study the attention was paid to the search for materials to be added to common glass ionomer cements available on the market, Fuji II GC, in order to improve its mechanical properties.
Nano-hydroxy and fluorapatite have been synthesized using a sol-gel technique in an ethanol base.
The results showed that after 1 and 7 days, the nano-HA/fluorapatite added to cements howed greater hardness to compression (CS) 177-179 MPa, a higher hardness to diametrical tension (DTS) 19-20 MPa and a higher hardness to biaxial flexibility (BFS) 26-28 MPa, compared to the control group (160 MPa in CS, 14 MPain DTS and 18 MPa in BFS) (Tab. 1). Therefore, glass ionomers containing nano-bioceramics are very promising restorative dental materials with improved mechanical properties and strong binding to dentin, and may very soon replace GIC currently on the market. From these studies it seems to emerge, in fact, an unmistakable statistical datum: modified GICs with the above listed substances possess much higher capacity than traditional materials.
Moshaverina et al. (2008) (2), have extensively studied the effects of incorporating hydroxyapatite and fluorapatite (FA) in a conventional glass ionomer cement (Fuji II GC). The addition of synthesized nano-HA and FA in Fuji II improves the mechanical properties (to compressive, diametral tensile and biaxial flexural forces) of the resulting cement and its bonding strength to dentin. These bioceramics are, therefore, considered promising additives for glass ionomer cements used as restorative materials. However, perhaps due to the low solubility value of FA, the FA-containing samples showed very high values, after 7 and 30 days, in both mechanical properties and binding tests, as compared to compounds containing HA and to GIC.
In recent years, attention has focused towards the synthesis of new compounds of nano-HA. This is the case in the study on the remineralizing effects of zinc carbonate nano-HA ($ZnCO_3$/n-HAP) performed by Tschoppe et al. in 2011 (3). In the research, 35 bovine incisors were taken; from these teeth, 70 experimental blocks of enamel and 85 samples of dentin were obtained. A quarter of all the samples were coated with a special acid resistant paint, in order to act as control group. Enamel lesions were obtained by dipping the blocks in a solution (5l) containing 6 pM of MHDP, 3 mMCa Cl_2 dihydrate, 3 mM KH_2PO_4 and 50 mM acetic acid, at a pH of 4.95, in an incubator (37° C, BR 6000; Heraeus Kulzer) for 14 days. The lesions in dentin were prepared by dipping

Table 1. The synthesized nano-ceramic particles were incorporated in a powder of commercial glass ionomer (Fuji II GIC).

	Control Group	Nano-HA/Fluoroapatite Group
CS	160 MPa	177-179 MPA
DTS	14MPA	19-20 MPa
BFS	18MPa	26-28 MPa

the samples in a solution containing 0.0476 mMNaF, 2.2 mMCaCl$_2$ dihydrate, 2.2mM KH$_2$PO$_4$, 50 mM acetic acid and 10 mM KOH, at a pH of 5.0 (37° C) for five days. The pH value of the demineralizing solutions was constantly monitored. Afterwards, half of all demineralized surfaces were again covered with paint (baseline control demineralization). The samples were divided randomly into five groups (enamel n=14, dentin n=17) and were placed separately in remineralizing solution for two and for five weeks. In agreement with EN ISO 11609 standards, the respective toothpastes were diluted in a 1:3 ratio in the remineralizing solution, in order to obtain a homogeneous substance. Commercially available toothpastes containing ZnCO$_3$/n-HA or n-HA (all without fluorides) were used while the toothpaste containing amino fluorides was used for the control group. Then, the samples were manually brushed with a soft brush and with a minimum pressure; this procedure was performed every day for about 5 seconds and with a contact time with the solutions of 115 seconds, a total time of 120 seconds. We must, however, emphasize that this procedure has some significant limitations as it is highly operator dependent and difficult to standardize and empirically assessable. After each brushing, the samples were rinsed with deionized water for 10 seconds. Every two days for each group, the solutions were changed (250 ml). Finally, sections of 100 mm were performed and analyzed by means of microradiography and through an appropriate software (TMR for Windows 2.0.27.2; Inspektor Research System, Amsterdam, The Netherlands). Thirty samples of enamel and two of dentin were lost during preparation procedures. This complex *in vitro* study shows that toothpastes containing different types of nano-hydroxyapatite have the same remineralizing capabilities on enamel and dentin, and those containing fluoride have lower capacity than the first. We must, however, take into account the limits of an *in vitro* study, as it is far from simulating the conditions present in the oral cavity.

In the study by Huang et al. of 2009 (4), the authors analyzed the remineralizing effect of nano-HA on demineralized bovine enamel under cyclical conditions of pH, by the microhardness test, on cross-sections (CSMH) and on surfaces and through polarized light microscopy (PLM). Nano-HA and conventional HA (crystals in the order of micrometers, from 0.5 to 2 μm), were obtained by the National Incubation Base of Nano-Biomaterials Industrialization, Sichuan University. The demineralizing solution (DS) used to create lesions similar to caries had the following composition: acetic acid50 mM, Ca(NO$_3$)$_2$2.2 mM, KH$_2$PO$_4$ 2.2 mM and NaF 5.3 µM. The pH value of the DS was adjusted to 4.5 by the addition of a solution of KOH. The remineralizing solution (RS) used under conditions of cyclical pH contains instead: HEPES 20 mM, CaCl$_2$ 1.5 mM, KH$_2$PO$_4$ 0.9 mM, KCl130 mM and NaN$_3$ 1 mM. The pH value was adjusted to 7.0 with KOH. The dissolution of HA products was studied in preliminary experiments. In the study, incisors of 4 years old bulls were analyzed. The teeth, cut into blocks, were dipped in 8 ml of DS for 72 hours at 37° C. Finally, 70 teeth with a KHN (Knoop Hardness Number) value between 171.6 and 204.3 were selected. They were then divided into 10 groups exposed to different pH values. Afterward, the blocks were longitudinally sectioned in order to be studied by CSMH. The data were analyzed using SPSS 13.0 software. The remineralizing effect of nano-HA increased significantly when the pH was lower than 7.0.

One of the most important variables present in the mouth is the variation of pH. The assessment of this variable is missing in this study. In another study by Huang et al. of 2009 (5), they analyzed the mineralizing ability of nano-hydroxyapatite in cyclically variable pH conditions.

The high availability of calcium and phosphate in these conditions, causes, according to the author, a positive effect on the remineralization of lesions. This indicates that nano-HA is a better resource of free-Ca, and this is important for the defense from dental caries and erosion. The largest increase in mineralization was observed in the group with pH 4.0. The group with pH 7.0, however, showed the lowest degree of mineralization. An accumulation of many minerals in the lesions and a corresponding reduction of their depth were also observed. The effect of nano-HA is better than the effect of micro-HA at pH 7.0 and at the same concentrations. The concentration of calcium in solutions containing nano-HA was greater than that detected in solutions containing micro-HA. The Ca concentration increase leads to a growth in the saturation of oral fluids with HA, favoring the deposition of apatite minerals in the lesions and eventually promoting remineralization.

In terms of dental erosion, it is important to emphasize that its prevalence is increasing in young children and adolescents in developed and in developing countries. The main external cause of increased erosion is a higher consumption of acids in the diet and with drinks. In particular the use of sport drinks has recently increased, and these may cause erosion according to their acid content. With reference to this, is the interesting Min et al. in 2011 study (6), on nano-hydroxyapatite as an addition to sports drinks. In this study was examined the possible beneficial effects of additioning nano-hydroxyapatite to sports drinks. Powerade ® (PA) was taken as experimental solution and citric acid was added as acid. They prepared different solutions with the PA alone and with the addition of 0.05%, 0.10% and 0.25% nano-hydroxyapatite. 20 bovine teeth per group, cut in 3.5 mm x 3.5 mm blocks, were treated for 20 minutes three times a day, with 2 h and 40 min of interval between each treatment. Once the treatment process was finished, the samples were thoroughly rinsed with distilled water. Throughout the rest of the day, when not being treated, the teeth were immersed in a solution containing artificial saliva with the following composition: gastric mucin 0.22%, KCl 14.93 mM, KH$_2$PO$_4$ 5.42 mM NaCl 6.51 mM and CaCl$_2$ dihydrate 1.45 mM.

The cyclic pH process was repeated for 7 days. The potential erosion was determined by changes in the surface microhardness (SMH), and the teeth were analyzed with the confocal laser scanning microscopy (CLSM) and with the scanning electron microscope (SEM). The prevention of dental erosions increased with the concentration of n-HA, and sports drinks containing 0.25% of the substance have obtained the best results.

The consumption of carbonic acid containing drinks is the main etiological factor for tooth erosion. An experimental study on 18 permanent teeth dental erosion caused by beer was conducted by Hangoo et al. (11) in 2011 (7). These elements were subsequently treated with a remineralizing substance nano-hydroxyapatite based. In the study they primarily measured the microhardness of 18 permanent teeth (Fig. 1). Then they did second measurement of the teeth, after dipping them in a solution containing 40 ml of beer (Behnoush Lemon Delester, Iran) for 5 minutes. The time was calculated according to actual studies on the permanence of beer in the mouth based on the amount drunk daily. The average microhardness primary values – i.e. prior to any type of manipulation of the teeth –, of the 18 cases was 340.24 ± 25.4² kgf/mm. This value reduced to 314.67 ± 33.89² (second value of microhardness) after immersion in beer; this is equivalent to 92.5% of the primary value of microhardness, and the "t" test analysis shows that this is statistically significant (p=6.20). The value of secondary microhardness of the 9 cases in water was 312.85 ± 36.79² kgf/mm; that reduces to 310.81 ± 31.44² (tertiary value of microhardness) after immersion in drinkable water. This is equivalent to 99.3%, a value that is not statistically significant (p=20.6). The secondary value of microhardness of the 9 cases dipped in NHAP was 315.18 ± 30.65 kgf/mm. This increases to 320.99 ± 24.74² kgf/mm (tertiary value of microhardness) after immersion in a solution with NHAP. This value is equivalent to 98.2%, which is statistically significant (p=0.012).

The results of this study demonstrate that there is a statistically significant increase in the microhardness of teeth demineralized by beer and then exposed to a solution of n-HA.

The aim of the study of Orsini et al. (8) is to evaluate the relative abilities of three desensitizing dentifrices to provide rapid relief of dentin hypersensitivity (DH). Using a double-mask, randomized design, three dentifrices: 1) containing 8% arginine and 1,450 ppm sodium monofluorophosphate; 2) containing 8% strontium acetate and 1,040 ppm sodium fluoride; and 3) containing 30% microaggregation of zinc-carbonate hydroxyapatite nanocrystals were compared after 3-day treatment. Participant's DH was evaluated at baseline and after 3 days using air-blast, tactile, cold water, and subjective tests. The final sample consisted of 85 individuals: 29 received the arginine-based dentifrice (group 1), 27 the strontium acetate-based dentifrice (group 2), and 29 the dentifrice based on zinc-carbonate hydroxyapatite (group 3). All dentifrices were mostly effective to reduce DH: the percentage of score reduction from baseline to 3 days was >30% for all tests (except for subjective test of group 2). The comparison among the three dentifrices showed that, after 3 days, there was an improvement in air-blast (mean percentage of reduction, 39.2% in group 1, 42.0% in group 2, and 39.2% in group 3), cold water (41.5, 51.8, and 50%), tactile (50.3, 40.1, and 33.8%), and subjective (33.1, 17.4, and 31.4%) test scores, with differences being significant for cold water and subjective tests. For air-blast and tactile tests, there were no significant differences across groups at 3 days. Moreover, no significant differences at any test were observed in a subset of patients that were followed up to 8 weeks: all dentifrices were all highly efficacious. This study documents that the three tested dentifrices significantly reduced DH after 3-day treatment, supporting their use in clinical practice. To the best of the authors' knowledge, this is the first report documenting the rapid relief from DH of a zinc-carbonate hydroxyapatite dentifrice. The study by Browing et al. of 2011 (9), finally focused attention on the search for a material that could reduce tooth sensitivity after bleaching. For this purpose, nano-hydroxyapatite was tested. It was noted that the teeth sensitivity after bleaching increased in the presence of enamel defects. Using a randomized clinical trial, the efficacy of a paste containing n-HA in reducing this type of sensitivity was analyzed. A paste containing n-HA (Renamel AfterBleach, Sangi Co. Ltd, Tokyo, Japan) and a placebo (zero-HA) were randomly assigned to 42 participants. A 7% hydrogen peroxide gel was used for 14 days, in association with a desensitizing paste used immediately for the 5 minutes afterwards. A diary was completed daily to note the effect of desensitization and the eventual sensitivity, on a VAS (Visual Analog Scale). Three aspects of the sensitivity of the teeth were analyzed: percentage of participants, number of days and intensity level. Color change was evaluated. For zero-HA and n-HAgroups, respectively, 51 and 29% of participants reported dentinal sensitivity (p=0.06) (Tab. 2). The days of sensitivity were 76 and 36 respectively (p=0.001). The changes in VAS score from baseline have an upward trend in the zero-HA group (p=0.16) (Tab. 3). The color change was equivalent for both groups. The conclusions showed that the group treated with n-HA had lower sensitivity levels.

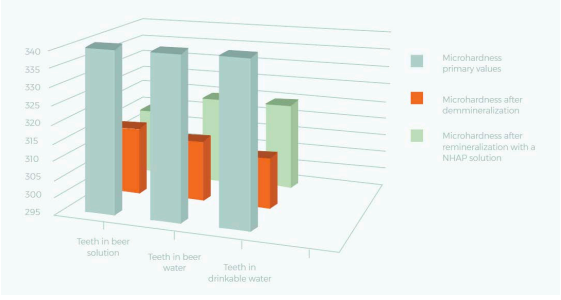

Figure 1. Microhardness values after demineralization and remineralization.

Table 2. Number (percentage) of participants with sensitivity to any assessment.

Number of assessments	Group	Participants with sensitivity	Participants without sensitivity
Baseline	Zero-HA	3 (14%)	18 (86%)
First week whitening	Zero-HA	14 (67%)	7 (33%)
Second week whitening	Zero-HA	7 (33%)	13 (65%)
First week after whitening	Zero-HA	5 (25%)	15 (75%)
Baseline	n-HA	2 (10%)	19 (90%)
First week whitening	n-HA	8 (38%)	13 (62%)
Second week whitening	n-HA	4 (19%)	17 (81%)
First week after whitening	n-HA	4 (19%)	17 (81%)

n-HA=nano-sizedhydroxyapatite, zero-HA=placebo
There is no significant difference between the groups during bleaching active phase (chi-square, p=0.06)

Table 3. Percentage of days associated with sensitivity.

Number ofassessments	Group	Days with sensitivity	Days without feeling
Baseline	Zero-HA	9 (6%)	138 (94%)
First week whitening	Zero-HA	50 (34%)	97 (66%)
Second week whitening	Zero-HA	26 (19%)	114 (81%)
First week after whitening	Zero-HA	16 (11%)	124 (89%)
Baseline	n-HA	7 (5%)	140 (95%)
First week whitening	n-HA	20 (14%)	127 (86%)
Second week whitening	n-HA	16 (11%)	131 (89%)
First week after whitening	n-HA	14 (10%)	133 (90%)

n-HA=nano-sized hydroxyapatite, zero-HA=placebo
Participants in the group with n-HA experience significantly more days with sensitivity during the active phase of bleaching (chi-square, p=0.001)

Analysis of the scientific literature (in oral surgery)

Many researchers have focused on the use of nano-HA as a co-adjuvant material in oral surgery, especially regarding the improvement of the dental implant characteristics. In the work of Masahiro et al. of 2012 (10), they analyze a new compound with nano-polymorphic crystalline HA applied to microrough titanium surfaces through a combination of flame spray and calcination at a low temperature. It was then analyzed for the biological capacity to increase bone-implant integration. The sandblasted microrough titanium implants and the titanium implants linked to HA and sandblasted were analyzed via biomechanical histomorphometric methods in rats.
In the study the HA used as an addition to implants surface is 55% crystalline and causes an increase of the surface area by 70% when compared to uncoated microrough surfaces. Furthermore, hydroxyapatite is free of impurities, with a calcium/phosphate ratio of 1.66, thus being equivalent to the stoichiometric value of HA. Titanium cylinders (1 mm in diameter and 2 mm long) were obtained from Grade 5 titanium alloy (Ti-6Al-4V). In order to create the micro-rough-ness, titanium samples were sandblasted with alu-minum oxidemicro-powder. The HA coating was ob-tained by flame spray on blasted surfaces. The flame was created by means of acetylene and oxygen, and the air was used as a high-speed carrier. All exam-ined surfaces were inspected by scanning electron microscope (SEM) (XL30; Philips, Eindhoven, The Netherlands) and by atomic force microscope (AFM) (SPM-9500J3; Shimadzu Corporation, Tokyo, Japan). Male Sprague-Dawley rats, about eight weeks old, were used for this study. The implant sites were prepared at about 10 mm from the distal edge of the femur using a 0.8 mm round bur and widening by means of reamers (# ISO 090 and 100). A cylindrical implant that had been machined and sandblasted, or sandblasted and coated with HA was inserted into each hole prepared on the femur. The muscles and skin were then sutured separately with absorbable suture. The total number of animals used was78 (54 animals for testing machined implants, sandblasted, sandblasted and coated with HA; analyzed at 2, 4 and 8 weeks; 24 animals for histological analysis for the groups of sandblasted and for sandblasted and coated with HA; analyzed at 2 and 4 weeks). HA-coated implants showed an increased percentage of bone-implant contact and an increase in bone volume within 50 µm close to the implant surface. On the contrary, around implants coated with HA, the bone volume outside the boundary of 50 µm was low. In particular, this study demonstrated that nano-crystalline hydroxyapatite is indisputably effective in increasing osteoconductivity and inhibiting the infiltration of soft tissue around the implant, but the effect is quite limited to the microenvironment around the implant.
Ceramic nanoparticles represent an encouraging class of bone graft substitutes due to their improved

osteointegrative properties. Nano-crystalline hydroxyapatite binds bone and stimulates bone healing encouraging osteoblastic activity. In the study conducted by Singh et al. in 2012 (11), the authors examine the clinical and radiographic results obtained with NCHA bone graft (Sybograf®) associated with collagen membranes (Periocol®), compared with OFD (Open Flap Debridement) in the treatment of intrabony periodontal defects.

In the work on the comparison between NCHA associated bone grafts with collagen membranes and traditional OFD technique, Singh et al. (11) designed a controlled randomized clinical trial in parallel groups. Eighteen intrabony defects in 14 patients aged between 25 and 65 years were randomly assigned to a test group and to a control group. The plaque index, the gingival index, the PPD (probing pocket depth), the clinical attachment level (CAL) and the gingival recession (REC) were recorded at baseline, and were reassessed 6 months later (Tab. 4). Furthermore, bone grafts were evaluated through digital software. In the test sites they have placed NCHA bone graft with collagen membranes, while in control sites they performed only OFD. Recall appointments were set at 7 days, 30 days, and then at 3 months and 6 months (Tab. 4). The association between resorbable membranes, derived from fish, and NCHA bone grafts is particularly positive as concern to the improvement of periodontal indices. This work has clearly shown that the additional use of resorbable membranes derived from fish in combination with NCHA bone grafts is clinically, radiographically and statistically significant compared to OFD alone, in terms of reduction of PPD, CAL gain and percentage of bone filling.

In the study by Qu et al. in 2010 (12), the use of a bioactive and osteoconductive composite formed by nano-hydroxyapatite and polyamide 66 (nHA/PA66) was tested, for the creation of a new asymmetric porous membrane to be used for guided bone regeneration (GBR). Regarding the membranes, they analyzed the cytotoxicity of the material, the response of the surfaces to bone formation, the morphology, proliferation and cell cycle progression of bone marrow stromal cells (BMSCs) in rat culture. The polygonal and fusiform shape of BMSCs was observed using a scanning electron microscope (SEM). In the research the PA66 with an average viscosity, molecular weight (Mv) 18 k Dawas used, derived from BASF (Ludwigshafen, Germany). The suspension of nano-hydroxyapatite used for the compound was prepared with a wet method and hydrothermal treatments. The proliferation of BMSCs culture on nHA/PA66 membranes has been tested with the MTT method (MTT: [3 - {4,5-dimethylthiazol-2YL}-2,5-diphenyl-2H-tetrazoliumbromide]), and it was found to be higher than the negative control group after 1 and 4 days of incubation and, moreover, did not appear to have any significant differences after 7 and 11 days of culture. The results of the cell cycle suggest that the membrane has no negative influence on cell division. The membrane has an asymmetric porous structure, in which pores smaller than 10 microns are distributed on one side (microporous layer), while pores ranging between 30 and 200 μM are located on the opposite side (macroporous layer). The microporous layer of the membrane prevents the migration of fibrous connective tissue in bone defects, being also able to permeate sufficient nutrients for regenerating tissue. The results show that n-HA/PA66 membrane is a 3D porous structure with a dense microporous layer on one side and with a spongy microporous layer on the other side. *In vitro* experiments show that nHA/PA66 membrane has a good affinity for attaching to BMSCs, and a non-negative effect on cells viability and proliferation. The results of *in vitro* and *in vivo* studies indicate that the nHA/PA66 membrane has an excellent biocompatibility and is indicated for use in guided tissue regeneration (GTR) or GBR.

Table 4. The plaque index, the gingival index, the PPD (probing pocket depth), the clinical attachment level (CAL) and the gingival recession (REC).

	Control group	Test group
PPD reduction	3.22 ± 1.09 mm p = 0.007	4.33 ± 0.5 mm p = 0.007
CAL gain	2.77 ± 1.09 mm p = 0.006	3.77 ± 0.66 mm p = 0.006
REC increase	0.55 ± 0.72 mm p = 0.025	0.49 ± 0.52 mm p = 0.046
Main gain in filled radiografic defect	2.07 ± 0.67 mm p = 0.007	0.91 ± 0.21 mm p = 0.008

Recent studies suggest that nano-crystalline hydroxyapatite (nano-HA) paste represents a promising class of grafting bone substitutes. The study of Kasai et al. of 2008 (13) was conducted to investigate the proliferation of human periodontal ligament (PDL) in cell cultures, in the presence of a nano-HA paste, and to analyze cells' associated signal paths. In this experimental research on the consequences of the application of nano-HA in cell culture with human periodontal ligament (PDL), PDL cells were stimulated with pastes of nano-HA and with an enamel matrix derivative (EMD) in a soluble form. The proliferation of PDL cells was determined by analyzing the incorporation of bromodeoxyuridine (BrdU) in the DNA of proliferating cells. In order to understand the mechanism that underlies the increase in cell proliferation of PDL cells exposed to nano-HA, the phosphorylation of serine/threonine protein kinase Akt was analyzed using phospho-specific antibodies. The nano-HA paste showed a potential for proliferation, two times lower than EMD, but both substrates significantly increased the proliferation rate ($p<0.05$) in comparison to the negative control group. In conclusion, it seems that the growth and proliferation rate of PDL cells in the presence of nano-HA paste is mechanically connected to the activation of the receptor for the epidermal growth factor receptor (EGFR) and its downstream targets ERK1/2 and Akt. In conclusion, these studies suggest that nano-HA paste is a potent stimulator of cell proliferation, which probably

contributes to the fundamental process of periodontal tissue regeneration.

Final remarks

The nano-hydroxyapatite is a revolutionary material with a wide use in dentistry. With regard to restorative and preventive fields, nano-hydroxyapatite has remarkable remineralizing effects on initial lesions of enamel, certainly higher than traditional fluorides used until now for this purpose. Nano-hydroxyapatite is, in fact, a better source of free Ca, and this is a key element as regards the remineralization, the protection against caries and dental erosion. With regard to the latter point, of fundamental importance in dentistry, the road leading to the addition of small percentages of nano-HA (0.25%) in beverages such as mineral supplements for sports activities, in order to prevent tooth erosion caused by those drinks, seems very promising. Nano-HA has also been used as a supplementary material, in order to improve the dental materials already existing and widely used. This is the case of the experimental addition to traditional GIC, a procedure that has led to significant improvements in the mechanical properties of these substances.

A continuing interest in the nano-crystalline structure of hydroxyapatite has prompted many researchers to look for new combinations that could improve existing materials or create new ones that could meet their needs. This has led to new complex compounds, such as nano-HA associated with zinc carbonate, which seems to be an excellent material for the remineralization of initial lesions involving enamel and dentin, or as the nano-HA associated with the polyamide 66, used in order to create a new periodontal membrane with improved properties.

Noteworthy are the applications of nano-HA in fields other than strictly restorative or preventive. Because of its unique properties, such as the ability to chemically bind to bone, without inducing toxicity or inflammation and stimulating bone growth through a direct action on osteoblasts, nano-HA has been widely used in periodontology and in oral and maxillofacial surgery. Collagen membranes associated with nano-HA are used to fill bone defects, since this substance leads to a clear improvement in periodontal indices.

Its use in the field of implantology, instead, is a practice widely used and has been consolidated in recent years, since the nano-hydroxyapatite has excellent osteoinductive capacity and improves osteointegration in bone-implant interface. It is therefore natural to expect for the coming years an increased interest in this revolutionary substance and a growing number of scientific articles on the subject.

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Comparative efficacy of a hydroxyapatite and a fluoride toothpaste for prevention and remineralization of dental caries in children

Bennett T. Amaechi[1], Parveez Ahamed AbdulAzees[1], Dina Ossama Alshareif[1], Marina Adel Shehata[1], Patrícia Paula de Carvalho Sampaio Lima[1], Azadeh Abdollahi[1], Parisa Samadi Kalkhorani[1] and Veronica Evans[1]

OBJECTIVE: This in situ study compared the effectiveness of two toothpastes containing hydroxyapatite or 500 ppm fluoride in promoting remineralization and inhibiting caries development.

MATERIALS AND METHODS: Two enamel blocks (human primary teeth), one sound and one with artificially-produced caries lesion, were exposed to toothpaste containing either 10% hydroxyapatite or 500 ppm F^- (amine fluoride) via intra-oral appliance worn by 30 adults in two-arm double blind randomized crossover study lasting 14 days per arm (ClinicalTrials.gov: NCT03681340). Baseline and post-test mineral loss and lesion depth (LD) were quantified using microradiography. One-sided *t*-test of one group mean was used for intragroup comparison (baseline vs. post-test), while two-sided *t*-test of two independent means was used to compare the two toothpaste groups.

RESULTS: Pairwise comparison (baseline vs. test) indicated significant ($p < 0.0001$) remineralization and LD reduction by either toothpaste; however, when compared against each other, there was no statistically significant difference in remineralization or LD reduction between the two toothpastes. No demineralization could be observed in sound enamel blocks exposed to either toothpaste. While F^- induced lesion surface lamination, HAP produced a more homogenous lesion remineralization.

CONCLUSIONS: 10% hydroxyapatite achieved comparable efficacy with 500 ppm F^- in remineralizing initial caries and preventing demineralization. Thus the HAP toothpaste is confirmed to be equal to the fluoride toothpaste in this study.

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INTRODUCTION

Although preventable, dental caries continues to be one of the most prevalent chronic diseases among children in the U.S. and the world, and one of the most common unmet healthcare needs of poor children.[1] As much as 80% of caries incidence is experienced by only 20–25% of children, with 10% having untreated cavities, and those from low socioeconomic and minority groups experiencing significantly higher rates and at younger ages.[2,3]

It is well documented that saliva has a caries protective effects due to its supersaturation with Ca^{2+} and PO_4^{3-} ions in a bioavailable form and enrichment with various proteins playing multiple roles in maintenance of hard tissue integrity throughout life.[4] Furthermore, the supersaturation of saliva with Ca^{2+} and PO_4^{3-} ions, at physiological pH, ensures that these ions are bioavailable to diffuse into mineral deficient lesions to induce remineralization.[5] However, the natural caries protective and remineralizing effects of saliva is not only a slow process but obviously insufficient to protect individuals against caries and remineralize existing lesions without additional agents to enhance its effects.

Although fluoride interventions seem to have the most consistent benefit in preventing caries development and remineralizing initial lesions with the highest level of supporting evidence,[6–8] caries still develop in high risk individuals of all ages, irrespective of the dose of fluoride used.[9,10] There are limitations to what application of fluoride alone can achieve in relation to caries prevention and remineralization.[11] These limitations may be associated with facts that fluoride becomes less effective below a pH of about 4.5;[12] fluoride still needs Ca^{2+} and PO_4^{3-} ions in a bioavailable form in saliva and other sources to be effective; and fluoride remineralization of initial lesions is most effective at the outer 30 µm of the lesion,[13,14] thus leading to surface-zone remineralization at the expense of the lesion body, making full remineralization difficult to achieve.[15,16] Furthermore, although the efficacy of fluoride is dose-dependent and increases with increase dose,[7] there is a limit to which you can increase the dosage of fluoride to avoid the risk of fluorosis in children[17] and toxicity in all ages.[18,19] The effect of dose limitation on fluoride effectiveness may be more pronounced in children below 6 years, since the fluoride dose recommended for this group is even lower than the regulatory 1000–1500 ppm fluoride concentration in non-prescription toothpastes, and as such probably suboptimal for effective remineralization of initial lesions.

The above mentioned limitations of the saliva homeostatic mechanisms and fluoride-based strategies in caries prevention and remineralization, especially in highly cariogenic oral environments justify the need for new-age strategies that could work either better than or as effective as fluoride but can permit increasing dosage for increase effectiveness without safety concerns. It is envisaged that the presence of additional extrinsic

[1]Department of Comprehensive Dentistry, School of Dentistry, University of Texas Health San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, USA
Correspondence: Bennett T. Amaechi (amaechi@uthscsa.edu)

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sources of stabilized Ca^{2+} and PO_4^{3-} ions could augment the natural caries preventive and remineralization potential of saliva by increasing diffusion gradients favoring faster and deeper subsurface remineralization.[20] One of the new caries remineralizing technologies is the biomimetic systems, among which are the synthetic hydroxyapatite (HAP; $Ca_5(PO_4)_3(OH)$ applied in microcluster or nanocrystalline forms in oral care products.[21,22] HAP is a bioactive and biocompatible material with similar chemical composition to the apatite crystals of human enamel.[21–24] Several in vitro and in situ studies have provided evidences supporting the caries remineralization and prevention potential of HAP in oral care products based on its demonstrated ability to strongly adsorb to tooth surfaces, plaque components and bacteria.[24–29] Randomized controlled clinical trials, some of which have led to the approval of HAP as an anti-caries agent in Japan in 1993 and in Canada in 2015, have demonstrated its non-inferiority and equivalence to fluoride.[30–32] Therefore, the objective of this in situ study was to determine whether Karex Kid's toothpaste containing 10% HAP microclusters (Kinder Karex Zahnpasta, Dr. Kurt Wolff GmbH and Co. KG, Bielefeld, Germany) is as effective as Elmex Kid's toothpaste containing 500 ppm fluoride as amine fluoride (Elmex Kinder Zahnpasta, CP GABA GmbH, Hamburg, Germany) in promoting the remineralization and inhibiting the development of carious lesions. We hypothesized that (1) each of the two toothpaste formulations promotes remineralization and lesion depth reduction that is significantly greater than zero, and (2) neither toothpaste is inferior to the other with respect to promoting the remineralization and inhibiting the development of carious lesions.

MATERIALS AND METHODS

This is a double-blind, randomized, crossover, single center, controlled in situ study to establish the equivalence of two children toothpaste formulations, containing either 10% HAP microclusters (crystallite size: length ≈ 80 nm (median) × width ≈ 30 nm (median)) or 500 ppm fluoride provided as amine fluoride (AMF), in inducing the remineralization and inhibiting the development of initial caries lesions. The primary outcomes to be examined were (1) the percentage remineralization and lesion depth reduction measured relative to the baseline mineral loss and lesion depth for initial caries, and (2) the amount of mineral loss and lesion depth for the sound enamel. The study was conducted at the clinical research facility of the University of Texas Health San Antonio (UTHSA) School of Dentistry. The UTHSA Institutional Review Board (IRB) approved the study (protocol #: HSC20180416H), and the study was registered with ClinicalTrials.gov (NCT03681340). The study was conducted in accordance with the ethical standards outlined in the 1964 Declaration of Helsinki and its later amendments, and in compliance with the International Conference on Harmonization (ICH) Good Clinical Practice Guidelines. The participants were recruited from among different ethnic origins and varied socio-economic status in the local San Antonio area, written informed consent was obtained from all participants prior to their participation in the study.

Participant recruitment

Fifty subjects aged from 18 to 60 years were given screening examination that included sialometry, medical/dental history, and oral examination (Fig. 1). Thirty two subjects qualified and were enrolled in the study. Inclusion criteria were age of 18 through 60 years; normal salivary function with unstimulated and stimulated salivary flow rates ≥0.2 ml/min and ≥0.7 ml/min, respectively, measured according to the Sreebny and Valdini[33] procedure. Other inclusion criteria were not taking any antibiotics or medications which could affect saliva flow rate; the presence of at least 20 natural uncrowned teeth (excluding third molars); a past history of dental caries but no clinically active caries; willing to wear the in situ appliance and use only assigned products for oral hygiene throughout the duration of the study; the ability to read and understand English; ability to provide informed consent; and no self-reported history of allergy to personal care/consumer products or any ingredient in the test products. Exclusion criteria were the presence of advanced periodontal disease or other oral pathology; medical condition that requires premedication prior to dental procedures; use of antibiotics one month prior to or during this study; self-reported pregnancy or breastfeeding; and use of tobacco products.

Creation of artificial initial caries and fabrication of the in situ appliance

Following consent from the donors, freshly extracted primary teeth were collected from the pediatric clinics of the UTHSA School of dentistry and stored in 0.1% thymol solution at 4 °C prior to use. The teeth were examined with a transilluminator, and thirty two teeth without caries, cracks, or enamel malformations were selected and cleaned with pumice using electronic toothbrush. Using a water-cooled diamond wire saw, 4 tooth blocks were produced from buccal and lingual surfaces of each of the selected teeth, with each block measuring ~2 mm length × 2 mm width × 1.5 mm thickness. Two of the 4 blocks were retained as sound enamel blocks for demineralization inhibition assessment while the other two blocks targeted for remineralization assessment had artificial initial caries produced in them as follows. All surfaces of each block were painted with two coats of acid resistant nail varnish except buccal or lingual on which an initial caries lesion was created by subjecting this exposed surface to 7 days demineralization in an acidified gel system (0.10 M lactic acid, 0.10 M sodium hydroxide, 6% $^{w}/_{v}$ hydroxyethyl cellulose, pH 4.5). Following lesion formation, the nail varnish was carefully removed with acetone. A tooth section (~150 µm thick) was cut from each tooth block for the measurement of the baseline mineral loss (Δz_1) and lesion depth (LD_1) of each produced initial caries lesion, and for selection of the suitable lesions for the remineralization assessment. The sections were processed for transverse microradiography (TMR) as follows. Both sides of the sections were polished using adhesive back lapping film in a MultiPrep™ Precision Polishing machine (Allied High Tech, USA) to achieve planoparallel surfaces, as well as reduce the thickness of the slice to 100 µm (the appropriate thickness for TMR). Following this, the sections were microradiographed on a type IA high resolution glass X-ray plate (Microchrome Technology, CA, USA) using a Phillips X-ray generator system set up for this purpose. The plates were exposed for 10 min at an anode voltage of 20 kV and a tube current of 10 mA, and then processed. Processing consisted of a 5 min development in Kodak HR developer and 15 min fixation in Kodak Rapid-fixer before a final 30 min wash period. After drying, the microradiographs were examined under a Leica DMR optical microscope linked via a Sony model XC-75CE CCTV camera to a personal computer. Using TMR2006 version 3.0.0.11 image analysis software (Inspektor Research Systems, Amsterdam, Netherlands), the enhanced image of the microradiographs were analyzed under standard conditions of light intensity and magnification along with the image of a step wedge as described by de Josselin de Jong et al.[34] 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, 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. It is pertinent to mention that the baseline measurements were not conducted for the sound tooth blocks to be used for demineralization-prevention study because the TMR does not measure the mineral density of sound tooth tissue rather the software uses the known mineral density of sound enamel or dentin to determine the amount of mineral loss in a demineralized tissue.

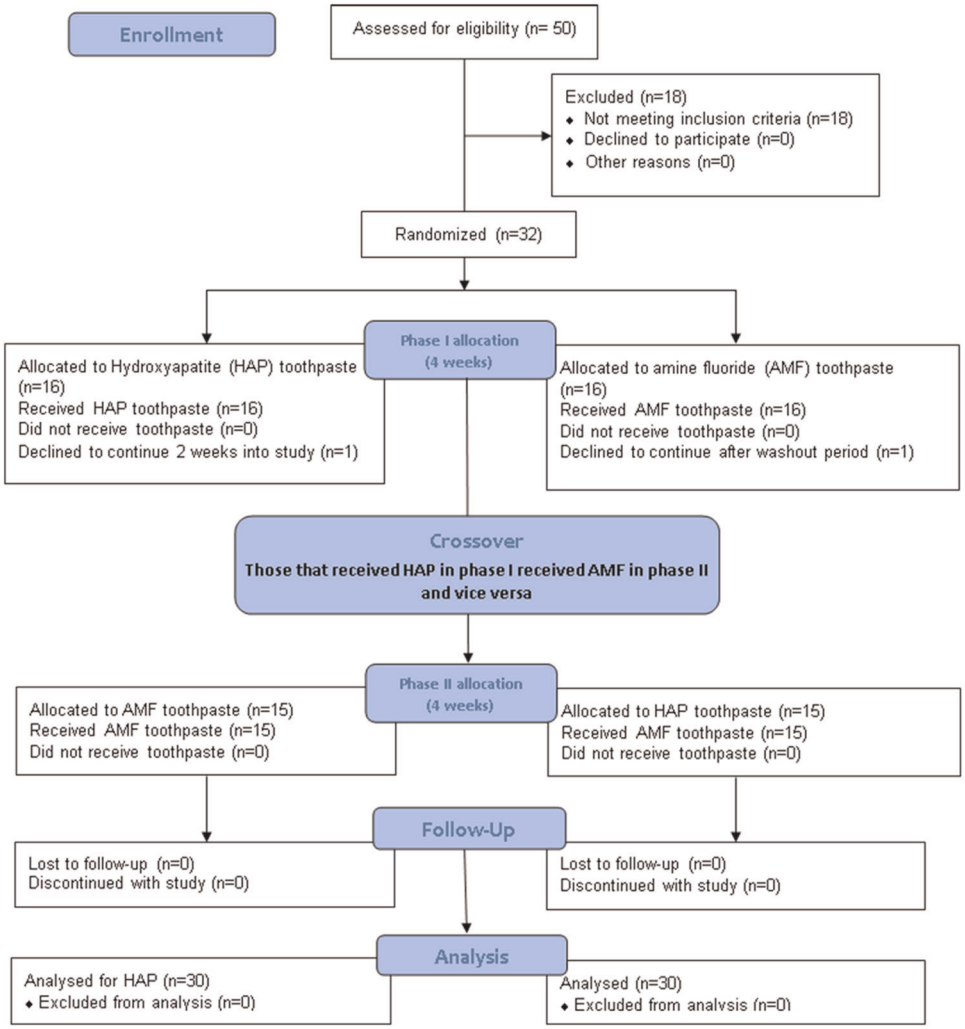

Fig. 1 Flow Diagram detailing the stepwise methodology. This is a crossover study so the 30 completers received the two intervention in a crossover design as phase I and II.

As stated above, the four tooth blocks from each tooth were distributed as follows**:** two lesion-bearing blocks for remineralization assessment and two sound blocks for demineralization inhibition assessment. These four blocks were used to fabricate the in situ appliances as follows. Each block was covered with polyester gauze (Bard Peripheral Vascular, Inc., Tempe, AZ, USA) and mounted within an in situ appliance, a customized orthodontic bracket.[27] The polyester gauze facilitated plaque retention on the surface of the tooth blocks on intra-oral exposure. The appliance consists of an orthodontic molar pad with retentive mesh backing (American Orthodontics Corp., Sheboygan, US), which has a ring of 0.7 mm orthodontic wire welded to it so that the ring closely encircles each test-block. The block was retained within the bracket with fluoride-free intermediate restorative material (IRM). All appliances were sterilized with gamma irradiation prior to delivery to the subject.

Study treatment

The study was performed in two distinct treatment phases during which subjects were exposed to one of the following two treatments in a randomized crossover design; (A) Karex Kid's toothpaste containing 10% HAP microclusters (Kinder Karex, Dr. Kurt Wolff GmbH and Co. KG, Bielefeld, Germany), and (B) Elmex Kid's toothpaste containing 500 ppm fluoride as AMF (Elmex Kinder zahnpasta, GABA GmbH, Hamburg, Germany). Each phase started with one week of washout period and then 4 weeks of treatment, consisting of two 2-week periods during which each subject used his/her assigned treatment under the following conditions. First 2-week period, with the subject wearing an in situ appliance with sound enamel block, and the second 2-week period, with the subject wearing an in situ appliance with lesion-bearing tooth block.

Subjects who satisfied enrollment criteria were given a specially manufactured washout toothpaste with neither fluoride nor HAP (Dr. Kurt Wolff GmbH and Co. KG, Bielefeld, Germany) and an adult soft-bristled toothbrush to use for a washout period of one week. The washout period allows for attenuation of any residual effect of the subject's previously used toothpaste. There was no washout period between individual 2-week treatment periods within each phase since the subjects used the same product for the 4 weeks. During the washout period, subjects were instructed to use the toothpaste and toothbrush for 2 min twice a day (morning after breakfast and night last thing before bed) in place of their normally used toothpaste and toothbrush, and as their only oral hygiene product. Subjects were given no restriction on dietary habit.

At the end of the washout period, patients returned to the clinic, and were assigned to a group to use either HAP or AMF by the Study Coordinator using randomization numbers generated by a computer program designed and operated by our

biostatistics team. However, to ensure that both the operators and the subjects were blinded as to product assignment, all toothpaste tubes were packaged identically and coded (A, B, or washout) by the manufacturing/packaging company, who retained the code until the completion of the study and data interpretation. Following randomization, the 4 block-bearing in situ appliances originating from one tooth were assigned to one subject. Then 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 bonded by a qualified dentist licensed in the state of Texas, who was different from the laboratory technician that process and analyze the samples to produce the final data. To bond the appliance, the buccal surface of the tooth chosen was carefully etched for 30 s, washed with water spray and dried for a further 30 s, and isolated using cotton rolls. The bottom of the appliance was loaded with Transbond™ XT light-cure adhesive paste (3M Unitek, Monrovia, CA, USA), and carefully positioned to avoid causing occlusal interference and to avoid soft tissue irritation. The excess composite material that spilled out from the sides of the appliance was used to cover the sides, beveling it to present a comfortable streamlined (non-catching) surface when the slab comes in contact with a soft tissue surface (e.g., tongue). The adhesive paste was cured using an Ortholux XT visible Light Curing Unit (3M Unitek, Monrovia, Ca, USA) applied for 20 s.

Following bonding of the appliance, each subject was given his/her respective test toothpaste and a soft-bristled toothbrush designed for use with orthodontic brackets with shorter bristles at the center to accommodate the bracket. Subjects were instructed to continue with the routine of brushing two times daily, morning after breakfast and last thing before bed, for 2 min before rinsing with only 10 ml of water. Subjects were also given special instruction on dispensing of the toothpaste, and were advised not to brush directly on the appliance but rather to brush around it to prevent disruption of the dental plaque on the surface of the tooth block. Subjects were restricted from eating nor drinking for at least 30 min after brushing. A timer and measuring cup were provided to each subject. As a method of monitoring compliance, a diary was provided to each subject for recording the time of each brushing episode, and in addition, toothpaste tubes were weighed at the time of randomization and at each study visit. Subjects were instructed to maintain their normal dietary habits and were prohibited from using any other oral hygiene product (e.g., mouthwash, chewing gum) or tooth-whitening product for the duration of the study. Immediately after bonding of the first appliance, each subject used the test product under the supervision of the Study Coordinator, and for the remainder of each treatment phase, subjects completed the procedure at home and as instructed by the Study Coordinator

Subjects returned to the clinical research facility after 2 weeks without using the product that morning, and the first appliance was detached and sent to the laboratory for analysis. The appliance for the second 2-week treatment period of the phase was bonded, the dairy checked, the toothpaste weighed, and safety evaluation performed. Upon completion of the second 2-week treatment period, the subject again returned to the clinic for detachment of the second appliance, and was given washout toothpaste and a soft-bristle toothbrush to undergo another 7-day washout period without an appliance in preparation for his/her phase 2 of the study. After completion of the washout period without an appliance, subjects return to the clinic, and the procedure of phase 1 was repeated until the second 2-week treatment period was completed, and each subject has gone through the two arms of the study.

Safety monitoring

At all visits, the dental examiner visually examines the oral cavity and peri-oral area, and this examination included an evaluation of the soft and hard palate, gingival mucosa, buccal mucosa, mucogingival fold areas, tongue, sublingual and submandibular areas, salivary glands, and the tonsillar and pharyngeal areas. In addition to oral examination, subjects were screened for adverse events using a questionnaire.

Post-treatment processing and study outcomes

Following intra-oral exposure, a tooth section (~150 µm thick) was cut from each tooth block, both sound and lesion-bearing blocks. The sections were processed for microradiography as described above for the control sections used for baseline data. Although the lesion-bearing control sections have been microradiographed and analyzed for selection of the appropriate lesions, they were microradiographed again together with the post-test sections and both analyzed together for quantification of the Δz and LD of the lesions as described for baseline sections. This step enabled both control and test sections from same block to be microradiographed and analyzed under the same conditions. For the lesion-bearing sections, this process yielded the pre-test mineral loss (Δz_1) and lesion depth (LD_1), the post-test mineral loss (Δz_2), and lesion depth (LD_2), and the pre-test and post-test microradiograms of the lesions. For the sections from sound tooth blocks, the process yielded the post-test mineral loss (Δz) and lesion depth (LD) if any lesion developed, and the microradiograms. Using the microradiograms, the pattern and the extent of remineralization produced within each lesion by each treatment arm was examined by comparing the pre-test and post-test images side-by-side. For each participant the post-treatment mineral loss was subtracted from the pre-treatment mineral loss, and then standardized across participants by dividing that difference by the pre-treatment mineral loss to obtain the % remineralization. The lesion depth pre-treatment and post-treatment was handled the same way to obtain the % lesion depth reduction. The two products were compared using the % remineralization and the % lesion depth reduction.

Power analysis and sample size calculation

The sample size calculations, which were based on a power analysis, were performed using nQuery Advisor software (Statistical Solutions, Cork, Ireland). Based on previous studies in which the mean % remineralization was equal to 30.3 with a standard deviation equal to 16.3,[27,35–37] and for the hypothesis that each of the two toothpaste formulations promotes remineralization and lesion depth reduction that is significantly greater than zero, an effective sample size of 30 subjects will have power greater than 0.95 with a 0.05 one-sided significance level to detect a difference between a hypothesis mean of zero and a sample mean % remineralization equal to or greater than 10% using a two-sided *t*-test of two independent means. However, 32 subjects were enrolled to make provision for 5% dropout.

Statistical analysis

For measurements by both mineral loss and lesion depth, three endpoints were analyzed. (1) The mean amount of remineralization and mean amount of lesion depth reduction were determined for Karex toothpaste as a percentage of pre-treatment mineral loss and pre-treatment lesion depth respectively, and these percentages were compared to a value of 0%, which is what would be expected if the toothpaste had no effect. The statistical test used was a one-sided *t*-test of one group mean. (2) In the same way, the mean amount of remineralization and mean amount of lesion depth reduction was determined for Elmex toothpaste, and also compared to 0%. (3) Finally, the primary endpoint was to compare the means of Karex toothpaste to Elmex toothpaste to check for non-inferiority/equivalence of the HAP toothpaste to the fluoride toothpaste, using the two-sided *t*-test of two independent means. Non-inferiority/equivalence was established if the difference between the two toothpaste formulations for any one measurement method was not

Table 1. Mean rates of remineralization (%) and lesion depth reduction (%) for each toothpaste.

Measurement	Karex	Elmex	*p*-value, two means
% Remineralization	55.8 (s.d. 13.8)	56.9 (s.d. 14.9)	0.81
p-value, One group:	<0.0001	<0.0001	
% Lesion depth reduction	27.1 (s.d. 10.6)	28.4 (s.d. 9.8)	0.68
p-value, One group:	<0.0001	<0.0001	

Table 2. Mean (Standard deviation) values of mineral loss (vol%μm) and lesion depth (μm) in the two study groups before and after treatment and their differences (with confidence intervals).

Treatment	Before treatment	After treatment	Difference (95% CI)	*P* value[a]
	ΔZ [Mean (SD)]			
Karex (10%HAP)	2357.5 (454.63)	1013.5 (273.59)	1344 (1119.93–1568.06)	<0.0001
Elmex (500 ppm)	2378.5 (593.16)	1009 (392.48)	1369.5 (1117.40–1621.59)	<0.0001
	LD [Mean (SD)]			
Karex (10%HAP)	92.89 (17.15)	67.07 (11.79)	25.82 (19.79–31.84)	<0.0001
Elmex (500 ppm)	91.91 (17.94)	65.46 (13.64)	26.44 (21.15–31.74)	<0.0001

[a]Paired *t*-test ($n = 30$, $\alpha = 0.05$)

regarded to be clinically relevant and was set to $\Delta \leq 20\%$. The statistical package R, version 3.5.0, was used for analysis.

RESULTS

Of the 32 subjects recruited into this study, 1 subject declined to participate further during the washout period, and one other subject declined to participate midway into the first 2-week treatment while wearing his first appliance that bears sound tooth block (Fig. 1). Thirty subjects (19 females, 11 males) with a mean (SD) age of 39.5 (15.0) years completed the study. The unstimulated and stimulated saliva flow rates of the subjects ranged from 0.2 to 1.5 ml/min and 0.9 to 3.5 ml/min respectively. The ethnic distribution of the subjects was as follows: Hispanic 17 (57%), Black (not Hispanic) 3 (10%), White (not Hispanic) 7 (23%), Asian 1 (3%), and others 2 (7%).

The mean rates of remineralization and lesion depth reduction are shown in Tables 1 and 2. Each toothpaste had a mean percent remineralization in excess of 50%, and a mean percent lesion depth reduction better than 25%. For both toothpastes, the mean % remineralization and mean % lesion depth reduction were statistically significantly greater than 0% ($p < 0.0001$). When compared against each other, there was no statistically significant difference in remineralization ($p = 0.81$), or in lesion depth reduction ($p = 0.68$). The 95% confidence interval of the difference between Karex™ (HAP) and Elmex (AMF) for remineralization was −8.8% to +6.5%, and the 95% confidence interval of the difference for lesion depth reduction was −6.8% to +4.1%. Therefore, in this study the HAP toothpaste is confirmed to be non-inferior to the fluoride toothpaste in effectiveness.

On analysis of the sound tooth blocks that examined the ability of the two toothpastes to inhibit demineralization of sound tooth surface, there was no evidence of demineralization in any of the tooth blocks following intra-oral exposure to either toothpaste (Fig. 2a, b) and (Fig. 3a, b). Critical examination of the microradiograms from the lesion-bearing samples exposed to HAP (Fig. 4a, b) and AMF (Fig. 5a, b) toothpastes in comparison with their respective control microradiograms, shows that while HAP induced a more homogenous remineralization distributed throughout the entire thickness of the subsurface lesion (Fig. 4b), the remineralization induced by AMF was denser in the out half (surface zone) of the lesion (lesion surface lamination) i.e., two zones of contrasting density can clearly be observed (Fig. 5b). These remineralization patterns in Fig. 4b and 5b were consistent in all specimens exposed to HAP and AMF respectively. There were no incidences of adverse effects reported by subjects or ascertained clinically.

DISCUSSION

Despite being a preventable disease and amid the presence of fluoride in oral dentifrices, dental caries prevalence in children continues to increase globally,[1] and at a faster rate among children from low socioeconomic backgrounds.[38] This indicates that although fluoride interventions have the highest level of supporting evidence as having the most consistent benefit in preventing caries and remineralizing initial caries lesions;[6–8] additional remineralizing and preventive agents are often needed to enhance fluoride effects in high caries risk individuals.[9,10] One may suggest increasing the dose of fluoride since its effectiveness is dose-dependent,[7] unfortunately, there is a limit to fluoride dose allowed in oral care products to avoid the risk of fluorosis in children[17] and toxicity in all ages.[18,19] The fluoride dose recommended for toddlers and children is even lower than the regulatory 1000–1500 ppm fluoride concentration in non-prescription toothpastes, which is probably suboptimal for effective remineralization of initial lesions; thus the effect of dose limitation on fluoride effectiveness may be more pronounced in toddlers and children below 6 years. Besides safety concerns, higher fluoride dose in remineralization materials results to surface-zone remineralization at the expense of the lesion body, thus preventing fuller and homogenous remineralization of the lesion.[15,16] It is envisaged that an agent as effective as fluoride, but can permit increasing dosage for increase effectiveness without safety concerns, may offer a more effective choice, especially for children. Hydroxyapatite, a bioactive and biocompatible material with wide applications in both medicine (e.g., bone substitute) and dentistry, is currently used in nanocrystaline or microcluster forms in toothpaste and mouthrinses in varying concentrations for caries prevention and remineralization.[23–25] The equivalence (non-inferiority) of 10% HAP in microcluster forms to 500 ppm fluoride provided as AMF in remineralization of initial caries lesion and

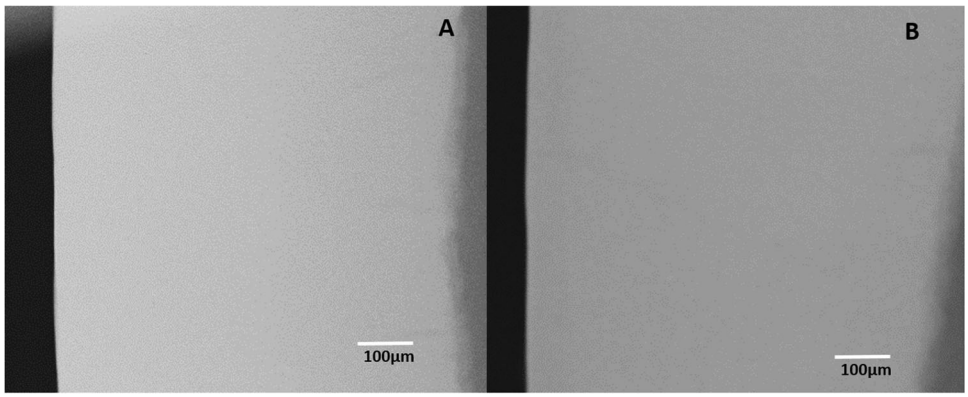

Fig. 2 Representative microradiographic images of sound tooth tissue before (**a**) and after (**b**) intra-oral exposure for demineralization while the research subject is using Karex Kid's toothpaste (10% HAP microclusters).

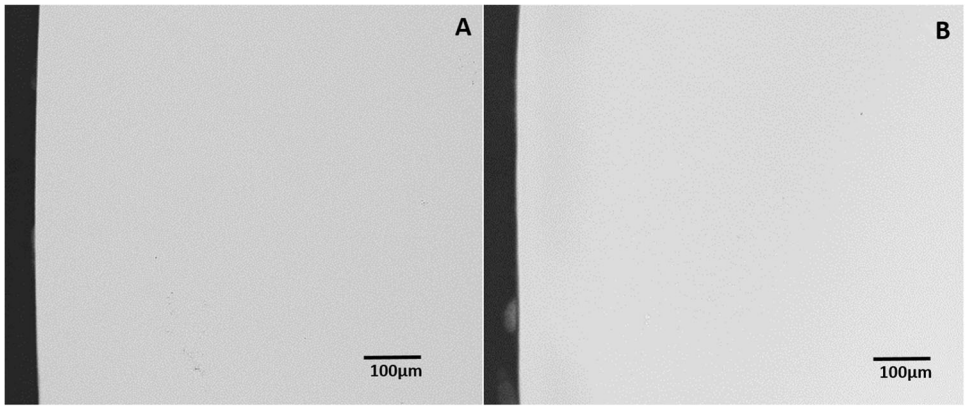

Fig. 3 Representative microradiographic images of sound tooth tissue before (**a**) and after (**b**) intra-oral exposure for demineralization while the research subject is using Elmex Kid's toothpaste (500 ppm fluoride as AMF).

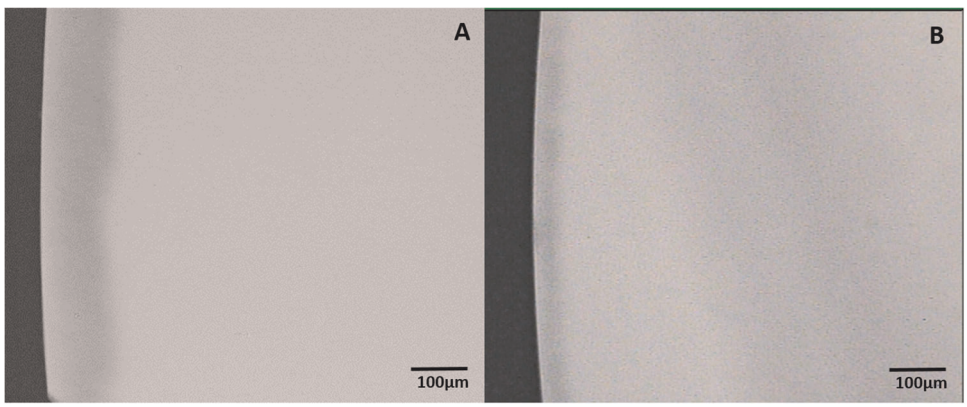

Fig. 4 Representative microradiographic images of enamel subsurface lesions (Initial caries lesions), before (**a**) and after (**b**) in situ remineralization via treatment with Karex Kid's toothpaste (10% HAP microclusters).

inhibition of sound enamel demineralization was investigated in this study. The findings of the present in situ study accepted the two hypotheses that each of the two toothpaste formulations promotes remineralization and lesion depth reduction that is significantly greater than zero, and that neither toothpaste is inferior to the other with respect to promoting the remineralization and inhibiting the development of initial caries lesions. Although this study was conducted with human primary teeth, similar result was observed in a previous in situ study that used human permanent teeth.[27] A randomized controlled clinical trial in children and adolescents at high caries risk undergoing orthodontic treatment also reported similar results and non-inferiority of microcrystalline HAP to 1400 ppm fluoride provided as AMF and stannous fluoride.[32] Effectiveness of AMF in these reports and this study is in agreement with the long-established fact that fluoride in varying concentrations are effective in preventing caries development and remineralizing initial caries,[9,39,40] and that the various fluoride salts were equally effective.[41] Furthermore, Hellwig et al.[42] in an in situ study demonstrated the remineralization of initial caries lesions of

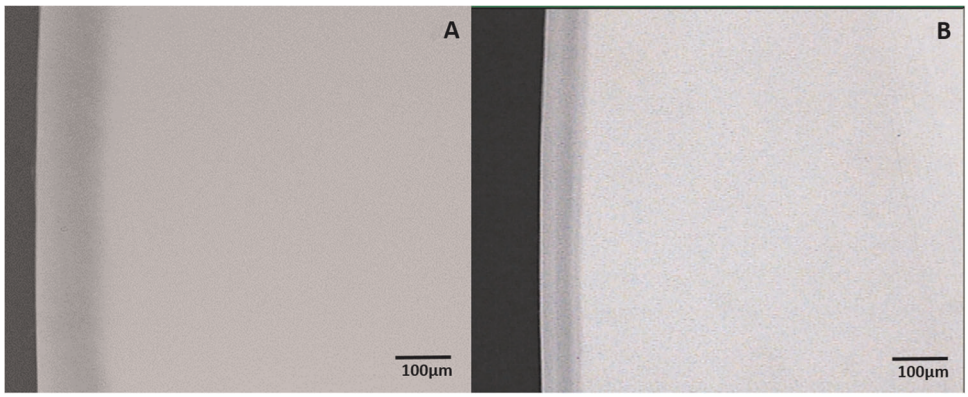

Fig. 5 Representative microradiographic images of enamel subsurface lesions (Initial caries lesions), before (**a**) and after (**b**) in situ remineralization via treatment with Elmex Kid's toothpaste (500 ppm fluoride as AMF).

permanent teeth, and concluded that remineralization of primary teeth with fluorides may be possible in the same way as the permanent teeth. The effectiveness of the HAP toothpaste in this study is in agreement with previous clinical and in vitro studies, and it is not surprising considering the various mechanisms through which HAP has been demonstrated to effect remineralization of initial caries.[25,27,28,32,43–47] Based on its strong affinity and adsorption to tooth surfaces,[24,26,43] HAP has been shown to induce remineralization of initial caries lesions by directly filling micropores in demineralized tooth surfaces,[26,48] where it acts as a crystal nucleus, and promotes crystal deposition and growth by continuously attracting large amounts of calcium and phosphate ions from the surrounding remineralization solution.[48,49]

The absence of any evidence of demineralization in all the sound tooth blocks following intra-oral exposure to either HAP or AMF toothpaste further demonstrated the inhibition of demineralization by both toothpastes. The caries prevention potential of HAP, which has been established in previous studies,[31,32] has been shown to be based on multiple mechanisms. HAP in toothpaste has been reported to elevate calcium and phosphate ions concentrations in saliva, plaque and tooth surfaces;[50] thus acting as a calcium and phosphate reservoir, helping to maintain a topical state of supersaturation of these ions with respect to tooth minerals.[43,51] The discussed mechanism must have applied in this study considering that the surface of the tooth blocks was covered with polyesther gauze, which encouraged and maintained plaque accumulation over the tooth surface, providing a nest for the accumulations of the mineral ions. The high potential of HAP to adsorb to bacterial cell wall has been shown to facilitate an antibiofilm effect by inducing coaggregation of bacteria within the HAP particles,[46] thus aiding biofilm removal from the tooth surfaces,[52,53] and hindering oral biofilm formation.[29,46,47,54] Again, this mechanism may have contributed the present findings by limiting the virulence of the biofilm on the surface of the tooth blocks.

The findings in this study further confirmed the surface zone remineralization by the fluoride agents.[13,14,55] Two zones of contrasting density can clearly be seen in Fig. 5b, showing the remineralization induced by AMF to be denser in the out half of the lesion (surface zone). Observation of such surface zone remineralization with only 500 ppm fluoride present in the toothpaste used in this study actually demonstrated that this 'lesion lamination' effect is not limited to materials with high fluoride concentration, rather it is based on the established fact that fluoride remineralization of initial lesions, irrespective of the concentration, is most effective at the outer 30 µm of the lesion.[13,14,55] This is supported by the findings of a previous study, which reported that higher fluoride concentrations did not produce any further significant increase in remineralization, rather laminations (surface zone remineralization) were apparent in lesions subjected to the 250-ppm and 500-ppm F^- solutions.[55] Another in situ study demonstrated no significant difference in the effectiveness of 500, 1000, and 1500 ppm fluoride in remineralizing initial caries in primary teeth,[42] further confirming the effect of lesion lamination. The findings of these previous studies and the lesion lamination effect demonstrate that the dose-dependent effect of fluoride effectiveness that reflects as increased effect of high fluoride toothpaste[41] or addition of further fluoride sources,[56] has limit at which it plateaus and further increase may not improve the effectiveness. In contrast to the effect of AMF, Fig. 4b shows that HAP induced a more homogenous remineralization distributed throughout the entire thickness of the subsurface lesion, and this may indicate that increasing the dose of HAP or continued usage of the toothpaste may result to increased remineralization of the lesion, and ultimately lead to complete or fuller remineralization of the initial lesion.

Based on above discussions one may suggest that HAP-containing toothpaste may be a better choice for children and individuals at high caries risk since the dosage can be increased to obtain higher efficacy without any safety issue such as the risk of fluorosis in children associated with high fluoride dose. Furthermore, the use of HAP in oral care products may eliminate the need of combining fluoride and antimicrobials in a dentifrice, as well as having different dosages for infants, children and adults. It is logical but scientific that since the remineralizing efficacy of topical fluorides is strictly dependent on the availability of calcium and phosphate ions, HAP dentifrices may be a more effective for xerostomic patients with diminished amounts of saliva. This may need to be confirm through a clinical trial on patients suffering from xerostomia.

In this study, there was no incidences of adverse effects reported by subjects or ascertained clinically. Previous clinical studies observed similar findings, and reported there is no safety issue with HAP in oral care products.[27,32,57] The fact that these studies investigated varying doses of HAP in toothpastes, and none reported any safety issue, confirms that the dose of HAP in oral care dentifrices can be safely increased for an increase effectiveness when necessary.

CONCLUSION

- This study confirmed hydroxyapatite toothpaste is equivalent or non-inferior to the fluoride toothpaste with respect to remineralization of initial caries lesions and prevention of carious lesion development.

- Future research should be large multicenter clinical trials to further establish the effectiveness of HAP dentifrices and its equivalence to fluoride.

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ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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Enamel and dentine remineralization by nano-hydroxyapative toothpastes

Peter Tschoppe [a], Daniela L. Zandim [a], Peter Martus [b], Andrej M. Kielbassa [a,c,*]

[a] Department of Operative Dentistry and Periodontology, University School of Dental Medicine, CharitéCentrum 3, Charité - Universitätsmedizin Berlin, Aßmannshauser Strasse 4-6, D-14197 Berlin, Germany

[b] Department of Biometry and Clinical Epidemiology, CharitéCentrum 4, Charité - Universitätsmedizin Berlin, Hindenburgdamm 30, D-12203 Berlin, Germany

[c] Centre for Operative Dentistry and Periodontology, University of Dental Medicine and Oral Health, Danube Private University (DPU), Steiner Landstraße 124, A-3500 Krems, Austria

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Abstract

Objectives: This *in vitro* study evaluated the effects of nano-hydroxyapatite (n-HAp) tooth-pastes on remineralization of bovine enamel and dentine subsurface lesions.

Methods: Specimens were demineralized, randomly divided into five groups, and exposed to an aqueous remineralizing solution for two and five weeks (37 °C). Brushing procedures were performed with the respective toothpaste/storage solution slurry twice daily (2 × 5 s; total contact time of the slurries 2 × 120 s/d): storage in remineralizing solution only (0); additional brushing with B (20 wt% zinc carbonate nano-hydroxyapatite, $ZnCO_3$/n-HAp); BS (24 wt% $ZnCO_3$/n-HAp); E (0.14 wt% amine fluoride); or A (7 wt% pure n-HAp). Differences in mineral loss (ΔΔZ) before and after storage/treatment were microradiographically evaluated. *Results:* Dentine groups 0, B, BS, and A showed significantly higher ΔΔZ values compared to E ($p < 0.05$; ANOVA). Enamel ΔΔZ values of group A were significantly higher compared to group E ($p < 0.05$), whilst no significant differences of these groups could be observed compared to 0, B, and BS ($p > 0.05$). *Conclusions:* With the *in vitro* conditions chosen, toothpastes containing n-HAp revealed higher remineralizing effects compared to amine fluoride toothpastes with bovine dentine, and comparable trends were obtained for enamel.

1. Introduction

The process of de- and remineralization is governed by the degree of saturation of oral fluids (saliva and plaque) with respect to apatite minerals.[1] Given an appropriate change in conditions, remineralization may become the predominant process, thus leading to lesion repair.[2,3] To enhance lesion remineralization, increase of calcium or fluoride concentrations in the oral fluids would seem reasonable.[4]

For this purpose, fluorides have traditionally been used in various formulations, and the concomitant cariostatic mechanisms can be explained by an increased driving force for fluoridated apatites.[5] The decline in dental caries experienced in most industrialized countries can be attributed largely to the widespread use of fluorides,[6] and this preventive effect is mainly due to the formation of calcium fluoride-like precipitates hampering demineralization, whilst fluoride levels needed for remineralization are assumed to be higher than those to prevent lesion formation.[7]

* *Corresponding author*. Tel.: +43 2732 70478; fax: +43 2732 70478 7060.
E-mail address: andrej.kielbassa@dp-uni.ac.at (A.M. Kielbassa).

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Nano-hydroxyapatite (n-HAp) is considered one of the most biocompatible and bioactive materials, and has gained wide acceptance in medicine and dentistry in recent years.[8] Whilst former attempts to use hydroxyapatites clinically did not succeed, synthesis of nano-scaled zinc carbonate hydroxyapatite ($ZnCO_3$/n-HAp) yielded a significant progress, and showed considerable affinity to the enamel surface.[9] Nano-sized particles have similarity to the apatite crystals of tooth enamel in morphology and crystal structure.[10] Recently, a few reports have shown that n-HAp has some potential to repair dental enamel,[11–15] but no information is available for established dentine lesions. To date, it can be summarized that for remineralization of subsurface lesions by n-HAp containing products, different formulations have been developed, and early data have suggested remineralizing properties.[8] However, evidence is still incomplete to substantiate claims by manufacturers,[16,17] and, so far, none of these products has been shown to be more effective than fluorides.

Therefore, the aim of the present study was to evaluate the effects of daily treatment with different n-HAp toothpastes on the remineralization of bovine enamel and dentine subsurface lesions stored in a remineralizing solution. An amine fluoride toothpaste was used as a reference for comparative reasons. We hypothesized (H_0) that additional brushing with n-HAp or fluoride toothpastes would result in equal remineralizing effects compared to a pure remineralizing solution (positive control). This null hypothesis was tested against the alternative hypothesis of a difference between products.

2. Materials and methods

2.1. Specimen preparation and demineralization

From 35 bovine incisors 70 enamel specimens ($6 \times 4 \times 4$ mm^3) were prepared from the labial aspects. Dentine specimens ($n = 85$) derived from the cervical regions ($4 \times 3 \times 4$ mm^3), and were prepared as described previously.[18] One quarter of each specimen's surface was covered with acid-resistant nail varnish (Jet-Set; Loreal, Karlsruhe, Germany) to serve as sound control. Following earlier studies, enamel lesions were prepared by immersion in a solution (5 l) containing 6 μM methylhydroxydiphosphonate (MHDP), 3 mM calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), 3 mM potassium dihydrogen phosphate (KH_2PO_4), and 50 mM acetic acid (CH_3COOH) (Merck, Darmstadt, Germany) at pH 4.95 in an incubator (37 °C; BR 6000; Heraeus Kulzer) for 14 days.[19] Dentine lesions were prepared by immersion in a solution containing 0.0476 mM sodium fluoride (NaF), 2.2 mM calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), 2.2 mM potassium dihydrogen phosphate (KH_2PO_4), 50 mM acetic acid (CH_3COOH), and 10 mM potassium hydroxide (KOH) (all chemicals from Merck) at pH 5.0 (37 °C) for five days.[20] The solutions were not stirred or replaced during the demineralization period. The pH values of the demineralizing solutions were monitored daily (pH-electrode GE 100 BNC connected to pH-meter GMH 3510; Greisinger, Regenstauf, Germany), and slight elevations were corrected with small amounts of hydrochloric acid (HCl) to maintain a constant pH value between 4.94 and 4.96 for enamel as well as 4.99 and 5.01 for dentine during the demineralization period. Standard buffer solutions (Sigma–Aldrich, Steinheim, Germany) with nominal pH values of 4.0 and 7.0, respectively, and with an accuracy of 0.01 units were used to calibrate the pH metre.

2.2. Solution preparation and treatment of the specimens

Subsequently, half of each demineralized surface was covered with nail varnish (control of baseline demineralization) again. Specimens were randomly divided into five groups (enamel $n = 14$; dentine $n = 17$), and were separately stored in a remineralizing solution[21,22] for two and five weeks (37 °C). In accordance with EN ISO 11609 (European standards for preparing artificial saliva/toothpaste slurries), the respective toothpaste (Table 1) was diluted in three parts (1:3) of the remineralizing solution to obtain a homogeneous slurry. Test products were commercially available toothpastes with either $ZnCO_3$/n-HAp or n-HAp (all without any fluorides) as active ingredients; a toothpaste containing amine fluorides served as reference group (Table 1). The pH values of the slurries were measured directly after preparation.

Subsequently, specimens were manually brushed by hand with a soft toothbrush (Meridol; GABA, Lörrach, Germany), and with minimum pressure; brushing procedures were carried out in each subgroup twice daily for 5 s each (with an additional contact time with the slurry of 115 s, thus resulting in a total contact time of 120 s). After each brushing treatment, specimens were washed with deionized water (10 s). Every two

Table 1 – Treatment products, regimes and specimen grouping.

Treatment products	Code	Active compound	Concentration	Treatment	pH
(Remineralizing solution)	0	Calcium and phosphate	1.5 mM	Only storage no further treatment	7.00
$ZnCO_3$/n-HAp[a] batch no. 928751019	B	Zinc carbonate-nano-hydroxyapatite	20 wt%	Slurry (ratio 1:3) from toothpaste with the remineralizing storage solution and brushing for 5 s with a total contact time of 120 s twice daily in all groups	7.39
$ZnCO_3$/n-HAp[a] batch no. 90001091_26-01-2010	BS		24 wt%		7.34
Fluoride[b] batch no. 435909	E	Aminefluoride	0.14 wt%		5.24
n-HA[c] batch no. 20.10.11	A	Nano-hydroxyapatite	7 wt%		6.94

[a] BioRepair and BioRepair Sensitive; Dr. Kurt Wolff, Bielefeld, Germany. [b] Elmex Kariesschutz; GABA, Lörrach, Germany.
[c] ApaCare; Cumdente, Tübingen, Germany.

days the remineralizing solutions were replenished (250 ml per group each time), and pH values were checked. After two weeks, half of the exposed surfaces were nail-varnished to evaluate interim effects (effect after two weeks).

2.3. Transverse microradiography

After *in vitro* exposure, thin sections (100 μm) were prepared. Following, contact microradiographs of the specimens were obtained by transverse microradiography, and these were evaluated using a dedicated software (TMR for Windows 2.0.27.2; Inspektor Research System, Amsterdam, The Netherlands) as described previously;[23,24] ethylene glycol ($C_2H_4(OH)_2$) (99%; Sigma–Aldrich, Munich, Germany) was used to avoid shrinkage of dentine lesions.[25] The investigator was blinded with respect to group allocation.

Mineral density profiles were evaluated from which integrated mineral loss (ΔZ) and lesion depth (LD) values were calculated following initial demineralization (ΔZ_{Demin}, LD_{Demin}) and after treatment for either two ($\Delta Z_{Effect\ 2}$, $LD_{Effect\ 2}$) or five weeks ($\Delta Z_{Effect\ 5}$, $LD_{Effect\ 5}$). Each pair of values was corrected by subtracting the respective values for sound enamel (ΔZ_{Sound} and LD_{Sound}) before data analysis. Changes in mineral loss ($\Delta\Delta Z_2 = \Delta Z_{Demin} - \Delta Z_{Effect\ 2}$, $\Delta\Delta Z_5 = \Delta Z_{Demin} - \Delta''' Z_{Effect\ 5}$) and lesion depth ($\Delta LD_2 = LD_{Demin} - LD_{Effect\ 2}$, $\Delta LD_5 = LD_{Demin} - LD_{Effect\ 5}$) were analyzed for treatment differences. Positive and negative values of ΔΔZ or ΔLD indicated net remineralization and net demineralization, respectively.

2.4. Statistical analyses

Normal distribution of ΔΔZ and ΔLD was tested (Kolmogorov–Smirnov). For overall comparison of solutions one-way ANOVA was applied; pairwise comparisons used Tukey's *post hoc* tests. Comparisons of changes in mineral loss and lesion depth before and after storage/treatment were performed by adjusted paired *t*-test (Bonferroni; correction factor ×5). Level of significance was set at $\alpha = 0.05$ (two-sided). Statistical analyses were performed using PASW for Windows (version 18.0; SPSS, Chicago, IL).

3. Results

Thirteen enamel and two dentine specimens were lost with preparation procedures. All de- and remineralized specimens developed subsurface lesions consistently revealing a surface layer that was more mineralized than the body of the lesion, and none of the treatment regimens yielded surface erosions. Baseline ΔZ_{Demin} and LD_{Demin} values (after demineralization) did not differ significantly between the various groups ($p > 0.161$; ANOVA). With dentine, specimens of group E revealed a hypermineralized surface layer (with an increased thickness), and subsurface lesions could be found with all groups (Fig. 1). The pH values of the remineralizing solutions remained stable for the experimental period.

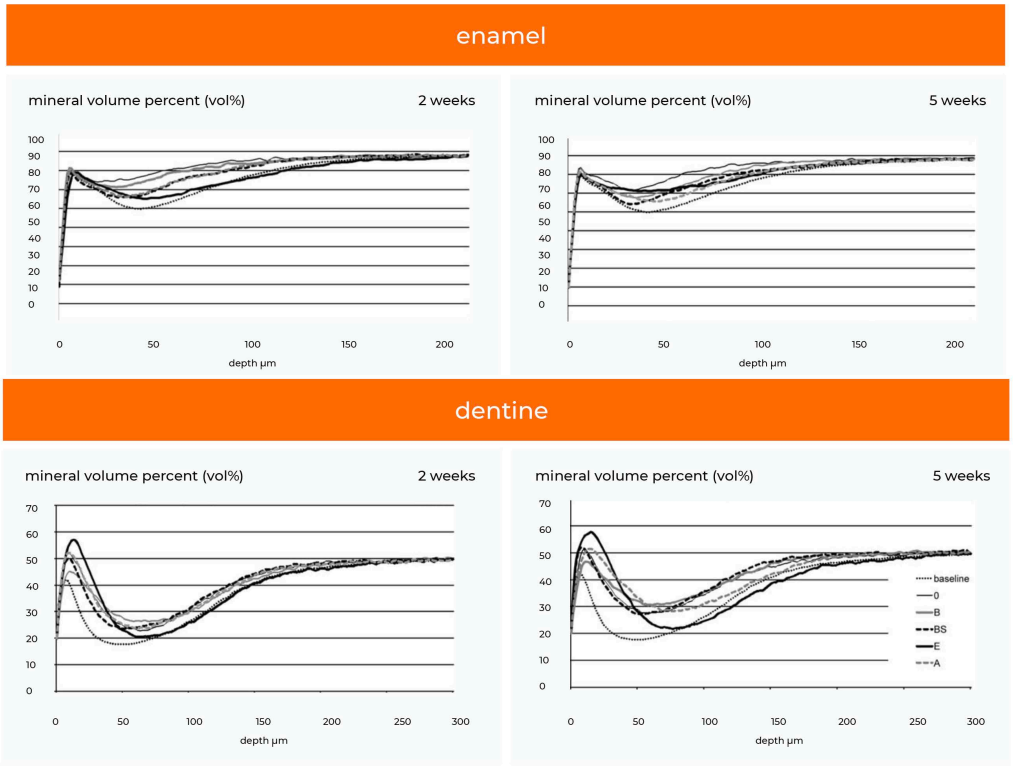

Fig. 1 – Mean mineral density profiles (enamel and dentine) after two and five weeks with or without additional toothpaste treatment (0 = no further treatment; B = $ZnCO_3$/n-HAp 20 wt%; BS = $ZnCO_3$/n-HAp 24 wt%; E = amine fluoride 0.14 wt%; A = n-HA 7 wt%) compared to baseline. Lesion bodies and surface layers of baseline lesions consistently remineralized; hypermineralization of dentine surface layer occurred with group E, but without any decrease of lesion depths.

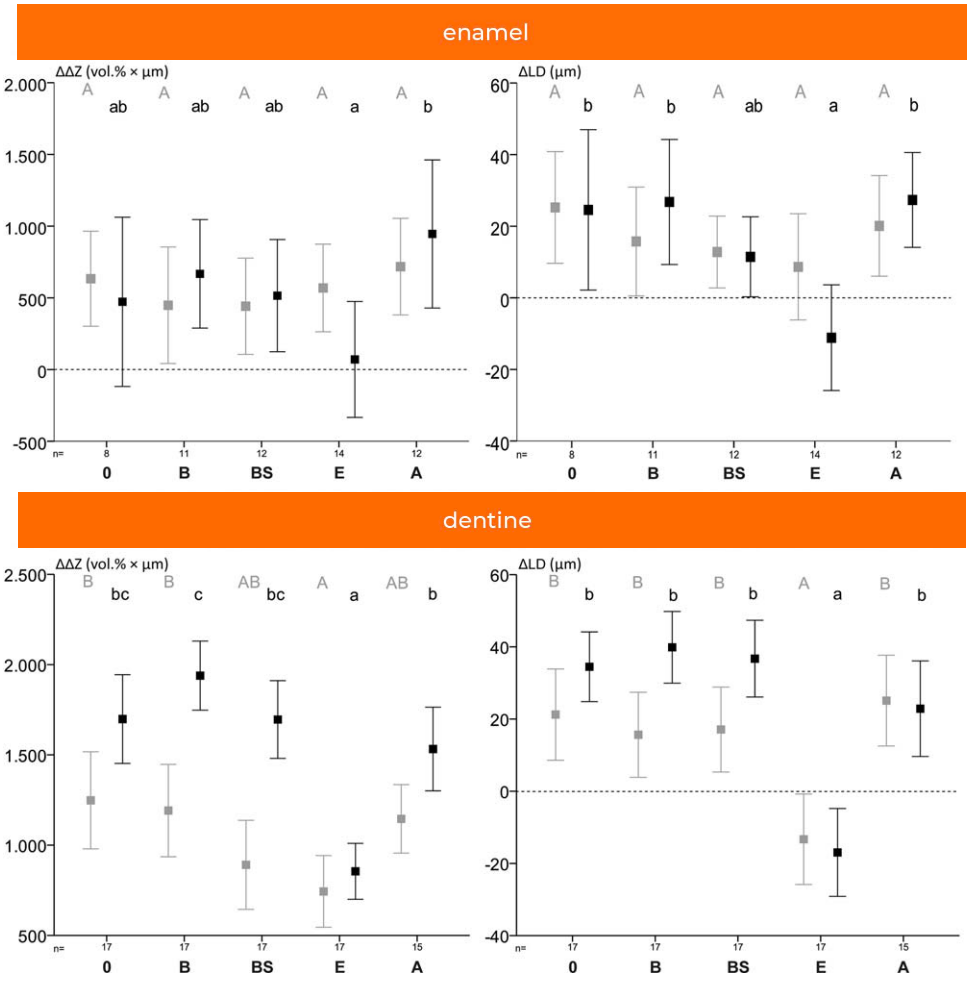

Fig. 2 – Means and confidence intervals (95%; enamel and dentine) of differences in mineral change (DDZ; vol% T mm) and lesion depth (DLD; mm) after two (grey) and five weeks (black) storage/treatment (0 = only storage and no further treatment; B = ZnCO3/n-HAp 20 wt%; BS = ZnCO3/n-HAp 24 wt%; E = amine fluoride 0.14 wt%; A = n-HA 7 wt%). Different letters indicate significant differences between groups within each storage/treatment period (p < 0.05; ANOVA, Tukey post hoc test).

Enamel $\Delta\Delta Z_{Effect\ 2}$ and $\Delta LD_{Effect\ 2}$ values did not differ significantly between groups ($p > 0.705$; ANOVA, Tukey; Fig. 2). $\Delta\Delta Z_{Effect\ 5}$ values of group A were significantly higher compared to group E ($p = 0.017$), whilst no significant differences of both groups could be observed compared to 0, B, and BS ($p > 0.221$). Comparable results were evaluated for lesion depths after both periods. With dentine, significantly higher $\Delta\Delta Z_{Effect\ 2}$ values could be observed for groups 0 and B compared to E ($p < 0.05$), whilst no differences could be seen compared to BS and A ($p > 0.101$). Groups 0, B, BS, and A showed significantly higher $\Delta\Delta Z_{Effect\ 5}$ and $\Delta LD_{Effect\ 2}/\Delta LD_{Effect\ 5}$ values compared to E ($p < 0.05$).

Enamel groups 0, E, and A showed significantly decreased $\Delta Z_{Effect\ 2}$ values compared with baseline demineralization ($p < 0.05$; adjusted *t*-test, Table 2); B and A significantly decreased $\Delta Z_{Effect\ 5}$ values ($p < 0.05$). Comparable $LD_{Effect\ 2}/LD_{Effect\ 5}$ values were observed. All dentine specimens revealed significantly decreased $\Delta Z_{Effect\ 2}/\Delta Z_{Effect\ 5}$ values if compared with baseline ($p < 0.05$). $LD_{Effect\ 5}$ values of groups 0, B, BS, and A decreased significantly compared with baseline ($p < 0.05$), whereas values increased for E ($p < 0.05$).

4. Discussion

The present *in vitro* study mainly showed that the different nano-hydroxyapatite toothpastes exert similar capacities to remineralize enamel and dentine subsurface lesions. Furthermore, the fluoride toothpaste displayed the lowest remineralizing effects on both hard tissues, along with an increase in lesion depths. Thus, the null hypothesis (stating that additional brushing with n-HAp or fluoride toothpastes would not result in significantly different remineralizing effects compared to control) was partially rejected.

Rationales for using bovine enamel and dentine specimens have been discussed previously,[26] and this source represents an accepted substitute for human dental hard substances.[27–29] Furthermore, several individual factors could have potential impact on remineralization (*e.g.*, behavioural changes, activity of the lesion, depth of the lesion, diet, stimulation of salivary flow, antibacterial and plaque removal strategies, brushing with fluoride toothpaste),[3,30,31] and these factors may modulate the natural process of lesion arrest (or repair).

Table 2 – Means with confidence intervals (CI 95%) of mineral losses (DZ; vol% T mm) and lesion depths (LD; mm) of enamel and dentine specimens after in vitro demineralization (DZDemin, LDDemin) and storage/treatment for two (DZEffect 2, LDEffect 2) and five weeks (DZEffect 5, LDEffect 5).

Enamel

Code	Mineral loss (vol% × μm)							
	ΔZ_{Demin}		$\Delta Z_{Effect\ 2}$			$\Delta Z_{Effect\ 5}$		
	Mean	CI 95%	Mean	CI 95%	*p*	Mean	CI 95%	*p*
0	1288	942;1633	655	420;889	0.015	816	410;1221	1.000
B	1572	1014;2131	1124	705;1543	0.170	905	309;1502	0.015
BS	1848	1236;2460	1407	623;2191	0.075	1333	838;1828	0.070
E	1633	1317;1948	1064	625;1503	0.005	1563	994;2131	1.000
A	2147	1547;2746	1429	817;2042	0.005	1202	737;1666	0.010

Code	Lesion depth (μm)							
	LD_{Demin}		$LD_{Effect\ 2}$			$LD_{Effect\ 5}$		
	Mean	CI 95%	Mean	CI 95%	*p*	Mean	CI 95%	*p*
0	83	67;97	58	45;71	0.030	58	39;78	0.180
B	86	70;102	70	52;88	0.220	59	40;78	0.035
BS	90	75;104	77	57;97	0.085	78	61;95	0.230
E	87	77;97	79	61;96	1.000	99	79;118	0.635
A	102	91;114	82	62;102	0.045	75	61;89	0.005

Dentine

Code	Mineral loss (vol% × μm)							
	ΔZ_{Demin}		$\Delta Z_{Effect\ 2}$			$\Delta Z_{Effect\ 5}$		
	Mean	CI 95%	Mean	CI 95%	*p*	Mean	CI 95%	*p*
0	3916	3540;4291	2667	2400;2935	0.0005	2217	1923;2511	0.0005
B	3919	3632;4205	2727	2448;3007	0.0005	1980	1739;2220	0.0005
BS	3708	3539;3878	2818	2579;3056	0.0005	2013	1802;2224	0.0005
E	3888	3605;4172	3145	2876;3413	0.0005	3033	2751;3316	0.0005
A	3870	3533;4207	2724	2465;2983	0.0005	2337	1961;2713	0.0005

Code	Lesion depth (μm)							
	LD_{Demin}		$LD_{Effect\ 2}$			$LD_{Effect\ 5}$		
	Mean	CI 95%	Mean	CI 95%	*p*	Mean	CI 95%	*p*
0	197	180;214	176	162;189	0.015	162	144;180	0.0005
B	191	181;201	176	162;189	0.065	151	140;163	0.0005
BS	185	172;198	168	152;184	0.035	149	140;157	0.0005
E	193	179;206	206	193;219	0.195	210	195;224	0.045
A	199	182;215	173	160;187	0.005	176	153;198	0.010

p-Values of differences between the values after demineralization and storage/treatment for either two or five weeks within each group as analyzed by adjusted paired *t*-tests (Bonferroni correction factor ×5) are given. Pairs differing significantly are highlighted (grey: demineralization; black: remineralization). Treatment code: 0 = only storage and no further treatment; B = $ZnCO_3$/n-HAp 20 wt%; BS = $ZnCO_3$/n-HAp 24 wt%; E = amine fluoride 0.14 wt%; A = n-HA 7 wt%.

The present set-up used abraded and polished specimens; a recent study reported that the *in vitro* demineralization pattern of unabraded samples more closely resembles the pattern of a natural white spot lesions. However, the inter-sample variation was greater than with abraded specimens,[31] and, therefore, we used abraded specimens for standardization reasons.[27] The current brushing procedure was accomplished by brushing the specimens with toothpaste/remineralizing solution slurry for 5 s (with 120 s total contact time of the slurry) twice daily. The specimens were manually brushed without any considerable force by the same operator. Indeed, this should not be considered as a completely standardized procedure (*i.e.*, using a brushing machine), even if slightly differing forces should have averaged during the study period.

Some specimens were lost during preparation for TMR. Main problems were surface losses due to sawing or polishing, and these brittle specimens were not suitable for further investigation. In some cases, thin section preparation could be repeated, but this procedure was limited, due to the small dimensions of the specimens. This problem can be only avoided by using non-destructive techniques (like T-WIM).[32] However, due to the surface misalignment in the outer ~15 μm, this method was not considered useful for the current experimental set-up.

In a clinical setting, toothpaste will be diluted, and this strongly depends on individual salivary secretion;[33] with the present experimental set-up, one part of toothpaste was dissolved in three parts (1:3) of remineralizing solution to obtain a homogeneous slurry. A major factor of the de- and reminer-

alization equilibrium of enamel is the ambient pH. For slightly acidic fluoride toothpaste slurries with a pH between 4.5 and 5.1, increased remineralization could be observed compared to higher pH values;[34] a pH of 5.24 was evaluated with the fluoride toothpaste (according to the manufacturer, the pH is 4.6 for 10% in water). However, only pH values higher than 5.5 have been assumed to promote lesion arrest and to facilitate remineralization.[30] In contrast, pH values of n-HAp toothpastes slurries have not been studied up to now, and it might be speculated that the higher pH values of the n-HAp slurries increased remineralization. Recently, for calcium phosphate based solutions a higher mineral gain could be observed with a pH of 6.5 compared to 5.5.[19] Moreover, a (constant) remineralization model was used to evaluate the effects of the different toothpastes (n-HAp or $ZnCO_3$/n-HAp versus fluoride). Whilst pH-cycling experiments (including demineralizing periods) might mimic the clinical dynamics more adequately, remineralization-only models offer the opportunity to effectively monitor caries-preventive regimens on dental hard tissues on a short-time basis, thus simulating a best-case scenario.[35] With the present approach, initial screening of the effects of hydroxyapatite was accomplished, thus highlighting the advantages of experimental control, even if the breadth of relevant biological aspects was limited.[36] Notwithstanding, the current results provide valuable information on n-HAp containing toothpastes, and are considered a sound basis for further experiments.

Dental enamel comprises by 85–90 vol% of a calcium-deficient carbonate hydroxyapatite, whilst dentine contains considerably lower amounts (~50 vol%).[37] With this in mind, in environments supersaturated with respect to apatites, quantity of dentine remineralization should be higher compared to enamel,[38] and this was corroborated by the present results. Thus, the current findings with dentine as substrate seem to indicate a meaningful direction, whilst the observations with enamel lesions are of predominantly confirmative value, but not less momentous.

Treatment of specimens with n-HAp or $ZnCO_3$/n-HAp toothpastes did not show any superior effects, but results were comparable to the pure remineralizing solution. From this outcome, one might speculate that n-HAp had no influence at all. However, it should be considered that the used remineralizing control (Buskes' solution) represents a solution with a substantial remineralizing potential,[21] and, therefore, allegorized a positive control. As a consequence, treatment with n-HAp toothpastes revealed no additionally beneficial effect on remineralization. Therefore, usage of a solution with a lower remineralizing potential[24] in combination with n-HAP toothpastes might have resulted in superior effects on mineralization compared to only storage under remineralizing conditions. Future pH cycling studies should elucidate these assumptions and are indicated to verify the observed results.

A direct incorporation of n-HAp or $ZnCO_3$/n-HAp particles into the lesions cannot be deduced from the present microradiographic data; however, from previous studies it is known, that crystal growth can be generated with CO_3/n-HAp particles.[9,39] Nonetheless, the present results are hardly comparable, since a control group (*i.e.*, only storage in artificial saliva) was missing in the mentioned papers. With the present set-up, dentine specimens of group B revealed the highest mineral gain of all groups after five weeks. Since the tested n-HAp toothpastes contained various active compounds (zinc carbonate nano-hydroxyapatite versus nano-hydroxyapatite), no direct inference seems derivable from the present data; additionally, from a recent paper, it is known that different n-HAp concentrations (>5%) seem to be of minor importance.[12] Consequently, it seems reasonable to assume that the higher pH value of group B slurry favoured remineralization by incorporation of n-HAp particles into the dentine lesions. Moreover, with other products (*i.e.*, CPP–ACP) a reduced fall in plaque pH following an immediate carbohydrate challenge has been reported[17] and this should be an interesting focus even for n-HAp toothpastes.

It should be emphasized that the used fluoride toothpaste (containing amine fluorides) is one of the well-known and widely used cariostatic products on the market (with documented remineralizing effects being higher than those of toothpastes containing sodium fluorides or monofluorophosphates),[35] and, therefore, has been included for comparative reasons. However, enamel and dentine specimens of group E revealed lower mineral gains compared to all other groups (including the controls). Additionally, dentine specimens treated with E revealed a hypermineralization of the surface layer (with an increased thickness), and it might be surmised that a distinct calcium fluoride-like layer on the specimens' surfaces should have been established by this regimen.[40] Moreover, when preparing the slurry, the degree of saturation with respect to calcium fluoride should have increased, and calcium-fluoride-like precipitates should have been favoured.[41] Such precipitates may have blocked any further ion transport into deeper lesion parts by decreasing the pore volume of the surface layer and obstructing the diffusion pathways,[30] and this could have inhibited further remineralization.[42] Furthermore, the observed hypermineralization of the surface layer was accompanied by an increase in lesion depth. Most likely, the low pH (prevailing during brushing with the slurry of group E) caused a redistribution of calcium and phosphates, and minerals situated at the bottom of the lesion should have diffused outwards and re-precipitated at the surface layer. This would be in accordance with the observation that fluorides can drive demineralization further into enamel by making the surface less soluble.[43]

Because of the different active toothpaste compounds, the pH of the amine fluoride toothpaste slurry was nearly two units lower compared to the hydroxyapatite toothpastes (5.24 and 6.94–7.34, respectively). Due to the lower pH, surface layer mineralization should have increased compared to a higher pH.[44] Groups treated with hydroxyapatite toothpaste revealed remineralized subsurface lesions compared to baseline, but without any hypermineralization. The used nano-sized particles (20 nm in size, with granular dimensions up to 100–150 nm)[9] as well as the calcium arising from storage solution should have followed a concentration gradient (with the solution higher than the subsurface lesion), thus leading to a remineralizing effect in deeper lesion parts.[38]

5. Conclusions

The prevention of tooth decay and the treatment of lesions are ongoing challenges in dentistry, and nanotechnology has been claimed as one of the most revolutionary approaches in this

field. Notwithstanding, at the moment, the applied and marketable dental products have been studied rarely.[8] Interestingly enough, within the limitations of the present *in vitro* set-up, the different nano-hydroxyapatite toothpastes revealed similar remineralizing capacities with enamel and dentine lesions. For dentine, even higher remineralization effects could be achieved with n-HAp or $ZnCO_3$/n-HAp toothpastes compared to the amine fluoride dentifrice. From the present outcome, we therefore speculate that nano-hydroxyapatite in dental products might help to promote remineralization. However, it is pertinent to note, that this experimental study did not take into account all oral factors; in particular, the complexity of any tooth–pellicle–plaque–saliva interface was not simulated. Hence, the current findings should be confirmed in future *in vitro* pH-cycling studies and clinical settings.

Declaration of interests

The authors declare that they have no conflict of interest.

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Comparison of CPP-ACP, Tri-Calcium Phosphate and Hydroxyapatite on Remineralization of Artificial Caries Like Lesions on Primary Enamel - An *in vitro* Study

Meghna Bajaj*
Poornima P**
Praveen S***
Nagaveni NB****
Roopa KB*****
Neena IE ******
Bharath KP*******

Objectives: To compare CPP-ACP, Tri-calcium phosphate and Hydroxyapatite on remineralization of artificial caries like lesions on primary enamel. Study design: Ten extracted Primary molars coated with nail varnish, leaving a window of 2x4 mm on buccal and lingual surface were immersed in demineralizing solution for 96 hours and sectioned longitudinally to obtain 40 sections (4 sections per tooth) and were randomly divided into 4 groups (A to D) n=10; Group A: negative control, Group B: CPP-ACP, Group C: Tri-calcium phosphate, Group D: Hydroxyapatite. Sections were subjected to pH cycling for 10 days and were evaluated by polarized light microscope before and after treatment. Results: Intra group comparison of demineralization and remineralization was done by paired t-test. One way ANOVA was used for multiple group comparisons followed by post HOC TUKEY'S Test for group wise comparisons. Remineralization was found more with Group D followed by Group B, C and A. Conclusion: Hydroxyapatite showed better remineralization when compared to CPP-ACP and Tri-calcium phosphate.

Key words: *CPP-ACP, Demineralization, Hydroxyapatite, Polarized light microscopy, Remineralization, Tri-Calcium phosphate.*

Introduction

Dental caries is a pathological process of localized destruction of tooth tissue by microorganisms. Over the last few decades, fluoride in various forms has been proven to reduce caries in both the primary and permanent dentitions when used in a variety of ways. [1] The enamel of primary teeth is more susceptible to caries development than that of permanent teeth due to lower mineral content and higher organic contents. [2] Crystals at the tooth surface regularly go through natural periods of mineral loss (demineralization) and mineral gain (remineralization).[3] The process of caries development is dynamic in which demineralization of the enamel is followed by remineralization which have a crucial impact on the hardness and strength of tooth enamel.[2]

From the College of Dental Sciences, Department of Pedodontics and Preventive Dentistry,Karnataka, India
*Meghna Bajaj, Postgraduate student .
**Poornima P, MDS, Professor and Head of Department .
***Praveen S, MDS, Reader.
****Nagaveni NB, MDS, Reader.
*****Roopa KB, MDS, Reader.
******Neena IE, MDS, Reader.
*******Bharath KP, MDS, Reader.

Send alll correspondence to
Meghna Bajaj
College of Dental Sciences, Department of Pedodontics and Preventive dentistry,Davangere-577004, India.
Phone: 7829099647
E-mail: meghnabajaj1@gmail.com

Various methods have analyzed tooth demineralization and remineralization, which includes both direct and indirect techniques. Several techniques have been used in Remineralization experiments like Scanning Electron Microscopy,[4] Microradiography,[5] Quantitative light-induced fluorescence,[6] Microhardness[7] and Polarized light microscopy.[8] The polarized light microscopy is a sensitive technique for assessing de- and remineralization in *in vivo* and *in vitro* studies.

CPP-ACP is derived from bovine milk protein, casein, calcium and phosphate, has been demonstrated to have anticariogenic potential in laboratory, animal, and human *in situ* experiments.[9,10] Tri-calcium phosphates has remineralization properties with the advantage of the calcium phosphate system, that is stable in aqueous environment and does not affect the fluoride activity when added to dentifrices.[11]

Hydroxyapatite is one of the most biocompatible and bioactive materials formed of nano-sized particles similar to the apatite crystals of tooth enamel morphology, crystal structure and crystalinity.[12] The aim of the present *in vitro* study evaluated and compared CPP-ACP, Tri-calcium Phosphate and Hydroxyapatite on remineralization potential of artificial caries like lesions on primary enamel.

Materials and Method

Ten extracted non-carious primary molars were collected, thoroughly cleaned free of debris and calculus using hand scalers and stored in 10% formalin.

De/Remineralizing solution preparation

The buffered remineralizing and demineralizing solu-tions were made from top-grade chemicals and deionized water. The demineralizing solution contained 2.2 mM $CaCl_2$, 2.2 mM KH_2PO_4, 0.05M acetic acid had the pH adjusted to 4.4 with 1 M KOH. The remineralizing solution contained 1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4, 0.15 M KCL had a pH of 7.0. This solution approximates to the super saturation of apatite minerals found in saliva.

The teeth were coated with an acid resistant nail varnish, leaving a narrow 'window', approximately 2x4 mm wide, on the intact surface on buccal or lingual surfaces. Each tooth was immersed in the demineralizing solution (10 ml) for 96 hours to produce artificial caries like lesions.

These demineralized teeth were mounted on an acrylic mold using self-cure acrylic resin with buccal and lingual half embedded in acrylic resin to obtain longitudinal sections through the lesion to produce four sections out of each tooth, approximately 150–200 µm thick using a hard tissue micro-tome. Forty enamel sections were made and stored in deion-ized water. Each section was mounted over the glass slide and examined for demineralized area at 40x magnification under a Polarized Light Microscopy (Olympus BX 51, Japan). The images were captured for each section and the depth of the lesion was measured using an image analyzer (Software Image Pro plus version 4.1.0.0 for Windows 95/NT/98,Media Cybernetics, USA). The sections showed a clear demarcation between sound enamel and the initial lesion. The depth of the lesion was determined at three different points from the outer surface to the deepest part of the demineralized area.

Forty sections (specimens) were randomly assigned to 4 groups (A to D) and were kept separately in deionized water -

Group A: sections with no treatment (negative control group)

Group B: sections were treated with CPP-ACP (GC tooth mousse, Recaldent TM, GC company, positive control group)

Group C: sections were treated with Tri-calcium Phosphate (Clinpro tooth crème, 0.21% sodium fluoride with fTCP, 3M ESPE company)

Group D: sections were treated with Hydroxyapatite (Remin-Pro, VOCO company)

Toothpaste and Tooth Mousse supernatant for Group B, C and D were prepared by suspending 15 g of the respective toothpaste/tooth mousse in 45 ml of deionized water, in order to achieve 1:3 (toothpaste: deionized water) ratio.

The sections were tied and tagged with 10 cm floss for ease of use and identification. These sections were then placed in the pH cycling system for 10 days. Total time for each cycle was 8 hrs /day. Each cycle involved three hours of deminer-alization twice a day with two hours of remineralization in between. Specimens in Group B, C and D were treated for 60 seconds with toothpaste supernatant (5 ml /section) both before and after the first and second demineralizing cycles. After the completion of each cycle per day, the sections were kept in deionized water, until required for use next day.

After the completion of pH cycle, sections were kept in acetone for 3 hours and then cleared in xylene and mounted on the glass slides with the DPX mounting medium.

Evaluation technique (for measurement of the depth of the lesion)

After imbibition of the sections in water, these were examined at 40x magnification under polarized light micros-copy to qualitatively evaluate the body of the lesions and the images were captured. Depth of the lesion was measured using an image analyzer (Software Image Pro plus) at three different points in each sample and values were compared before and after the experiment.

Results

The results were subjected to appropriate statistical anal-ysis; mean ± standard deviation of lesion depth was calculated for each group. Among the three groups; group D (9.41 µm) showed the highest amount of remineralization compared to (4.61 µm) group A (Figure 1 and 2).

Intra group comparison of demineralization and remineralization was done by paired t-test. There was statistically highly significant value found (p-value of 0.001) following the remineralization in individual group (Table 1).

One way ANOVA was used for multiple group compari-sons where Mean ± SD values were not significant (p-value 0.98) following demineralization but were highly significant following remineralization (p-value 0.001) (Table 2).

Post HOC TUKEY'S Test for group wise comparisons were not significant ($p>0.05$) after demineralization in each group. Following remineralization, there was a statistically significant difference found between Group A and B and highly significant difference between Group A and D (Table 3).

Discussion

The recent approach in caries management is the non invasive method. This method can transform a lesion from an active to an inactive state. Non-cavitated and cavitated lesions extending upto dentinoenamel junction can be arrested if the cariogenic challenge of certain microenvironment are sufficiently controlled and if therapeutic agents are applied for tissue healing. Professional delivery methods, such as toothpastes, gels, varnishes, fluoride releasing materials are commonly applied to remineralize high-risk areas. Comple-menting traditional diagnostic methods with advanced, more sensitive methods will improve caries diagnostic efficiency and hence the dental care and treatment of patients. [9,11]

In a primary tooth, enamel caries progress rapidly into the underlying dentin. For a carious process to proceed, the pH and the ionic activities of calcium and phosphate in plaque fluid are critical because they determine the stability of the tooth structure under cariogenic attack. Topical agents prevent and inhibit caries progression by inhibition of demineralization: and/or enhancement of remineralization, so creating a more caries resistant surface due to the remineralized crystals. [2, 13]

We investigated the changes in demineralization that indirectly reflected remineralization in advanced enamel lesions with similar depths as natural white spot lesions. Our

Figure 1: Polarized microscope under 40X magnification, showing Demineralized area of enamel in Group D

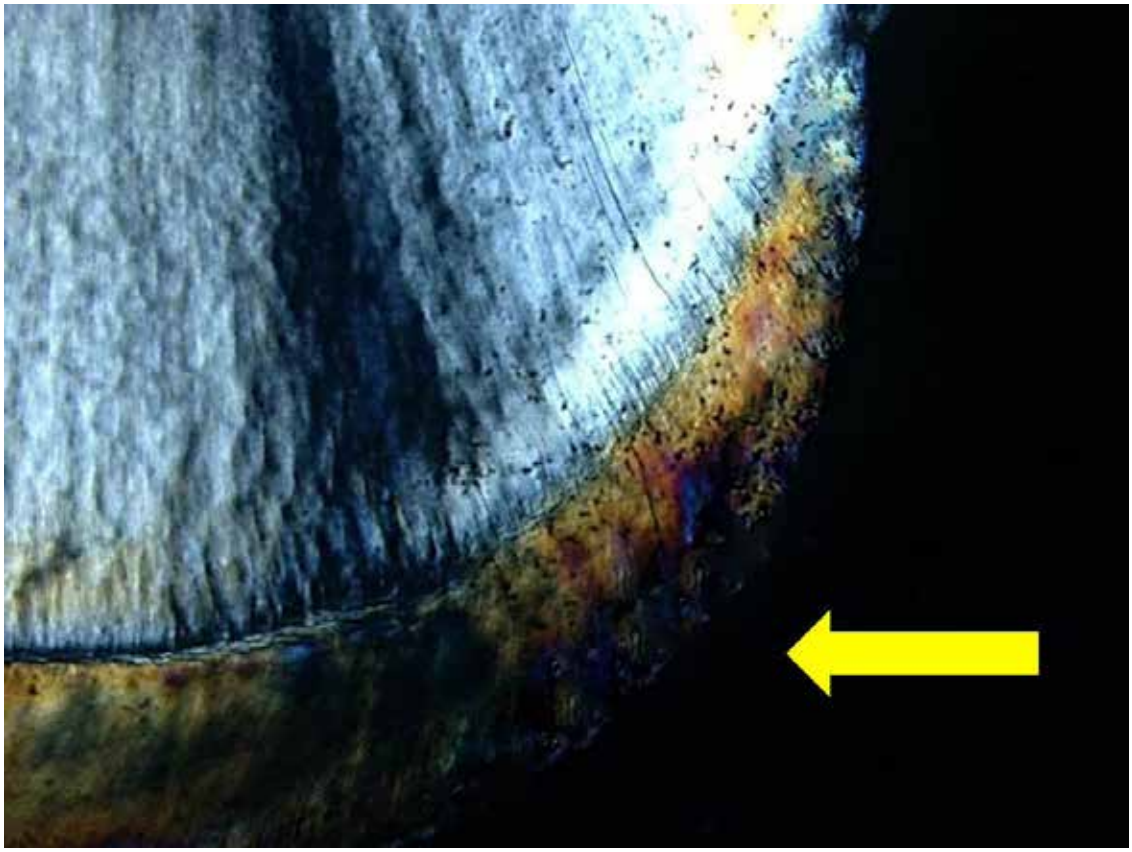
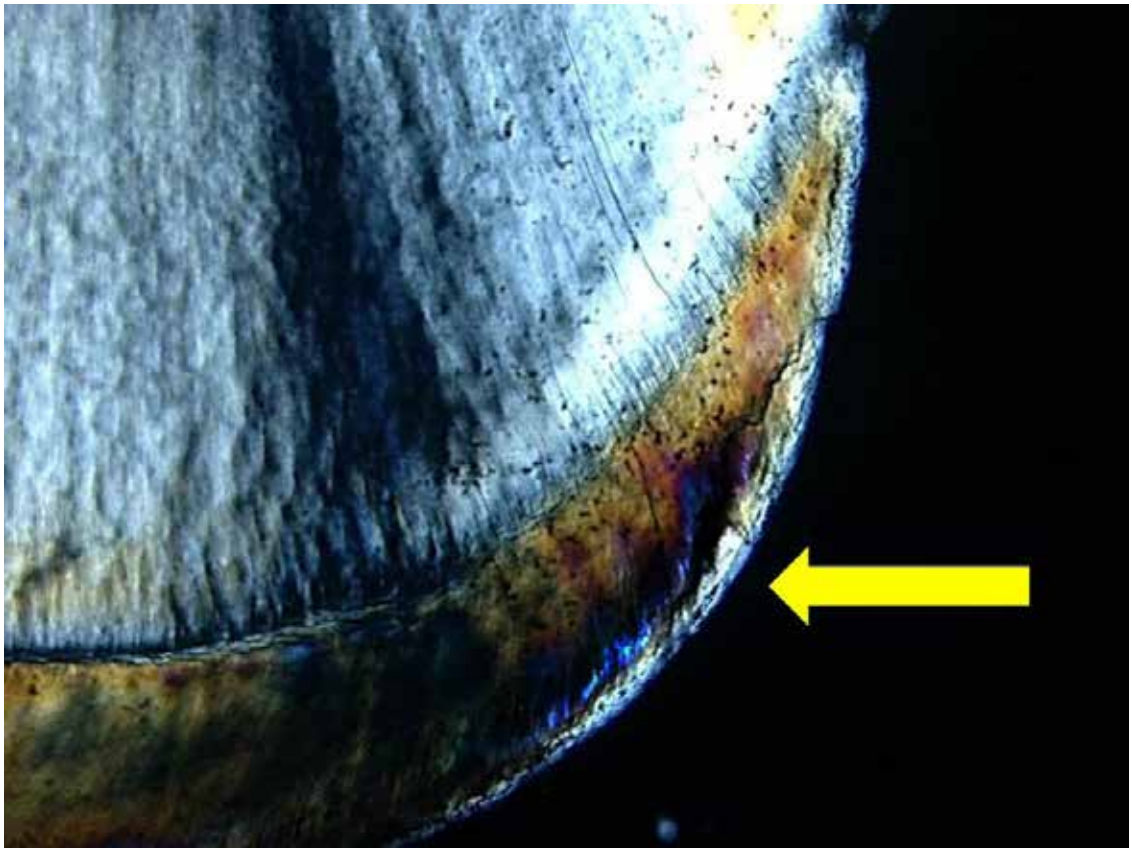


Figure 2: Polarized microscope under 40X magnification, showing Remineralized area of enamel in Group D



results showed that all agents had a significant reduction in the demineralized area and that differed significantly from each other. The remineralizing agents used in the present study were Hydroxyapatite (Remin pro), CPP-ACP (GC Tooth mousse) and Tricalcium phosphate (Clinpro tooth crème).

The tooth sections were subjected to a chemical caries model for the production of artificial caries lesion and pH cycling for testing the efficacy of remineralizing agents, which were sequentially exposed to demineralizing and remineralizing solutions with intermediary treatments with the agents. These methods have improved understanding of the mechanism of demineralization and remineralization. Further, they provide information about the effects of caries preventive agents on de/remineralization dynamics at the surface of the teeth.[15] Polarized light microscopy (PLM) analysis was chosen as it is extremely sensitive to changes in hard tissues. With respect to demineralization and remineralization, it can quantitatively show the areas of mineral loss and mineral gain represented by the visualization of areas with different porosities and birefringence.[14,16]

Table 1: Mean lesion depth and Standard deviation values for all the four groups after Demineralization and Remineralization.

Groups		Demineralization (µm)		Remineralization (µm)	
		Mean	SD	Mean	SD
Group A	No treatment	103.54	8.45	4.61	1.36
Group B	CPP	103.95	9.64	8.10	3.26
Group C	TCP	102.98	7.79	7.22	2.09
Group D	HA	104.35	7.78	9.41	2.95

*Student's paired t-test

Table 2: Comparison of Mean ± SD values of depth of lesion in experimental and control groups.

Groups		Demineralization (µm)	Remineralization (µm)
Group A	No treatment	103.54 ± 8.45	4.61 ± 1.36
Group B	CPP	103.95 ± 9.64	8.10 ± 3.26
Group C	TCP	102.98 ± 7.79	7.22 ± 2.09
Group D	HA	104.35 ± 7.78	9.41 ± 2.95
ANOVA	F	0.05	6.45
	P	0.98, NS	0.001**, HS

One way ANOVA test

**p< 0.001=HS Highly significant

Table 3: Comparison of difference between groups

Groups	P value of difference in Demineralization	P value of difference in Remineralization
A-B	NS	0.02*, S
A-C	NS	0.11, NS
A-D	NS	0.001**, HS
B-C	NS	0.86, NS
B-D	NS	0.66, NS
C-D	NS	0.23, NS

Post-hoc Tukey's Test
*P < 0.05, S
**P < 0.001, HS
P > 0.05, NS

The early enamel caries could be divided into four zones based on its histological appearance under polarized light microscope, i.e. - translucent zone, body of lesion, dark zone and surface zone. There is a translucent zone at the inner advancing front of the lesion, while a dark zone may be found superficial to this. The body of the lesion which is the major part is the third zone lying between the dark zone and the surface enamel. The surface zone lies above on the outer side. Dark zone and surface zone are the positively birefringent zones and showed signs of remineralization which was similar in a study done by Bansal *et al* 2010.[17] The process of remineralization was observed starting from the outer surface of the lesion towards inner surface.

CPP-ACP is the acronym for a complex of casein phosphopeptides (CPPs) and amorphous calcium phosphate (ACP). It has been proposed that the anticariogenic mechanism of CPP-ACP is due to localization of ACP at the tooth surface which then buffers the free calcium and phosphate ion activities, thereby helping to maintain a state of supersaturation with respect to enamel, so depressing demineralization and promoting remineralization.[18]

The remineralizing potential of CPP-ACP has been shown in animal studies,[18] *in vitro* [9,19,20] and *in vivo* studies.[10,21] Several papers have also shown that higher concentration of CPP-ACP elicit higher remineralization.[10] The concentration of CPP-ACP used in the trials varied from 2% to 10% w/w. It has been reported that CPP-ACP produce a similar remineralization effect as that of 2800 ppm F at 2% w/w concentration,[22] and could efficiently promote enamel remineralization at 3% w/w.[23] CPP-ACP used for the present study was 10% w/w in concentration We found an increased enhancement of remineralization by CPP-ACP when compared to Tricalcium phosphate with 950 ppm F, whereas decreased enhancement seen when compared to hydroxyapatite with 1450 ppm F.

In a previous *in vitro* study, CPP-ACP when used in combination with fluorides showed better results and lower caries score than when used individually.[1,24] This was probably due to the ability of CPP-ACP to interact with fluoride ions to produce an additive anticariogenic effect through the formation of a stabilized amorphous calcium fluoride phosphate phase. Conversely, research also suggests that CPP-ACP cream is not as effective as fluoride in remineralizing early enamel caries at surface level. Combination of fluoride and CPP-ACP does not provide any additive remineralization potential compared to fluoride alone. [25]

Rehder Neto *et al* in 2009 compared the remineralization lesion potential of CPP-ACP and CSP (calcium sodium phosphosilicate) containing paste on acid softened enamel. They compared 4 products with control, (i) CPP-ACP (MI paste) (ii) CPP-ACP + Fluoride (MI paste plus) (iii) CSP (tooth revitalizing paste) (iv) Fluoridated dentrifices (FD Sensodyne cool gel) (v) control, and concluded that, the increase in surface microhardness in CPP-ACP group did not differ significantly and was higher than the control group.[26] However, in the present study, although CPP-ACP was used alone without the combination of fluoride, it showed significant increase in remineralization and decrease in lesion depth when compared to tricalcium phosphate containing fluoride. The advantage of using CPP-ACP as a supplement to fluoride-containing products is still unclear. High-quality, well designed clinical studies in this area are still required before definitive recommendations are made.[27]

Tricalcium phosphate (TCP) has been considered as one possible means for enhancing the levels of calcium in plaque and saliva. Combining calcium phosphate and fluoride ions in oral care products is problematic and can lead to loss of bioavailable fluoride ion due to a reaction between the calcium phosphate phase and the fluoride ion. In an approach to overcome this incompatibility of calcium phosphates and fluoride ions, new technologies have been developed.[22,28] This technology supports functional tricalcium phosphate (fTCP) where tricalcium phosphate particles have been ball milled with sodium lauryl sulfate, and has been included in a tooth crème with sodium fluoride marketed as Clinpro tooth crème (3 M ESPE).[28]

In a previous *in vitro* study, NaF (5000 ppm) showed the highest degree of remineralization when observed from GC MI paste plus (CPP-ACP + 900 ppm of fluoride) and Clinpro tooth crème (TCP + 950ppm of fluoride) which were found to be comparable.[14, 29] This observed response showed that F-deposition during treatment depends on lesion depth. With elevated external F-levels, the F-gradient will be higher, driving the fluoride deeper into the lesion, in spite of the F-diffusion being slowed by adsorption onto and reaction with hydroxyapatite crystallites in the pore walls. [30]

Vanichvatana *et al* tested the efficacy of two calcium phosphate pastes (Tooth mousse plus with 900 ppm of fluoride and Clinpro tooth crème with 950ppm of fluoride) fluoride toothpaste (Colgate Palmolive with 1000ppm of fluoride) on remineralizing artificial caries using polarized light microscopy and concluded that Clinpro tooth cream showed similar benefits compared to fluoride toothpaste and had no additional benefits of tooth mousse plus.[31] However, Clinpro tooth crème in the present study showed least amount of remineralization effect when compared to CPP-ACP and Hydroxyapatite.

Hydroxyapatite (HA) is one of the most biocompatible and bioactive materials and is widely applied to coat artificial joints and tooth roots.[24] Nano-sized particles have similarity to the apatite crystal of tooth enamel in morphology, crystal structure and crystallinity.[32] In the present study hydroxyapatite (Remin pro) was used as remineralizing agent, which is a water- based cream, containing hydroxyapatite, fluoride and Xylitol. Hydroxyapatite which is the main constituent of Remin Pro fills the superficial enamel lesions and the tiniest irregularities that arise from erosion. Fluoride, which is also one of the content of Remin pro gets converted to fluorapatite when it comes in contact with saliva; thus strengthens the tooth and renders it more resistant to acid attacks.[33] Since the surface area and proportion of the atomicity increase with decreasing particle size, nano-HA has bioactive and biocompatible properties.[34]

Uday *et.al* 2013 assessed the effect of Remin pro on bleached enamel and concluded that Remin pro causes an increase in the microhardness. The author believes this is due to the presence of 1450 ppm fluoride, which is 61% higher than other available agents today.[33] In the present study hydroxyapatite (Remin pro) showed significant result in surface remineralization when compared with CPP-ACP and tricalcium phosphate.

One must bear in mind that remineralization *in vitro* may be quite different when compared with dynamic, complex biological system, which occurs naturally in the oral cavity. Thus, direct extrapolations to clinical conditions must then be exercised with caution However, there is a need for further long term research under clinical conditions to prove the efficacy of these agents.

Conclusion

All the three groups viz. CPP-ACP, TCP and HA showed remineralization under *in vitro* pH cycling model.

HA group showed significantly m ore remineralization compared to CPP-ACP and TCP.

Remineralization was observed from the surface towards the lesion.

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In Vitro Effects of Nano-hydroxyapatite Paste on Initial Enamel Carious Lesions

Fabíola Galbiatti de Carvalho, DDS, MSc, PHD[1]
Basílio Rodrigues Vieira, DDS[2]
Rogério Lacerda dos Santos, DDS, MSc, PHD[3]
Hugo Lemes Carlo, DDS, MSc, PHD[4]
Pablo Queiroz Lopes, MSc[5]
Bruno Alessandro Silva Guedes De Lima, MSc[6]

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Abstract: *Purpose: The purpose of this study was to analyze the protective effect of remineralizing agents on enamel caries lesions using surface Knoop microhardness testing (KHN) and atomic force microscopy (AFM). Methods: Forty-eight human enamel blocks were assigned to four groups (N=12): (1) control (without agent); (2) fluoride varnish (Duraphat); (3) nano-HAP paste (Desensibilize Nano P); and (4) casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) paste (MI Paste Plus). Incipient caries-like lesions were artificially developed. Cario- genic challenge (pH-cycling) was performed for seven days. The pastes were applied before each immersion in demineralization solution, and the varnish was applied only once. KHN values were obtained at baseline, after incipient enamel lesion, and after challenge. The percentage of surface hardness recovery (% SMHR) was performed, and the surface morphology was evaluated by atomic force microscopy (AFM). ANOVA, Tukey's, and student paired t tests were applied at P<.05. Results: After the cariogenic challenge, the nano-HAP group showed significantly higher KHN and %SMHR values than varnish. The CPP-ACP group showed no increase in KHN. The nano-HAP group showed, via AFM, a protective layer formation with globular deposits on the surface. Conclusion: SMHR and AFM morphology revealed that nano-hydroxyapatite paste showed a protective effect against in vitro enamel caries development.* (Pediatr Dent 2014;36:E85-E89) Received October 12, 2013 | Last Revision February 23, 2014 | Accepted February 24, 2014

Although dental caries continues to be the most common oral infectious disease in childhood,[1] many studies have shown that initial enamel caries lesions can be remineralized by topical application of fluoride agents, including dentifrices, mouthwash solutions, gels, and varnishes.[2-5]

It is known that the application of highly concentrated fluoride agents is considered an effective method for reducing enamel demineralization, because the formation of a calcium fluoride (**CaF_2**)-like layer on the demineralized surface partially reduces enamel mineral loss by subsequent acid attack.[2,6] It has been demonstrated that more concentrated fluoride agents, which form thicker CaF_2 layers, may offer greater protection against dental demineralization.[2,7]

Fluoride varnishes have been extensively used to treat and prevent incipient enamel caries lesions due to their high fluoride concentration, adhesion capacity to tooth enamel, and safety when applied to children's teeth.[4,8-11] However, new tooth remineralization 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 substrate, such as phosphopeptides from milk protein casein and nano-hydroxyapatite (**nano-HAP**).[12-18]

Casein phosphopeptides (**CPPs**) can stabilize the nanoclusters of amorphous calcium phosphate (**ACP**) in a metastable solution.[12] CPPs bind to the nanoclusters of ACP in the supersaturated solutions, preventing the precipitation of calcium and phosphate ions.[12] The casein phosphopeptide–amorphous calcium phosphate (**CPP-ACP**) complex also acts as a reservoir for storing bioavailable calcium and phosphate and maintains the solution supersaturated, hence facilitating remineralization.[12] Several studies have demonstrated the efficacy of the CPP-ACP technology in inhibiting demineralization and promoting remineralization of enamel and dentin.[13-16,19,20] The CPP-ACP complex is commercially available in paste or mousse form, and the product MI Paste Plus (GC America Inc., Alsip, Ill., USA) contains CPP-ACP and fluoride (900 ppm).

Nano-HAP is considered one of the most biocompatible and bioactive materials; it has been studied as a biomimetic material that has demonstrated the potential effect of remineralizing initial enamel caries under dynamic pH-cycling conditions.[17,18] The nano-HAP paste is a bioactive agent that contains calcium nanophosphate organized in a crystalline form of hydroxyapatite and 9,000 ppm of fluoride (Desensibilize Nano P, FGM, Joinville, Santa Catariuna, Brazil). This paste is indicated for desensitization and/or remineralization of the enamel and is commercially available for professional use. According to the manufacturer, up to four applications of the paste are recommended, but the estimated number of sessions may vary according to the clinical case.

The nano-crystals of phosphate are smaller than 100 nm, improving the bioactivity of agent due to the increase in the superficial area and wettability of hydroxyapatite nanoparticles. The calcium, phosphate, and fluoride ions released might increase the saturation level with respect to dental hard tissue in the liquid adjacent to the surface, thus promoting remineralization. Based on these mechanisms, it would be interesting to evaluate the protective effect of nano-HAP paste against enamel caries development, since no data regarding its remineralizing effect in comparison with fluoride varnish and CPP-ACP paste are available.

[1]Dr.Carvalho is an adjunct professor of pediatric dentistry, Department of Clinical and Social Dentistry; [4]Dr. Carlo is an adjunct professor of restorative dentistry, Department of Operative Dentistry [5]Dr. Lopes is assistant professor of Pharmaceutical Sciences, Department of Pharmaceutical Sciences; and [6]Mr. Lima is a PhD student of Mechanical Engineering, Department of Materials Engineering, all at Federal University of Paraíba, João Pessoa, Paraíba, Brazil. [2]Dr. Vieira is an undergraduate student, and [3]Dr. Lacerda is anadjunct professor of Pediatric Dentistry, both at Department of Dentistry, Federal University of Campina Grande, Campina Grande, Paraíba, Brazil.

Correspond with Dr. Carvalho at fabigalbi@yahoo.com.br

Table 1.	COMPOSITION OF THE REMINERALIZING AGENTS INVESTIGATED IN THE STUDY
Agent	Composition (batch number)
MI Paste Plus (GC America Inc.)	Water, glycerol, casein phosphopeptide-amorphous calcium phosphate, D-sorbitol, carboxymethylcellulose propylene glycol, silicone and titanium dioxide, xylitol, phosphoric acid, flavor, sodium saccharin, ethyl propyl butyl p-hydroxybenzoate, 900 ppm F (090813M)
Desensibilize Nano P (FGM)	Calcium nanophosphate organized in crystalline form of hydroxyapatite, potassium nitrate, water, surfactant, tensoative, flavor, 9,000 ppm F (170610)
Duraphat (Colgate)	Alcohol, natural resins, wax, saccharine, flavor, 22,600 ppm F (P008203LA)

The purposes of this study were to: (1) investigate the protective effect of fluoride varnish, nano-hydroxyapatite, and casein phosphopeptide–amorphous calcium phosphate pastes on artificial enamel caries lesions by means of microhardness testing; and (2) evaluate the enamel surface morphology by atomic force microscopy. The null hypothesis tested was that there would be no difference among the effects of the remineralizing agents on enamel microhardness and surface morphology after cariogenic challenge.

Methods

Specimen preparation and selection. Following proper approval from a Research Ethics Committee of Federal University of Campina Grande, Campina Grande, Paraíba, Brazil, 30 sound human third molars were selected for this study. Teeth were stored in 0.1 percent thymol at four degrees Celsius and used within one month after extraction. From each tooth, two enamel specimens from the buccal and lingual surfaces, without visible defects at a magnification of 30×, were prepared (four by four by two mm) using a water-cooled, low-speed diamond saw (Isomet, Buehler Ltd., Lake Bluff, Ill., USA). The specimens were embedded in acrylic resin (Vipi Flash, Pirassununga, São Paulo, Brazil), and the enamel surfaces were ground flat with water-cooled silicon carbide paper discs (400, 600, and 1,200 grit; Buehler Ltd.) and polished with one µm alumina suspension (Erios Corp., São Paulo, São Paulo, Brazil).

After polishing, the baseline hardness of the enamel surface was determined by Knoop microhardness testing (**KHN**)) with five indentations on the specimens using 25 g-loads applied for 10 seconds (HMV II, Shimadzu Corporation, Kyoto, Japan). Specimens with KHN values between 300 and 380[21] were selected and randomized into four groups (n equals 12), according to the remineralizing agent: (1) Control–no treatment; (2) fluoride varnish (Duraphat, Colgate, São Paulo); (3) nano-HAP paste (Desensibilize Nano P); and (4) CPP-ACP paste (MI Paste Plus; Table 1). Specimens without pre-established KHN values were discarded.

Incipient caries lesion formation. Initial caries development was induced to obtain incipient lesions before application of the remineralizing agents. A pH-cycling protocol was used according to Vieira et al.[22] The specimens were kept in a de- mineralizing solution (2.0 mmol L^{-1} calcium, 2.0 mmol L^{-1} phosphate in 0.075 mol L^{-1} acetate buffer, 0.02 µm F/mL, pH 4.7) for six hours (35.5 mL per specimen) and in a remineraliz- ing solution (1.5 mmol L^{-1} calcium, 0.9 mmol L^{-1} phosphate, 150 mmol L^{-1} KCl in 0.1 mol L^{-1} TRIS buffer, 0.03 µm F/mL, pH 7.0) for 18 hours (17.75 mL per specimen) for two days. After initial caries formation, post-lesion KHN tests were conducted with the same static load and time applied for obtaining the baseline measurements. Five KHN measurements were performed; the first indentation was performed in the center of the specimen and the other four at a distance of 300 µm from the first.[14]

Remineralizing agent applications and cariogenic challenge. Prior to the cariogenic challenge, all the remineralizing agents were maintained in different 0.3 mL insulin syringes (BD Ultra-fine, Franklin Lakes, N.J., USA) to standardize the quantity of agent applied on the enamel surface. The quantity of 20 µL of all agents was sufficient to cover the enamel surface of specimens. Thus, according to each group, 20 µL of agent was applied on the specimens, in accordance with the manufacturers' instructions. For Group 2, fluoride varnish was applied and specimens were stored in artificial saliva. After 24 hours, the varnish was removed with a scalpel blade, taking care to avoid touching the enamel surface[23]. For Group 3, nano-HAP 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 deionized water. For Group 4, CPP-ACP paste was applied and maintained in contact with enamel for five minutes and removed with deionized water.

The cariogenic challenge was conducted using the same de- and remineralizing solutions described for inducing initial caries development; however, specimens were kept in the demineralizing solution for three hours and in remineralizing solution for 21 hours.[15,24,25] This cycle was repeated for seven days. The de- and remineralizing solutions were changed daily to prevent depletion or saturation of the solution and accumulation of enamel dissolution agents. Before each immersion in demineralization solution, the pastes were applied according to each group. The varnish was applied only once to simulate the clinical situation of a single professional application.[23,24]

The post-treatment KHN tests were conducted with the same static load and time applied for baseline and post-lesion measurements.

Atomic force microscopy. After microhardness testing, three randomized specimens of each group were analyzed using atomic force microscopy (**AFM**; SPM-9600, Shimadzu, Kyoto, Japan). Each specimen was fixed to the microscope holder on a stub (two by three mm). The block surface morphology was probed in "contact mode." Imaging was performed with a standard geometry silicon nitride micro-cantilever (OMCL-TR, Olympus, Tokyo, Japan) and probed with 0.15 N/m constant elastic and 24 KHz resonant frequency. Images of 30 by 30 µm with a resolution of 512 by 512 pixels and an operating point of 1.5 V were collected at a very low scan rate to obtain details of the enamel structure and to avoid damaging the tip.

Statistical analysis. Data analysis was performed with GraphPad Instat 2.0 software (GraphPad Software, La Jolla, Calif., USA) at a α=0.05 level of significance. Since all the

Table 2. BASELINE, POSTLESION, AND POST-TREATMENT KNOOP MICROHARDNESS TEST VALUES FOLLOWED BY THE PERCENTAGE OF SURFACE HARDNESS RECOVERY (%SMHR) ON ENAMEL SURFACE, ACCORDING TO REMINERALIZING AGENTS APPLICATION

Groups	Baseline	Post-lesion	Post-treatment	% SMHR
Control	373.6±14.2^{a,A*}	116.9±5.5^{a,B†}	98.6±16.0^{d,B}	-7.1^{d}
Nano-hydroxyapatite paste	354.5±19.1^{a,A}	131.6±21.6^{a,C}	260.7±23.3^{a,B}	+57.9^{a}
Varnish	350.4±19.9^{a,A}	123.6±12.9^{a,C}	193.4±19.1^{b,B}	+30.8^{b}
Casein phosphopeptide-amorphous calcium phosphate paste	363.3±11.8^{a,A}	120.4±8.0^{a,B}	147.3±8.8^{c,B}	+11.1^{c}

* For each vertical column, values with different lowercase letters indicate a statistically significant difference ($P<.05$), according to one-way analysis of variance and Tukey's tests.
† For each horizontal row, values with different uppercase letters indicate a statistically significant difference ($P<.05$), according to student's paired *t* test.

variables tested satisfied the assumptions of equality and normal distribution (Bartlett and Kolmogorov-Smirnov tests, respectively), one-way analysis of variance and Tukey's tests were carried out for statistical comparisons of enamel hardness among remineralizing agents in each experimental period. The student's paired *t* test was carried out for statistical comparisons in the same remineralizing agent after different experimental periods. Additionally, the percentage of surface hardness recovery (**%SMHR**) was determined as follows: %SMHR equals 100 [(post-treatment - postlesion)/(baseline - postlesion)].[17]

Results

Table 2 shows the initial, post-lesion, and post-treatment KNH values followed by %SMHR for all groups. No statistically significant differences were observed among groups for baseline KHN values ($P=.84$). After initial caries development, all groups showed lower KHN values (post-lesion) compared with baseline; however, there were no statistically significant difference among groups ($P=.79$). After application of remineralizing agents and the cariogenic challenge (post-treatment), only nano-HAP and fluoride varnish groups showed a significant increase in KHN values ($P=.01$).

There were significant differences among groups post-treatment ($P=.01$), The nano-HAP group showed the highest KHN values (260.7±23.3), followed by the fluoride varnish group (193.4±19.1), CPP-ACP group (147.3±8.8), and control group (98.6±16.0; Table 2). The positive values observed for the %SMHR are indicative of mineral recovery of surface microhardness, while the negative value shown in the control group could be related to demineralization. The nano-HAP group showed significantly higher %SMHR (approximately 58 percent) compared with the fluoride varnish (approximately 31 percent) and CPP-ACP (approximately 11 percent) groups ($P=.01$). The control group showed the lowest %SMHR (approximately seven percent), and the negative value observed was indicative of the KHN value being reduced after pH-cycling (Table 2).

Figure 1 shows the AFM images after remineralizing agent application and cariogenic challenge. The control and CPP-ACP groups showed similar morphology with enamel areas without protective layer formation (Figures 1A and B). The nano-HAP group showed a surface interspersed with a homogeneous protective layer formation with globular deposits (Figure 1C). The fluoride varnish group showed a homogeneous protective layer on the enamel surface (Figure 1D).

Discussion

Although the effect of fluoride or nonfluoride-containing agents on enamel microhardness during caries development has been evaluated by some studies,[14-17,24] the innovative approach of the present investigation is related to the result of showing the protective potential of a highly concentrated fluoride agent, associated with a nano-HAP component, against enamel caries development.

Considering the importance of the enamel surface layer in caries progression, the purpose of this study was to evaluate the changes in the enamel surface after simulating caries development and then using different remineralizing agents. Thus, the KHN measurements were performed in three experimental periods: (1) sound enamel (baseline); (2) incipient caries lesion formation (such as white spot lesion/post-lesion); and (3) post-treatment (after the application of remineralizing agents and subsequent cariogenic challenge). The standardization of baseline KHN values (Table 2) made it possible to establish the comparisons of KNH and %SMHR among the groups after treatment. After incipient caries lesion formation, there was a decrease in KHN values for all groups. After post-treatment, only the fluoride varnish and nano-HAP groups showed the effectiveness of these agents in rehardening the initial enamel caries lesions at the surface level. However, the composition of each agent influenced this process differently.

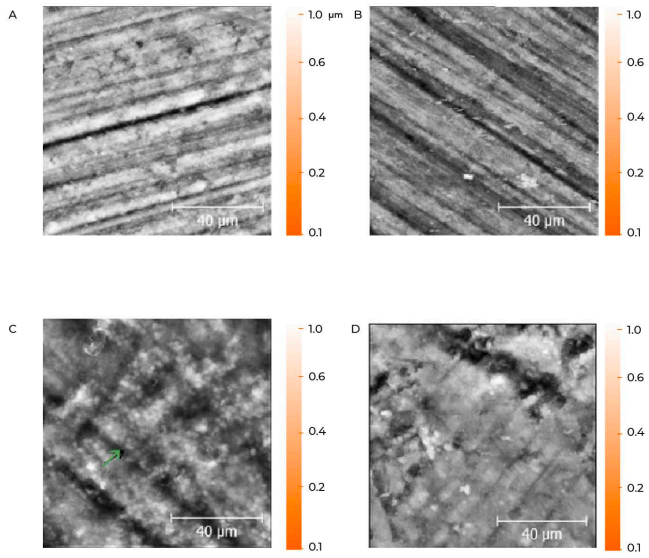

Figure 1. Atomic force microscopy images of enamel surface after cariogenic challenge and application of remineralizing agents. (A) No-agent application (control group). (B) Casein phosphopeptide-amorphous calcium phosphate paste application: surface enamel with "scratch" lines by polish procedure with no protective layer. (C) Nano-hydroxyapatite paste application: (→) protective layer formation with globular deposits of nano-hydroxyapatite crystals. (D) Fluoride varnish application: surface enamel with protective homogeneous layer formation.

According to the manufacturers' information, the remineralizing agents investigated contain different concentrations of fluoride: fluoride varnish—approximately 22,600 ppm F; nano-HAP paste—9,000 ppm F; and CPP-ACP paste—900 ppm F. It was expected that the varnish would show higher KHN and %SMHR values than the other agents, since it has the potential to decrease enamel solubility in acids in vitro[4,23,24,26] and contains the highest fluoride concentration among the studied agents. However, the fluoride varnish group showed intermediate KHN and %SMHR values compared with the nano-HAP and CPP-ACP groups (Table 2). The varnish probably reacted chemically with enamel during the 24 hours of contact, but this was not enough to reduce the mineral loss caused by the cariogenic attack. A higher frequency of application during pH-cycling could perhaps have enhanced the anticaries protection of the varnish, but it was applied only once to simulate the clinical situation of a single professional application every seven days, as described in previous studies.[23,24]

The nano-HAP group showed the highest %SMRH and KHN values post-treatment (Table 1). Although the nano-HAP paste contained a lower fluoride concentration (9,000 ppm F) than the varnish, the presence of calcium and phosphate ions organized as nano-HAP crystals and associated with fluoride enhanced its remineralizing potential. The deposits of nano-HAP crystals could be visualized in the AFM image (Figure 1C). It may be suggested that the composition and application form of this paste (10 seconds of friction) could have influenced the results. In white spot formation, there is dissolution of hydroxyapatite crystals from the subsurface, forming a subsurface lesion with a highly mineralized surface layer.[15,27] The calcium phosphate nano-crystals may have penetrated more deeply into the demineralized subsurface as a result of friction (10 seconds), forming a "reservoir-like" deposit of calcium and phosphate ions. This reservoir-like deposit associated with fluoride could make these ions available during a subsequent cariogenic challenge and help maintain a state of supersaturation with respect to enamel minerals.[17] Furthermore, the diffusion of mineral ions into deep regions of a lesion could be inhibited by the highly mineralized surface layer. This may explain why complete remineralization was not achieved by the application of nano-HAP paste.

Although the risk of developing fluorosis is mainly associated with the ingestion of fluoridated toothpaste by young children (one to three years old),[2] there is clearly a need for a differentiated treatment philosophy involving preventive and therapeutic measures to prevent and control initial caries development and to prevent the development of fluorosis. In this context, the rational use of fluoride is crucial and the professional application of fluoridated agents with reduced concentration of fluoride, but with a remineralizing effect, is desirable. For pediatric clinical practice, the results found in the present study also emphasize that fluoride associated with nano-HAP crystals could be an alternative to reduce the fluoride concentration in preventive agents without losing the remineralizing effect.

Among the remineralizing agents tested, CPP-ACP paste was the only one that was not effective in rehardening the early enamel caries, showing the lowest KHN and %SMRH values post-treatment (Table 2). Other studies have shown that CPP-ACP paste did not have any effect on reducing the progression of artificial or in vivo initial enamel carious lesion.[28,29] Reynolds[12] suggested that CPP-ACP molecules need an acid challenge to be activated, and ACP should be separated from the casein. In the present study, CPP-ACP may have been incorporated into the lesion, but it was not activated when it was necessary. This may be due to a different time between the release of ACP from CPP during the acid challenge and the timing of a gradient necessary for depositing calcium and phosphate in the lesion during remineralization.[28] Furthermore, it has been demonstrated that CPP-ACP paste can have higher remineralizing potential when used in combination with fluoridated toothpaste.[30]

AFM is able to provide surface morphology images with minimal sample preparation. The use of AFM made it possible to visualize the effects of the demineralization/remineralization processes on the enamel morphology in the presence of the remineralizing agents investigated. Nano-HAP paste and fluoride varnish were the groups that showed a significant increase in KHN values post-treatment, in addition to the formation of a homogeneous protective layer on the enamel surface (Figures 1C and D). The nano-HAP group showed the deposition of globular particles on the enamel surface, characterized as nano-HAP crystals (Figure 1C). The control and CPP-ACP groups showed the enamel surface without any protective layer formation (Figures 1A and B), and the "scratch" lines visualized were possibly due to the polishing procedure used to prepare the specimen.

The period for demineralization in the pH cycling phase (three hours) was based on early studies[15,24,25] to simulate the duration of demineralization that can occur in the oral cavity.[31] However, it is relevant to emphasize that there are numerous dissimilarities between cycling models and in vivo conditions. The pH-cycling model did not entirely simulate the oral conditions where the pH fluctuates frequently, and the levels attained depend upon the individual's eating habits, oral hygiene practices, fluoride usage, and the composition and quality of saliva and biofilm. Thus, the remineralizing agents tested in the present study should also be evaluated in vivo.

The null hypothesis was rejected because fluoride varnish and nano-HAP groups showed increased KHN and %SMRH values compared with the control and CPP-ACP groups. Furthermore, there were significant differences among the remineralizing agents investigated by AFM. Due to the lower fluoride concentration and the increase in KHN and %SMRH values after cariogenic challenge, the nano-HAP paste could be an alternative agent for treating initial enamel caries lesions.

Conclusions

Based on this study's results, the following conclusions can be made:

1. Fluoride varnish and nano-hydroxyapatite paste were effective in rehardening the initial enamel caries lesion after artificial cariogenic challenge.
2. Casein phosphopeptide-amorphous calcium phosphate paste with fluoride did not show any protective effect on artificial enamel caries development, showing the lowest Knoop microhardness test and percentage of surface hardness recovery values post-treatment.

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Effect of nano-hydroxyapatite concentration on remineralization of initial enamel lesion *in vitro*

S B Huang, S S Gao and H Y Yu[1]

State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu 610041, People's Republic of China

E-mail: yhyang6812@scu.edu.cn
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Abstract

The purpose of the research was to determine the effect of nano-hydroxyapatite concentrations on initial enamel lesions under dynamic pH-cycling conditions. Initial enamel lesions were prepared in bovine enamel with an acidic buffer. NaF (positive control), deionized water (negative control) and four different concentrations of nano-hydroxyapatite (1%, 5%, 10% and 15% wt%) were selected as the treatment agents. Surface microhardness (SMH) measurements were performed before/after demineralization and after 3, 6, 9 and 12 days of application, and the percentage surface microhardness recovery (%SMHR) was calculated. The specimens were then examined by a scanning electron microscope. The %SMHR in nano-hydroxyapatite groups was significantly greater than that of negative control. When the concentration of nano-HA was under 10%, SMH and %SMHR increased with increasing nano-hydroxyapatite concentrations. There were no significant differences between the 10% and 15% groups at different time periods in the pH-cycling. The SEM analysis showed that nano-hydroxyapatite particles were regularly deposited on the cellular structure of the demineralized enamel surface, which appeared to form new surface layers. It was concluded that nano-hydroxyapatite had the potential to remineralize initial enamel lesions. A concentration of 10% nano-hydroxyapatite may be optimal for remineralization of early enamel caries.

1. Introduction

Dental caries in enamel is unique amongst diseases as enamel is both acellular and avascular. Thus, in contrast to other tissues, enamel cannot heal itself by a cellular repair mechanism [1]. Nonetheless, it is now well established that the formation of incipient enamel caries is a reversible process where periods of progression alternates with periods of remineralization [2]. Given an appropriate change in conditions, remineralization may even become the predominant process, leading to apparent repair of the lesion [3].

Fluoride (F) has been a useful instrument and is one of the most effective remineralizing agents in caries prevention [4]. Over the last 25 years, the decline in dental caries experienced in most industrialized countries can be attributed largely to the widespread use of fluoride [5]. Nevertheless, some concern has been expressed that with the wide array of both prescription and over-the-counter fluoride products now being marketed in every country, the total fluoride intake has increased to perhaps harmful levels. Chronic low-level exposure to fluoride can present problems in organ systems (gastro-intestinal, genito-urinary and respiratory) of normal individuals [6]. The prevalence of dental fluorosis, on the other hand, has increased noticeably in non-fluoridated areas and to a lesser extent in optimally fluoridated areas [6–8]. Therefore, it is still necessary to seek alternative, effective non-fluoride agents that can provide a complete cure for caries.

Hydroxyapatite (HA) is one of the most biocompatible and bioactive materials and is widely applied to coat artificial joints and tooth roots [9]. Nano-sized particles have similarity to the apatite crystal of tooth enamel in morphology, crystal

[1] Author to whom any correspondence should be addressed.

structure and crystallinity [10]. In recent years, an increasing number of reports have shown that nano-hydroxyapatite has the potential to remineralize artificial carious lesions following addition to toothpastes, mouthwashes, etc [11–13]. Some studies have also reported that a 4% (wt%) nano-HA liquid suspension had good potential to remineralize incipient caries lesions [14, 15]. However, other studies have found no significant difference between NaF and 10% (wt%) nano-HA on the effect of remineralization of initial enamel lesions [16]. The divergence in these results is probably related to different methodologies (*in vivo* or *in vitro*, type of remineralizing agents used, time of application, etc). To date, there have been no reported studies regarding the concentrations of nano-hydroxyapatite required for protection, or which manifest the best protection under systems that reflect real-life situations. Another important factor to be considered is that when *in vitro* remineralization and demineralization cycle model systems are used to evaluate the efficacy of agents, different testing protocols may obtain distinct results. Therefore, the aim of this study was to determine the effects of nano-hydroxyapatite concentration on the initial enamel lesions over a range of time periods under dynamic pH-cycling conditions. In addition, our goal was to address the mitigating factors and thereby aid in a thorough evaluation of nano-hydroxyapatite for practical applications in the anti-caries area.

2. Methods and materials

2.1. Solution preparation

Demineralization solution: The demineralization solution used to initially form subsurface caries lesions and in the pH-cycling was a pH 4.5 acetic acid (50 mM) solution containing 2.2 mM $Ca(NO_3)_2$, 2.2 mM KH_2PO_4 and 0.1 ppm NaF.

Remineralization solution: The remineralization solution used in pH-cycling contained 20 mmol l^{-1} HEPES, 1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, 130 mM KCl and 1 mM NaN_3. The pH was adjusted to 7.0 with KOH; these solutions were similar to those used by Ten Cate and Duijsters [17].

Nano-hydroxyapatite power was purchased from National Incubation Base for Nano-Biomaterials Industrialization, Sichuan University, China. The nano-HA crystals were of nanometer grade and had a crystal size of 5–26.7 nm diameter by 30–84 nm in length, giving an aspect ratio of 3.1. These nano-HA crystals had also a similarity in crystallinity to apatite in bone and enamel as revealed by XRD [18–20].

Treatment solutions were 1000 ppm NaF aq. (positive control), distilled and deionized water (DDW, negative control); 1%, 5%, 10% and 15% (wt%) nano-HA suspension liquid in distilled water, pH adjusted to 7.0 using 2 M HCL.

2.2. Enamel specimen preparation

In this study, bovine incisors were used. The freshly extracted teeth were thoroughly cleaned of debris and inspected under a stereoscopical microscope for visibly observable cracks, hypoplasia or white spot lesions, and then stored in a 0.1% thymol solution until required. The crowns were separated from the roots by a diamond-coated band saw under continuous water cooling (Struers Minitom; Struers, Copenhagen, Denmark). Enamel blocks (4 mm × 4 mm) were embedded in polymethyl methacrylate. The superficial enamel surface was ground flat with water-cooled carborundum discs (1200 grit; Water Proof Silicon Carbide Paper, Struers, Germany) and polished with diamond paste (15 μm Diamond Paste, Struers), thereby removing approximately 100 μm of the outermost enamel layer and yielding a flat surface.

Table 1. The pH-cycling model in the experiment.

Time	Experimental solution
8:00 a.m.–8:03 a.m.	Treatment solutions
8:03 a.m.–9:00 a.m.	Remineralization solution
9:00 a.m.–9:03 a.m.	Treatment solutions
9:03 a.m.–11:00 a.m.	Remineralization solution
11:00 a.m.–1:00 p.m.	Demineralization solution
1:00 p.m.–3:00 p.m.	Remineralization solution
3:00 p.m.–3:03 p.m.	Treatment solutions
3:03 p.m.–4:00 p.m.	Remineralization solution
4:00 p.m.–4:03 p.m.	Treatment solutions
4:03 p.m.–8:00 a.m.	Remineralization solution

The treatment solutions, remineralization solution and demineralization solution were used as described above.

2.3. Baseline microhardness test

Baseline surface hardness of the sound enamel after polishing was performed with a microhardness tester (Duramin-1/-2; Struers, Copenhagen, Denmark) using a Knoop indenter at 10 g load for 15 s. 126 enamel blocks with baseline surface microhardness (SMH) between 469.6 and 488.0 Knoop hardness numbers (KHN) were selected for further study.

2.4. Preparation of early artificial caries lesions

Early artificial caries lesions were produced in the enamel, basically according to Ten Cate and Duijsters [17]. Each specimen was immersed in 8 ml of demineralization solution for 72 h at 37 °C. After artificial caries preparation, the SMH of the enamel blocks was again measured (SMH 1). Indentations were spaced at 100 μm from each other and baseline measurements were made. After artificial caries preparation, 70 blocks with baseline KHN values (SMH1) between 169.6 and 189.8 were selected for pH-cycling. One half of each specimen was covered with an acid-resistant varnish to maintain the baseline lesion.

2.5. pH-cycling model

The specimens were randomly divided into six groups (10 specimens/group) according to the treatment solutions. The cycling schedule was designed to approximate the pH dynamics of the oral environment and used the regime reported by White [21]. The de- and remineralization cycles consisted of the episodes shown in table 1. Each cycle involved 2 h of demineralization in order to simulate the daily acid challenges occurring in the oral cavity.

The treatment solutions, remineralization solution and demineralization solution were used as described above.

Table 2. Surface microhardness analysis of enamel blocks at various time periods according to the treatments.

Treatments	Baseline SMH	Before pH-cycling	After pH-cycling 3 days	After pH-cycling 6 days	After pH-cycling 9 days	After pH-cycling 12 days
NaF	484.7 ± 3.3[a]	187.3 ± 7.6[a]	270.6 ± 8.7	334.2 ± 8.3	351.3 ± 12.1[c]	362.0 ± 16.5[c]
1%Nano-HA	483.7 ± 6.7[a]	190.7 ± 9.9[a]	218.5 ± 5.0	226.2 ± 3.0	233.5 ± 2.4	236.6 ± 4.3
5%Nano-HA	481.0 ± 3.5[a]	189.6 ± 9.1[a]	230.1 ± 3.0[a]	252.9 ± 1.5	261.7 ± 3.4[c]	263.4 ± 4.3[c]
10%Nano-HA	475.7 ± 4.3[a]	188.9 ± 6.3[a]	236.5 ± 3.0[a,b]	306.1 ± 18.0[a,c]	309.2 ± 10.6[a,c]	313.5 ± 8.2[a,c]
15%Nano-HA	478.1 ± 6.0[a]	194.3 ± 3.2[a]	245.7 ± 12.3[b]	313.1 ± 14.6[a,c]	314.6 ± 6.7[a,c]	318.8 ± 3.5[a,c]
DDW	483.8 ± 9.1[a]	186.3 ± 7.9[a]	193.02 ± 4.2	204.1 ± 2.7[c]	203.3 ± 2.8[c]	204.7 ± 3.2[c]

[a,b] The same letter denotes values that are not significantly different within the same time period in different treatments ($p > 0.05$).
[c] Show no significant difference at different time periods for each treatment ($p > 0.05$).

The regimen was repeated for 12 days and temperature maintained at 37 °C. The de- and remineralizing solutions were freshly made every third day and the treatment solutions were made daily and used with continuous stirring throughout the experimental period. Nano-HA treatment solutions were ultrasonicated immediately after preparation, as ultrasonication is particularly effective in breaking up the aggregates and in reducing the size and polydispersity of nanoparticles [22].

2.6. Surface microhardness analysis

After 3, 6, 9 and 12 days of application, the SMH of the enamel blocks was again measured (SMHn). At each time point, five indentations were placed next to the previous measurement at 100 μm intervals, the mean values of all five measurements at different application times were then compared and the percentage SMH recovery was calculated as [after n(th) days
%SMHR = 100 (SMH n–SMH 1)/(SMH–SMH 1)] [23] (n = 3/6/9/12).

2.7. SEM examination

After the SMH analysis, representative specimens from the nano-HA groups, the positive control and the negative control groups were randomly selected for SEM sample preparation. These were then examined using a scanning electron microscope (S-2460 N, Hitachi, Tokyo, Japan).

2.8. Statistical analysis

Data were computerized and analyzed using SPSS 13.0 software. SMH and percentage surface microhardness recovery (%SMHR) among treatments were analyzed by repeated measures, followed by the LSD test. The significance level was set at 0.05.

3. Results

The results of SMH analysis of enamel blocks are shown in table 2. The enamel blocks in all treatment groups had rehardened significantly after pH-cycling. Fluoride had a significantly greater effect than all the other treatments ($p < 0.05$). All treatments except the 10% nano-HA and 15% nano-HA treatments were statistically different from each other after pH-cycling has been completed. In the nano-HA groups, %SMHR was significantly greater than that of the negative control group at each time point ($p < 0.05$). When the concentrations of nano-HA were under 10%, SMH and% SMHR increased with nano-HA concentration at each time point in the pH-cycling. The highest percentage SMHR was found for the treatment with 15% nano-HA and the lowest with 1% nano-HA. In addition, the %SMHR and time curve (figure 1) revealed that, for all treatment groups, the remineralization rate increased significantly in the first 6 days of pH-cycling. However, little further improvement happened beyond this point.

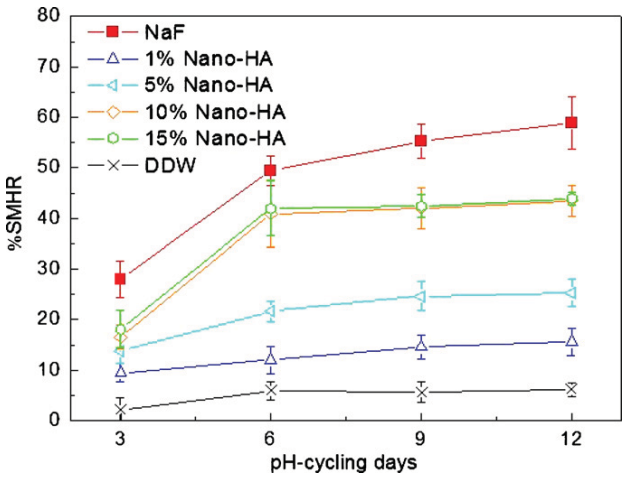

Figure 1. %SMHR of enamel blocks at various time periods according to the treatments. (This figure is in colour only in the electronic version)

Distinct surface coatings deposited by different agents were evident by SEM on the treated anatomical enamel surfaces of the specimens under different conditions (figure 2). As shown in figure 2(*a*), a smooth and intact surface was obtained in the normal anatomical enamel surface before demineralization; however, many micropores and cellular structures appeared on the surface of the initial lesions (figure 2(*b*)). After pH-cycling, in the nano-hydroxyapatite groups, acicular crystals had sedimented on the enamel surfaces after demineralization, and the cavities and defects of the enamel surface had decreased (figure 2(*c*)). The NaF group indicated formation of different-sized globular structures in

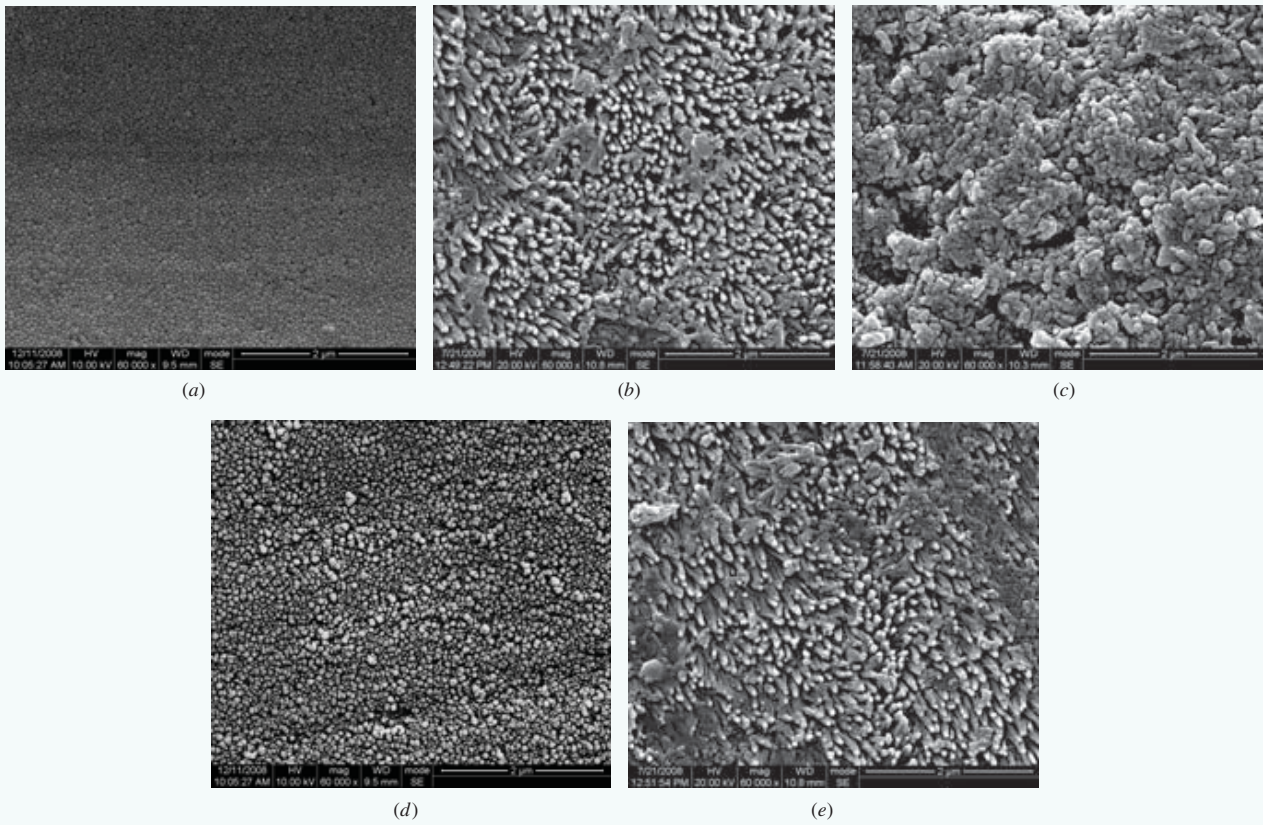

Figure 2. SEM images of a representative specimen in different groups before and after pH-cycling: (*a*) normal enamel before demineralization, (*b*) initial enamel lesion, (*c*) initial enamel lesion remineralized by nano-HA (10%), (*d*) initial enamel lesion remineralized by NaF, (*e*) initial enamel lesion remineralized by DDW.

the enamel surface (figure 2(*d*)); however, only a honeycomb structure was found in the DDW groups (figure 2(*e*)).

4. Discussion

In the current study, nano-hydroxyapatite was directly selected as a remineralizing agent and an *in vitro* pH-cycling model was used to evaluate the effect of four nano-HA concentrations on the initial enamel caries lesions. Although the data were obtained in a laboratory setup, the pH-cycling model provided a better simulation of the caries processes and more closely approached the oral environment, compared to separate de- and remineralization studies [24]. These results confirmed the ability of nano-hydroxyapatite to aid in remineralizing enamel; at each time point in the pH-cycling, the different concentrations were directly related to the distinct effects on remineralization. The most likely explanation of the increased remineralization effect is that it was due to the ability of nano-hydroxyapatite to promote remineralization.

In a previous study on the remineralization effect of nano-HA toothpaste on artificial caries, the solubility properties of nano-HA were found to play a significant role in remineralization when the demineralized specimens were subjected to the treatment solutions continuously for several days [11]. However, in the present study, nano-HA was applied for only a short period during pH-cycling, and due to the low solubility of pure hydroxyapatite, not enough Ca^{2+} and PO_4^{3-} were available to increase the stability of hydroxyapatite in the enamel and to prevent dissolution of the dental enamel.

Since the surface area and proportion of atomicity increase with decreasing particle size, nano-HA has bioactive and biocompatible properties [25]. As shown in figures 2(*a*) and (*b*), incipient lesions extended into the enamel and were significantly more porous than was sound enamel, which allowed a greater penetration of solution ion constituents and a larger surface area for a subsequent reaction of enamel mineral [26]. These factors increased the potential of nano-HA to directly fill up defects and micropores on demineralized teeth. If nano-HA penetrates the enamel pores, nano-HA will act as a template in the precipitation process and will continuously attract a large amount of Ca^{2+} and PO_4^{3-} from the remineralization solution to the enamel surface to fill the vacant positions of the enamel calcium crystals. This in turn will promote crystal integrity and growth.

The surface chemical properties and morphological structure of nano-HA has been claimed to play the most important part in the remineralization of early caries lesions. As the concentration increases, the rate and amount of nano-HA precipitation would also increase, along with the deposition of extensive amounts of Ca^{2+} and PO_4^{3-}, thus

significantly promoting the remineralization effect. Kim *et al* [12] also demonstrated that surface hardness of the demineralized enamel increased with increasing nano-HA concentration when nano-HA was added to a NaF mouthwash. In the nano-HA groups, 15% nano-HA showed a good effect on remineralization; however, this concentration is a little high for practical purposes in mouthwash or toothpaste, as concentrations in this range will cause a certain level of unavoidable aggregation. However, as the data obtained for 10% nano-HA were quite similar to those for 15% nano-HA, a 10% suspension may prove to be an optimal concentration for remineralization of early enamel caries.

From the %SMHR and time curve (figure 1), it was clear that remineralization continued over an extended period of time. However, the rate of remineralization in all treatments was fastest during the first 6 days of pH-cycling and then slowed and stabilized beyond this point. A previous report [28] on mineral deposition in artificial early caries lesions also supported the data of the current study.

When the concentration of nano-HA was under 10%, the remineralization effect increased significantly along with increase in the concentration while there was a sharp change between the remineralization effect at 5% and 10%. These findings also could be due to the possible mechanisms of nano-HA in remineralization as described above. As a result of remineralization of the outer enamel region, the deposition of nano-HA on the surface layer would probably block surface pores and restrict diffusion into the lesion over the short term of remineralization. However, this deposition would eventually come to a stable level even though the concentration increased, which would result in no significant difference between the 10% and 15% nano-HA groups. Research to date has shown that lesions can be rehardened by deposition of hydroxyapatite that is initially deposited near the surface layer, but then is gradually transferred inward and finally precipitated in the dark zone in the long-term remineralization [27]. It has been well established that nano-HA has a good potential for remineralization and promotes remineralization with regular daily usage from this perspective.

In the current study, the enamel surfaces in different treatments were examined by a scanning electron microscope. The different enamel surface morphologies in the corresponding treatments may be due to different mechanisms for promoting remineralization. After the application of the higher concentrations of fluoride, calcium fluoride-like material was preferentially formed in partially demineralized human enamel [28], while it seemed to cause crystal growth of surface apatite crystals (figure 2(*d*)). Since, in the current study, we used 1000 ppm NaF as a positive control, it may be assumed that the globular structures, which sedimented in the surface layer, consisted mainly of CaF_2.

In the nano-HA groups, since the enamel surface morphologies were similar among the different concentration groups after remineralization, we selected a representative result to describe the change in the crystal morphology of specimen after application of the agent. As shown in the figure 2(*c*), the entire enamel surface was covered with finely divided particles and the products appeared to coalesce and form a surface layer microstructure. At the same time, the globules themselves appeared to be agglomerates of still smaller particles. From the aspect of a mechanism for nano-HA remineralization, acicular crystals of nano-HA sedimented onto the enamel surfaces and directly filled up defects and micropores on demineralized teeth surfaces after demineralization. This resulted in the observed decreases in cavities and defects of the enamel surface and the increased surface hardness of the enamel surface. The results of the surface microhardness analysis were supported by the observed crystal morphology.

5. Conclusions

Nano-hydroxyapatite had the potential to remineralize initial enamel caries lesions under dynamic pH-cycling conditions. A suspension of 10% nano-hydroxyapatite appeared to be the optimal concentration for remineralization of early enamel caries. Nano-hydroxyapatite of proper concentration could therefore be beneficial in promoting remineralization with regular daily usage.

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Nano-hydroxyapatite vs. fluoride

KRISTINE BYRUM
Ohio '22

IT'S COMMON KNOWLEDGE that enamel is the hardest substance in the human body. To the general population, it is one of those random, good-to-know facts that might show up during a trivia night. What might not be as obvious, however, is why our tooth enamel is the hardest substance in the body. The answer is due to the presence of the naturally occurring mineral, hydroxyapatite.

What is hydroxyapatite?

Hydroxyapatite is the bioavailable form of calcium our bodies use to strengthen and rigidify bone and the hard tissues of teeth. Nano-hydroxyapatite is the synthetic version. Enamel, being roughly 97% hydroxyapatite by weight, is the most highly mineralized substance in the body. Due to this high level of mineralization, enamel is also the hardest substance in the body.

The layers upon layers of minerals, primarily hydroxyapatite, strengthen the tooth and serve to protect the inner tissues from decay and trauma. In the oral cavity, hydroxyapatite is also present in saliva, where it remineralizes and repairs the teeth. Just as amino acids are the building blocks for proteins, hydroxyapatite crystals are the building blocks for enamel.

Hydroxyapatite's "big bang" into the health care field

Hydroxyapatite's medical application begins with outer space. Hydroxyapatite was applied to NASA astronauts returning from space, whose bones and teeth suffered a significant loss in mass after experiencing a zero-gravity environment. Hydroxyapatite helped remineralize and repair the astronauts' bones and teeth. In 1978, the Japanese company Sangi Co. obtained the patent from NASA and advertised a new enamel-restoring toothpaste with hydroxyapatite as the main ingredient. Other toothpastes and products soon followed, due to the success of hydroxyapatite's regenerative and remineralizing capacities.

Fluoride vs. hydroxyapatite: Who wins?

While hydroxyapatite is the gold standard of oral health care for Japan, countries such the United States also have fluoride. Is one safer or more effective than the other? Hydroxyapatite and fluoride may appear to prevent tooth decay and harden enamel, yet they differ in subtle ways. Fluoride works to remineralize teeth by way of saliva. When fluoride is present in saliva and acids attack the teeth leaving them more vulnerable to decay, fluoride joins with the calcium and phosphate ions to create fluorapatite, a substance that is a great deal stronger and more resistant to dental decay. Nano-hydroxyapatite works directly on the tooth surface, mimicking natural enamel. The hydroxyapatite crystals repair and remineralize the enamel surface, bonding directly to the surface of the tooth to replace the sections of broken or dissolved enamel.

In addition to the differing mechanisms of action, patients claim fluoride and nano-hydroxyapatite have distinguishable tactile differences on the tooth surface. Product reviews show customers report their

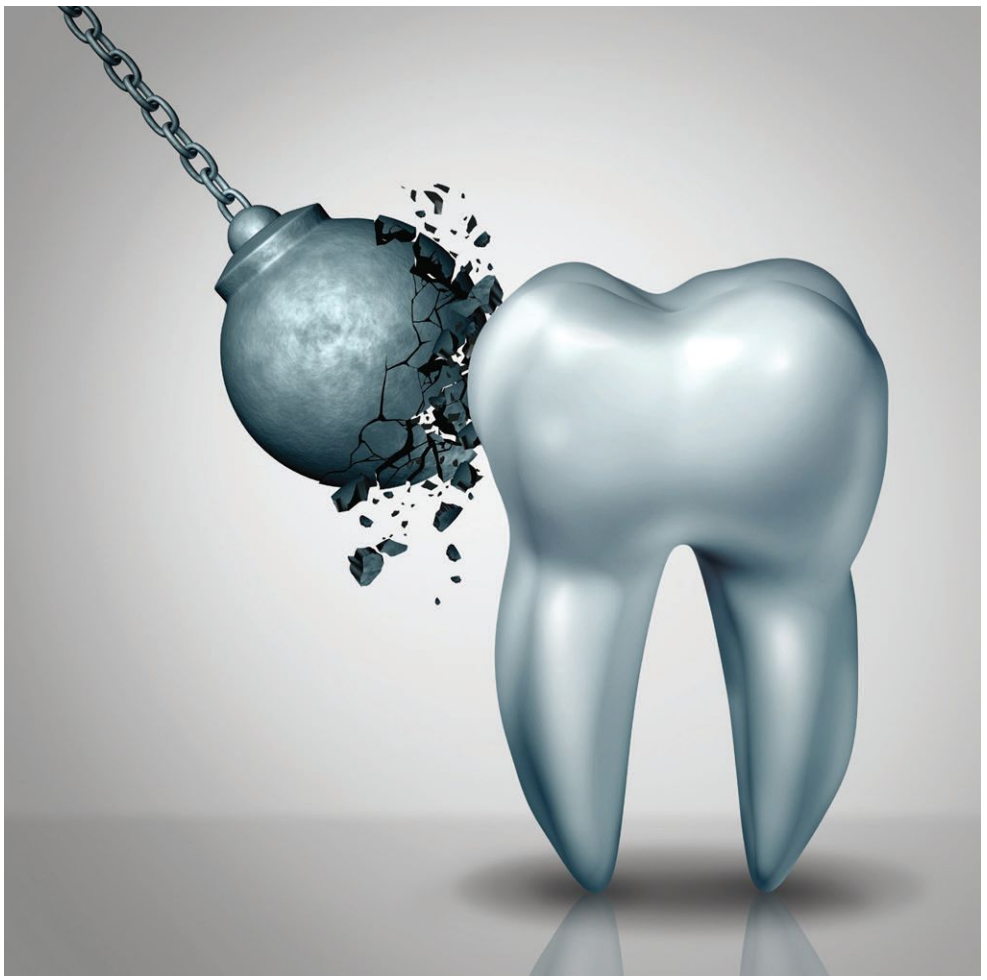
teeth feeling stronger, less sensitive to temperature changes, smoother, and healthier after brushing with a nano-hydroxyapatite toothpaste over fluoride toothpastes. One study published in the June 2011 Journal of Dentistry found "toothpaste(s) containing nano-hydroxyapatite had higher remineralizing effects compared to amine fluoride toothpastes." Another study published in the March Journal of Contemporary Dental Practice that analyzed remineralization, found "no significant difference between the fluoride varnish group and the nano-hydroxyapatite group."

As of today, nano-hydroxyapatite is not held superior to fluoride due to the lack of research evidence to support this claim. Rather, nano-hydroxyapatite is viewed as a valid alternative to fluoride and is even recommended over fluoride in cases involving dental hypersensitivity in which nano-hydroxyapatite can better maintain tooth integrity and avoid unwanted sensitivity.

The future

We live in a world where people consume more sugar and highly acidic food and beverages than any other time in history. This reality is a challenge for dentists who strive to prevent tooth decay before it causes irreversible damage. Nano-hydroxyapatite is another tool dentists can use to remineralize and protect teeth from decay in addition to fluoride products. It also offers some antimicrobial properties and fights soft tissue infection caused by pathogens, reduces tooth sensitivity and provide pain relief and makes the teeth whiter, glossier and more translucent. Because hydroxyapatite is a naturally occurring organic mineral in our bodies, nano-hydroxyapatite products are non-toxic and biocompatible, making them safer for human consumption than many other dental products on the market.

Product reviews show customers report their teeth feeling stronger, less sensitive to temperature changes, smoother, and healthier after brushing with a nano-hydroxyapatite toothpaste over fluoride toothpastes.



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Impact of a toothpaste with microcrystalline hydroxyapatite on the occurrence of early childhood caries: a 1-year randomized clinical trial

Elzbieta Paszynska[1,8]✉, Malgorzata Pawinska[2,8], Maria Gawriolek[1], Inga Kaminska[2], Justyna Otulakowska-Skrzynska[1], Grazyna Marczuk-Kolada[3], Szymon Rzatowski[1], Katarzyna Sokolowska[3], Aneta Olszewska[4], Ulrich Schlagenhauf[5], Theodor W. May[6], Bennett T. Amaechi[7] & Elzbieta Luczaj-Cepowicz[3]

The aim of this trial was to determine whether a toothpaste with microcrystalline hydroxyapatite is not inferior to a fluoride toothpaste in prevention of caries in children. This double-blinded randomized control trial compared two toothpastes regarding the occurrence of caries lesions using *International Caries Detection and Assessment System* (ICDAS) ≥ code 1 on the primary dentition within 336 days. The test group used a fluoride-free hydroxyapatite toothpaste three times daily while control group used a toothpaste with fluoride. 207 children were included in the intention-to-treat analysis; 177 of them finished the study per protocol. An increase in caries ICDAS ≥ code 1 per tooth was observed in 72.7% of the hydroxyapatite-group ($n = 88$), compared with 74.2% of the fluoride-group ($n = 89$). The exact one-sided upper 95% confidence limit for the difference in proportion of participants with ICDAS increase ≥ 1 (-1.4%) was 9.8%, which is below the non-inferiority margin of 20% demonstrating non-inferiority of hydroxyapatite compared to the fluoride control toothpaste. This RCT showed for the first time, that in children, the impact of the daily use of a toothpaste with microcrystalline hydroxyapatite on enamel caries progression in the primary dentition is not inferior to a fluoride control toothpaste (Clinical Trials NCT03553966).

Early childhood caries (ECC) is still one of the most prevalent diseases worldwide[1–3]. Children of any socioeconomic status can be affected by ECC[4,5]. Although a general trend in caries decline has been observed[6,7], current data show that caries is still a highly prevalent disease[8–11]. In Poland, for example, 76.9% of 5-year-old children and 89.4% of 7-year-old children are still affected by caries[8]. Even in Germany with a well-established health care system and a long-term record of declining caries prevalence figures still 13.7% of 3-year-old, 43.6% of 6–7-year-old, and also 21.2% of 12-year-old children have at least one tooth with a caries experience[9]. Other developed countries like Australia and the USA show a comparable high prevalence of ECC[10,11].

The prevention of dental caries in children and adults follows a multifactorial approach[12]. Besides promoting a healthy, low-sugar diet[13], a thorough preventive oral health care, i.e. toothbrushing with toothpaste, at home is advised to reduce the caries-risk[14].

Toothpastes should promote remineralization and inhibit demineralization of enamel and dentin to prevent dental caries[14,15]. Thus, fluoride provided as amine fluoride (e.g. Olaflur; $C_{27}H_{60}F_2N_2O_3$), sodium fluoride (NaF),

[1]Department of Integrated Dentistry, Poznan University of Medical Sciences, Poznan, Poland. [2]Department of Integrated Dentistry, Medical University of Bialystok, Białystok, Poland. [3]Department of Pedodontics, Medical University of Bialystok, Białystok, Poland. [4]Department of Facial Malformation, Pediatric Dentistry Clinic, Poznan University of Medical Sciences, Poznan, Poland. [5]Department of Conservative Dentistry and Periodontology, University Hospital Wuerzburg, Wuerzburg, Germany. [6]Society for Biometrics and Psychometrics, Bielefeld, Germany. [7]Department of Comprehensive Dentistry, School of Dentistry, University of Texas Health San Antonio, San Antonio, TX, USA. [8]These authors contributed equally to this work: Elzbieta Paszynska and Malgorzata Pawinska. ✉email: paszynska@ump.edu.pl

sodium monofluorophosphate (Na_2PO_3F), or stannous fluoride (SnF_2) are well-known for their caries preventing effect and consequently they are frequently used in many toothpaste formulations[6,14,16–18]. However, it is known that infants and toddlers swallow a substantial proportion of the applied toothpaste during toothbrushing[19–24], which can increase the systemic uptake of fluorides beyond a threshold associated with the occurrence of dental fluorosis and other unwanted side effects[19,25]. Therefore, in many countries toothpastes for children contain a reduced amount of fluorides compared to toothpastes for adults[17].

Besides fluorides, another approach for caries prevention is to focus on biomimetic and bio-inspired agents promoting remineralization and inhibiting demineralization of the dental hard tissue. One of these biomimetic agents is particulate hydroxyapatite (HAP; a calcium phosphate mineral; $Ca_5(PO_4)_3(OH)$)[26–32]. HAP has been studied in different fields of preventive oral health care[14,29–47]. Unlike fluoride, the accidental swallowing of HAP as a toothpaste ingredient is not associated with any relevant systemic health risks such as fluorosis, as hydroxyapatite is the main inorganic component of all human hard tissues, like teeth and bones[48].

In Germany, Italy, Japan, and other countries worldwide, HAP toothpastes are commercially available for many years[14,36,49]. The anti-caries efficacy of HAP-toothpastes could be shown e.g. in a placebo-controlled clinical trial in Japanese school children[38]. Based on this study, HAP was approved as anti-caries agent in Japan in 1993[14].

The caries-preventing efficacy of a HAP-toothpaste compared to fluorides has been evaluated in a randomized controlled 6-month trial[31] using *The International Caries Detection and Assessment System* (ICDAS)[31,50,51]. This study showed that in orthodontic patients the daily use of a HAP-toothpaste was not inferior to a fluoride toothpaste with 1400 ppm fluoride (amine fluoride and stannous fluoride) with respect to caries progression[31]. Due to the antibacterial fluoride counter ion (i.e. the ammonium salt), amine fluoride shows, in contrast to sodium fluoride and sodium monofluorophosphate, additional antibacterial/antibiofilm properties[52–54]. Additionally, a recent in situ study showed an effective remineralization of early caries lesions by a HAP toothpaste. Here, the remineralization effect was comparable to a toothpaste with 500 ppm fluoride (provided as amine fluoride). While the fluoride toothpaste showed a remineralization of mainly the surface-layer, the HAP toothpaste remineralized also the deeper enamel layers as revealed by transverse microradiography images[32]. While the optimal fluoride concentration of toothpastes for children is still subject to discussions (i.e. caries preventing efficacy vs. fluorosis-risk), the caries-inhibiting efficacy of children's toothpastes containing 500 ppm fluoride has been demonstrated by several clinical studies[16,17,55,56].

The objective of this clinical trial was to compare, for the very first time, the impact of the daily use of a HAP-containing children's toothpaste on the development of enamel caries in the primary dentition with use of a fluoride control toothpaste with proven caries preventive efficacy. Enamel caries development was monitored according to the criteria of the *International Caries Detection and Assessment System* (ICDAS II)[50,51]. The hypothesis of this study was that the impact of the evaluated fluoride-free biomimetic HAP-containing children's toothpaste on enamel caries development in the primary dentition is not inferior to the impact of a fluoride control toothpaste.

Subjects and methods

Study design and test centers. This multicenter, double-blind, randomized, active-controlled, parallel-group 336 days study was performed in children with an initial age of 3–7 years at the University Hospitals of Poznan and Bialystok, in Poland. The trial was approved by the ethics committees of the University Hospitals of Poznan and Bialystok and was registered at ClinicalTrials.gov (NCT03553966). Planning and conduct of the study were in accordance to the declaration of Helsinki and the principles of *Good clinical practice* (GCP). During the study, an external study monitor (Dr. Egmont Zieseniss, Inpharm-Consulting, Dortmund, Germany) regularly reviewed the case report forms to verify completeness, plausibility, data consistency, protocol adherence, and the progress of enrolment. He also ensured that study supplies were being stored, dispensed, and accounted for according to specifications.

Primary and secondary endpoints. *Primary endpoint.* Primary endpoint was the proportion of study subjects experiencing the development of at least one new enamel caries lesion ≥ ICDAS code 1 or the progression of an existing enamel caries lesion by at least one ICDAS score on any of the evaluated primary molars during the observation period of 336 days. The inspected areas were all enamel surfaces of the primary molars (i.e. buccal, distal, lingual, mesial, occlusal). In this study, ICDAS II was applied based on criteria described by Ismail et al.[50].

An enamel caries lesion with ICDAS code ≥ 1 or the progression of an existing enamel caries lesion by at least one ICDAS code in a given study participant was documented, when at least one of the assessed primary molars fulfilled one of the following conditions:

1. ICDAS code 0 on all surfaces at baseline [visit 2] AND ICDAS code ≥ 1 on at least one surface at follow-up [visit 3 to visit 6].
2. ICDAS code 1 (on at least one surface) at baseline [visit 2] AND ICDAS code ≥ 2 on at least one (not necessarily the same) surface at follow-up [visit 3 through visit 6].
3. ICDAS code 2 (on at least one surface) at baseline [visit 2] AND ICDAS code ≥ 3 on at least one (not necessarily the same) surface at follow-up [visit 3 to visit 6].
4. No filling (restoration) at baseline [visit 2] AND presence of a restoration (i.e. treatment of a caries lesion of ICDAS code ≥ 3) at the follow-ups [visit 3 to visit 6]

Secondary endpoints. Secondary endpoints were:

1. The percentage of study subjects experiencing the development of at least one new enamel caries lesions ≥ ICDAS code 2 on any of the evaluated primary molars (i.e. buccal, distal, lingual, mesial, occlusal surfaces) during the observation period (336 days),
2. The changes in the coverage of the assessed primary molars with bacterial plaque according to the criteria of the Plaque Control Record[57], and
3. The changes in the status of gingival health of the assessed primary molars according to the criteria of the Modified Gingival Index[58].

Inclusion criteria and exclusion criteria. The following exclusion and inclusion criteria were applied:

Inclusion criteria.

- Age 3–7 years
- Complete set of fully erupted primary molars (teeth 55, 54, 64, 65, 75, 74, 84, 85)
- Presence of a restoration (filling) due to caries on at least 1 primary molar
- Minimum of 4 primary molars without a restoration or sealed fissure/pit

Exclusion criteria.

- Untreated caries lesions of ICDAS code 3–6
- Known hypersensitivity to one of the ingredients of the toothpastes to be tested
- Systemic disorders interfering with salivary function or flow
- Regular medication intake interfering with salivary function or flow
- Need for antibiotic prophylaxis during dental treatments
- Participation in any other clinical study within the past 3 months or ongoing
- Lack of intellectual or physical ability to follow the instructions of the study protocol
- Any other reason that, in the opinion of the investigator, excludes the subject from eligibility for study participation

Treatment. Both test toothpaste (HAP) and control toothpaste (fluoride) were handed out to the parents of the study participants by study nurses, not being involved in the clinical assessment of the study parameters, using a computer-generated randomization list. Randomization was performed separately for each study center and was stratified for the number of restored primary molars at baseline.

Stratum A: Baseline number of primary molars with restorations ≥ 1 ≤ 2.
Stratum B: Baseline number of primary molars with restorations ≥ 3 ≤ 4.

Toothpastes and toothbrushes. To ensure blinding, both study toothpastes (HAP test, fluoride control) were provided in neutral plastic tubes of identical shape and color but differentiated with code numbers known to only the manufacturer. Both toothpastes were manufactured by a *Good Manufacturing Practice* (GMP) certified external laboratory.

The test toothpaste with 10% microcrystalline HAP[34] was identical in composition to a commercially available product (Kinder Karex Zahnpasta, Dr. Kurt Wolff GmbH & Co. KG, Bielefeld, Germany) and contained the following ingredients:

Aqua, Hydrogenated Starch Hydrolysate, Hydrated Silica, Hydroxyapatite, Xylitol, Silica, Cellulose Gum, Aroma, 1,2-Hexanediol, Caprylyl Glycol, Sodium Methyl Cocoyl Taurate, Sodium Cocoyl Glycinate, Sodium Sulfate, Limonene (pH in 1:10 aqueous solution was 7.7).

The fluoride control toothpaste with amine fluoride (500 ppm F^-) was also a commercially available product (elmex Kinder-Zahnpasta CP GABA GmbH, Hamburg, Germany) and contained the following ingredients in addition to amine fluoride: Aqua, Sorbitol, Hydrated Silica, Hydroxyethylcellulose, CI 77,891, Cocamidopropyl Betaine, Olaflur, Aroma, Saccharin, Limonene (pH in 1:10 aqueous solution was 4.8).

Toothbrushes. Next to the assigned toothpastes the study participants were also provided with a standardized electric toothbrush (Braun Oral-B Stage Power, P&G, Schwalbach, Germany).

Instructions were given to parents of the participants to brush the teeth of their children with the assigned toothpaste and the provided toothbrush for 3 min in the morning and in the evening over the observation period of 336 days.

Additionally, all study participants brushed their teeth themselves at noon for 3 min with the assigned experimental toothpaste using a manual children's toothbrush (elmex Kinder-Zahnbürste, CP GABA GmbH, Hamburg, Germany) and applying a horizontal scrub technique under the supervision of an adult. In total, brushing with the study toothpastes was performed 3 × daily. A brushing diary was used to monitor toothbrushing frequency.

Note that besides the study toothpastes, no other fluoride- and/or antiseptics-containing dental care products (mouthwashes, gels etc.) were used during this study. Furthermore, no professional tooth cleaning was performed.

Course of the study. During the course of the study 6 visits were scheduled at the clinics in Poznan and Bialystok, Poland.

Visit 1 (screening): 0–63 days before study start. Individuals potentially eligible for study participation and their parents were informed by the investigators about the aims, significance, and risks of study participation by a written patient information form and face to face interviews. Before study inclusion, the willingness of the child and the parents to properly follow the study protocol for the next 336 days was assessed. A child was included as study participant only after the parents had given their written informed consent. After informed consent was obtained, an initial examination took place to screen the potential subject for study eligibility (inclusion and exclusion criteria) and to document the subject's demographic data.

Visit 2 (baseline): study day 0, enrollment in the study, collection of baseline data. In case Visit 1 dated back more than 7 days, investigators reconfirmed that inclusion and exclusion criteria did not change and patients were still eligible for inclusion in the study.

Assessment of the 3 study parameters was done on all surfaces of the 8 molars in this following sequence:

1. Modified Gingival Index (GI): Gingivitis was assessed visually without touching the gingiva on the buccal and lingual marginal gingivae and interdental papillae of the included 8 primary molars. The gingiva was segmented into 6 sites per tooth (mesio-buccal, buccal, disto-buccal and mesio-lingual, lingual, disto-lingual), and gingival inflammation was recorded at each tooth site on a scale of 0 to 4 as described Lobene et al.[58]:
    - 0—Normal (absence of inflammation)
    - 1—Mild inflammation (slight change in color, little change in texture) of any portion of the gingival unit
    - 2—Mild inflammation of the entire gingival unit
    - 3—Moderate inflammation (moderate glazing, redness, edema, and/or hypertrophy) of the gingival unit
    - 4—Severe inflammation (marked redness and edema/hypertrophy, spontaneous bleeding, or ulceration) of the gingival unit
2. Plaque Control Record (PCR): Then PCR scores were assessed by touching the tooth surfaces with a blunt periodontal probe to evaluate the coverage of the assessed primary molars with bacterial plaque[57].
3. Afterwards, thorough teeth cleaning was performed by an experienced dentist in order to remove all dental plaque from the tooth surfaces. Professional teeth cleaning was not performed, as primary teeth and gingiva of those young children are very sensitive. Tooth brushing with a dentist was performed to ensure that all children started at the same baseline with respect to the coverage of the assessed primary molars with bacterial plaque.
4. Caries status (ICDAS): Caries assessment by ICDAS required the removal of adherent plaque by a toothbrush (see above).

    The teeth were first examined wet, then the surfaces were dried for 5 s with a dental air–water syringe, and again examined dry. All levels of caries lesions ranging from initial (non-cavitated) to cavitated lesions were visually identify on all surfaces (i.e. buccal, distal, lingual, mesial, occlusal surfaces) of each included primary molars, using the ICDAS II criteria (Scores 0–6) as described by Ismail et al.[50]:
    - Score 0: sound tooth surface;
    - Score 1: first visual change (opacity or discoloration) in enamel hardly visible on the wet surface but distinctly visible after air drying;
    - Score 2: distinct visual change (opacity or discoloration) in enamel, visible without air drying;
    - Score 3: localized enamel breakdown without visible dentin;
    - Score 4: underlying dark shadow from dentin without cavitation;
    - Score 5: distinct cavity with visible dentin;
    - Score 6: extensive distinct cavity with visible dentin.
5. After assessment of GI, PCR, and ICDAS score by the examiner, the study subjects received an electric toothbrush (for brushing in the morning and in the evening), a manual toothbrush (for brushing at noon), and the allocated toothpaste (test or control) from a trained study nurse not involved in the preceding recording of GI, PCR and ICDAS. Proper use of the assigned electric toothbrush, the manual toothbrush, and the issued toothpaste were also instructed by this study nurse or a dentist not involved in the clinical examinations. To confirm the subjects' understanding of the brushing instructions, they were requested to perform their first brushing episode there in the clinic under the supervision of the study nurse. Finally, subjects/parents received a brushing diary and new appointment date for the first follow-up visit (*visit 3*) at 84 days (± 14 days).

Visit 3: study day 84 (± 14 days), 1st follow-up examination; visit 4: Study day 168 (± 14 days), 2nd follow-up examination, and visit 5: study day 252 (± 14 days), 3rd follow-up examination.. At each follow-up visit, the GI, PCR and ICDAS were reassessed as described for the baseline visit. Subsequently, a study nurse not involved in the assessment of these study parameters (GI, PCR, ICDAS) collected back the used toothpaste tubes and toothbrushes given at the baseline visit, and supplied the subjects with new manual toothbrush, a new brushing head for the electric toothbrush, and a new supply of the assigned experimental toothpaste (test or control) for the next 84 days. Furthermore, for all visits, the study nurse checked the efficacy of the oral hygiene efforts (e.g. plaque removal) of the subjects by supervising another brushing episode there in the clinic, and if necessary, re-trained the subjects on effective brushing technique. Finally, subjects/parents received a new brushing diary and a new appointment for the subsequent visit.

Visit 6: study day 336 (± 14 days), final visit. 336 days after baseline visit, the GI, PCR, and ICDAS assessments were repeated as described for the baseline visit. Subsequently a study nurse not involved in the assessment of the study parameters (GI, PCR, ICDAS) collected back the used toothpaste tubes and toothbrushes given at the baseline visit. Subjects' parents were informed that subjects may now resume using their usual toothpaste.

Sample size calculation. It was assumed that the primary endpoint (development of at least one new enamel caries lesion ≥ ICDAS code 1 or progression of an existing enamel caries lesion by at least ICDAS code ≥ 1) would occur in about 70% of the study subjects during the observation period (48 weeks)[59]. The non-inferiority margin Δ was set to 20%[31]. A sample size of 2 × 75 study subjects was calculated to be sufficient to reject the null hypothesis that the test toothpaste is inferior to the control toothpaste, using a non-inferiority margin of Δ = 20% for the primary endpoint and one-sided, exact Fisher's Test (α = 5%, power = 90%). Assuming a drop-out rate of 25% of the study population, a total of 200 study subjects had to be included in the study to reach at least 150 study subjects for the analysis.

Statistical analysis. The primary outcome measure was analyzed primarily for the per protocol (PP) population and repeated for sensitivity reasons for the intent-to-treat (ITT) population. For the primary endpoint, non-inferiority was claimed if the exact one-sided upper 95% confidence limit (CI) for the corresponding difference between test and control toothpaste was less than the pre-set inferiority margin Δ (difference) of ≤ 20% [≤ 0.20][60]. In addition, logistic regression analyses were performed with the primary endpoint and the secondary endpoint of development of a new enamel caries lesion with ICDAS code ≥ 2 as dependent variable and toothpaste, center, stratum (1–2 filled molars vs. 3–4 filled molars), and age as independent variables (covariates). Analyses of covariance (ANCOVA) and non-parametric tests (two-sided Mann–Whitney test for between-group comparisons and Friedman tests for within-group comparisons) were performed for the secondary endpoints PCR and GI. IBM SPSS Statistics, version 25.0 (IBM Corp., Armonk, N.Y., USA) and SAS 9.4 software package (SAS Institute, Cary, NC, USA) were used for the statistical evaluations. Figures 2, 3, 4 and 5 were produced using the open software R (ggplot2)[61].

Note that the planned duration of the study was 336 day (48 weeks). But, in case that ≥ 70% of study participants would have experienced the primary study outcome (i.e. development of an enamel caries lesion ≥ ICDAS code 1 on one or more of the evaluated primary molars) already at Visit 4 (study day 168 [± 7 days], 2nd follow-up examination), the study would have been finished prematurely. The blinded interim analysis revealed that < 70% of study participants experienced the primary study outcome. Thus, the study was not prematurely finished.

Blinding and randomization. The study was designed as a double-blind trial. Using block randomization (provided by T.W.M.) with a block size of 4, a random list was generated to code the test and control toothpaste tubes with consecutive unique identification numbers. The randomization of toothpaste assignment was stratified by the study center and stratum (number of filled (restored) molars). Distribution of the experimental toothpastes to the study patients followed the sequence of the identification numbers and was performed by trained study nurses not involved in the examination of the study participants.

Inter-examiner reliability (ICDAS). All examiners (Poznan: 4 dentists, Bialystok: 5 dentists) involved in the practical assessment of ICDAS were trained before the onset of the trial by using the ICDAS e-learning course at the ICDAS website (www.iccms-web.com) as well as by intensive practical training of ICDAS assessment under the guidance of an experienced cariologist. Scoring skills of the investigators were trained at the start of study and retrained twice more during the course of the study using an additional internet-based ICDAS training tool (specially developed for this study; assessment of 26 clinical photographs of teeth with different ICDAS-scores; random sequence). Based on the results of this online tool, the linear weighted kappa coefficient was calculated as measure of the inter-rater reliability of ordinal scaled categories (ICDAS codes) between two raters. The inter-examiner reliability was calculated using the open source statistical software R with the package IRR (kappa2; option: weight = "equal "). In addition, the squared weighted kappa coefficients were calculated (kappa2; option: weight = "squared").

Results

Subjects. The demographic data of the per protocol (PP) population are shown in Table 1.

A total of 214 children were included in the study: 107 (50%) of them were assigned to the HAP toothpaste group and 107 (50%) to the fluoride toothpaste group. 202 (94.4%) subjects completed visit V6: HAP: *n* = 101 (50%), fluoride: *n* = 101 (50%). The study was prematurely terminated in 12 subjects (HAP: *n* = 6, fluoride: *n* = 6) for various reasons (Table 8). Due to major violations of the window for protocol-defined visits (> ± 4 weeks), 17 subjects (HAP: *n* = 9, fluoride: *n* = 8) were excluded from the per-protocol analysis. Furthermore, 8 subjects (HAP: *n* = 4, fluoride: *n* = 4) were excluded due to non-compliance from the per-protocol analysis. Non-compliance was defined based on subject diaries if application of toothpaste was ≤ 1 × daily in > 10% of study days (except last month of the study). Thus, 37 (17.3%) subjects were excluded from PP-population: HAP: *n* = 19, fluoride: *n* = 18. In total, 177 (82.7%) subjects were included in the PP set: HAP: n = 88 (82.2%), fluoride: n = 89 (83.2%).

All subjects who applied the test or control toothpaste at least once were included in the intention-to-treat analysis set (ITT). Missing values were replaced by last-observation-carried-forward (LOCF) provided that any follow-up visits (V3-V6) were performed. In 7 subjects the study was terminated already at visit V2 (Baseline), i.e. these subjects were not included in the ITT-analyses (HAP: *n* = 4, fluoride: *n* = 3). In total, 207 subjects were included in the ITT set: HAP: *n* = 103, fluoride: *n* = 104. The primary endpoint was analyzed for the PP set and, in addition, repeated for sensitivity reasons for the ITT set.

	Treatment					
	HAP toothpaste			Fluoride toothpaste		
	Stratum			Stratum		
	A	B	Total	A	B	Total
Sex						
Female						
N	36	12	48	40	12	52
%	52.9%	60.0%	54.5%	63.5%	48.0%	59.1%
Male						
N	32	8	40	23	13	36
%	47.1%	40.0%	45.5%	36.5%	52.0%	40.9%
Age (months)						
Mean						
	58.2	62.5	59.2	60.8	66.2	62.3

Table 1. Demographic data of the per protocol (PP) population.

Patient flow chart. The "Patient Flow Chart" according to the CONSORT Statement is shown in Fig. 1.

Primary endpoint. Table 2 summarizes the percentage of subjects who developed enamel caries lesions with ICDAS code ≥ 1 or a progression of an existing enamel caries lesion by at least one ICDAS code.

The exact one-sided upper 95% CI limit for the difference in proportion of subjects with ICDAS increase ≥ 1, per tooth as well as per surface was 9.5% which is clearly below the non-inferiority margin 20% (PP analysis) (Fig. 2). Thus, the test toothpaste (HAP) can be considered non-inferior to the control toothpaste (fluoride). This is also true for the exact two-sided upper 95% limit (11.6%). Superiority cannot be assumed as the confidence intervals are not below zero. The results of the ITT-analysis/Table 3) confirmed the results of the PP-analysis (Table 2).

In addition, a logistic regression analysis was performed with the primary endpoint as dependent variable and toothpaste, center, number of filled molars, and age as independent variables (covariates). The results for the PP population confirmed that the "risk" of development of new enamel caries lesion on ICDAS ≥ code 1 was not significantly dependent on "toothpaste" (HAP vs. fluoride).

Secondary endpoints. *Development of at least one new enamel caries lesions with ICDAS code ≥ 2.* The proportion of subjects with development of at least one new enamel caries lesion with ICDAS ≥ code 2 up to visit 6 (56.5%) was lower than the proportion of subjects with development of enamel caries lesions with ICDAS ≥ code 1 or the progression of an existing enamel caries lesion by at least increase in ICDAS ≥ code 1 (74.9%) (Tables 3 and 4).

The exact one-sided upper 95% confidence limits for the difference in proportion of subjects with ICDAS increase ≥ 2 up to visit (8.4%) were below the non-inferiority margin 20% [see primary endpoint] (PP analysis). This indicates that the test toothpaste (HAP) is non-inferior to the control toothpaste (fluoride) concerning this secondary endpoint (Fig. 3). This is also true for the exact two-sided upper 95% confidence limits. Superiority cannot be assumed as the confidence intervals are not below zero.

Plaque control record (PCR). The distributions of the Plaque Control Record (PCR) differentiated by toothpaste at visit 2 (baseline) to visit 6 (end of study of study, after 336 days) are shown in Table 5. The results indicate that the PCR scores only slightly differed between treatment groups (HAP vs. fluoride) but decreased in both treatment groups from (V2) to the end of study (V6) (Fig. 4).

The results of ANCOVA indicate the change/reduction of PCR (V6-V2) was not significantly different for both toothpastes ($p = 0.152$).

An additional ANCOVA that includes the interaction term "treatment x center" showed that effects of both toothpastes were not different in the centers Poznan and Bialystok. Moreover, a non-parametric test (Friedman Test) was performed to analyze whether PCR (coverage of the assessed primary molars with bacterial plaque according to the criteria) decreased during the whole observation period (visit V2 to visit 6). The Friedman test revealed that in each treatment group (HAP and fluoride) the decrease in PCR during the observation period (visit 2 to visit 6) was significant ($p < 0.001$).

Modified gingival index (GI). The modified Gingival Index (GI) was calculated as the mean of the modified GI scores of the eight included primary molars (teeth 54–85). Table 6 shows the descriptive statistics of modified GI score at visit V2 (baseline) to V6 (end of study) for both treatment groups/toothpastes. The results of ANCOVA indicate the change/reduction of modified GI (V6-V2) was not significantly different for both toothpastes ($p = 0.853$).

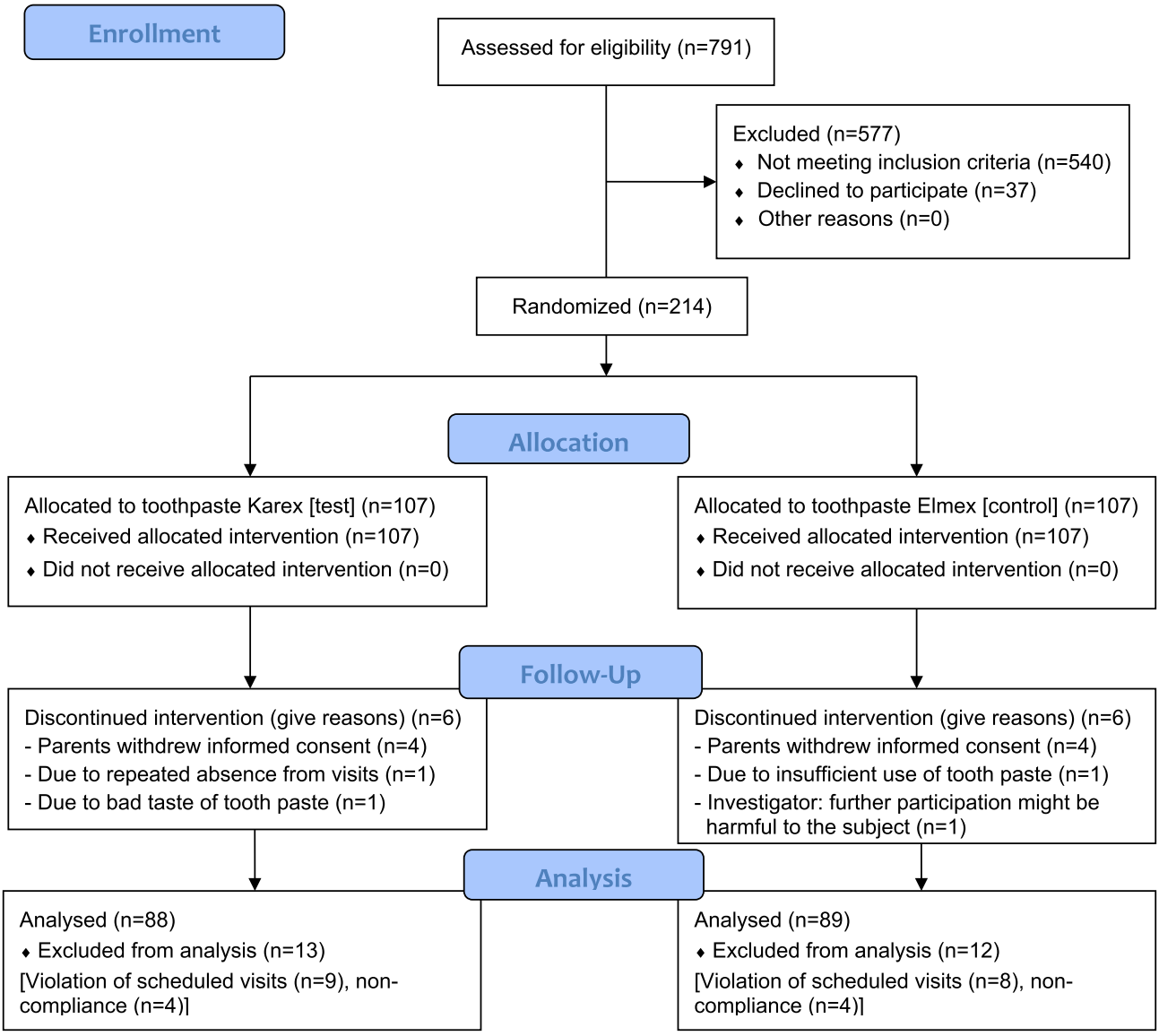

Figure 1. Patient flow chart.

	Treatment/toothpaste					
	HAP (test)		Fluoride (control)		Total	
PP-population	n	%	n	%	n	%
ICDAS increase ≥ 1 per tooth						
No	24	27.3%	23	25.8%	47	26.6%
Yes	64	72.7%	66	74.2%	130	73.4%
Total	88	100.0%	89	100.0%	177	100.0%
Primary endpoints	**Population**	**Difference HAP-fluoride**	**Lower CI95% one-sided**	**Upper CI95% one-sided**	**Lower CI95% two-sided**	**Upper CI95% two-sided**
Confidence Intervals for the "risk difference" HAP – fluoride						
ICDAS increase ≥ 1 per tooth	PP	– 1.4%	– .12.4%	9.5%	– 14.4%	11.6%

Table 2. Development of enamel caries lesions with ICDAS code ≥ 1 or the progression of an existing enamel caries lesion by at least one ICDAS code in the PP-population and confidence intervals for the "risk difference". CI95%, confidence interval; PP, per protocol; HAP, hydroxyapatite.

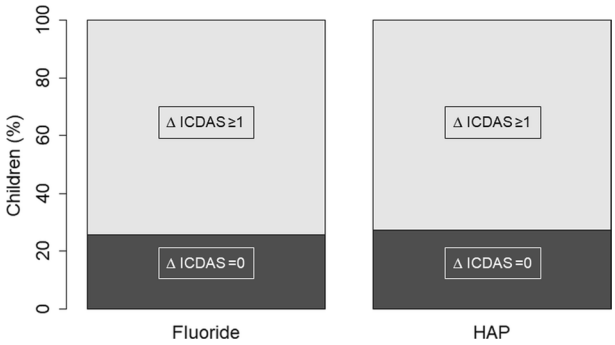

Figure 2. Increase in ICDAS ≥ Δ1 in the fluoride, and HAP-group. In both groups, the proportion of children who developed at least on one tooth a caries lesion of ICDAS ≥ Δ1 is not significantly different (more details are shown in Table 2).

	Treatment/toothpaste					
	HAP (test)		Fluoride (control)		Total	
ITT-population	n	% (column)	n	% (column)	n	% (column)
ICDAS increase ≥ 1 per tooth						
No	26	25.2%	26	25.0%	52	25.1%
Yes	77	74.8%	78	75.0%	155	74.9%
Total	103	100.0%	104	100.0%	207	100.0%
Primary endpoints	**Population**	**Difference HAP-fluoride**	**Lower CI95% one-sided**	**Upper CI95% one-sided**	**Lower CI95% two-sided**	**Upper CI95% two-sided**
Confidence intervals for the "risk difference" HAP-Fluoride						
ICDAS increase ≥ 1 per tooth (Version 1)	ITT	− 0.2%	− 10.2%	9.7%	− 12.1%	11.6%

Table 3. Development of enamel caries lesions with ICDAS code ≥ 1 or the progression of an existing enamel caries lesion by at least one ICDAS code in the ITT-population and confidence intervals for the "risk difference". CI95%, confidence interval; ITT, intent-to-treat; HAP, hydroxyapatite.

	Treatment/toothpaste					
	HAP		Fluoride		Total	
PP-population	n	%	n	%	N	%
Proportion of subjects with ICDAS increase ≥ 2 in at least one tooth up to visit 6 (after 48 weeks)						
No	40	45.5%	37	41.6%	77	43.5%
yes	48	54.5%	52	58.4%	100	56.5%
Total	88	100.0%	89	100.0%	177	100.0%
Secondary endpoint	**Population**	**Difference HAP-fluoride**	**Lower CI95% one-sided**	**Upper CI95% one-sided**	**Lower CI95% two-sided**	**Upper CI95% Two-sided**
Confidence Intervals for the "risk difference" HAP-Fluoride						
ICDAS increase ≥ 2 in at least one tooth up to visit 4 (after 24 weeks)	ITT	− 4.1%	− 16.4%	7.9%	− 18.3%	10.1%
ICDAS increase ≥ 2 in at least one tooth up to visit 6 (after 48 weeks)	ITT	− 3.9%	− 16.1%	8.4%	− 18.5%	10.7%

Table 4. Development of at least one enamel caries lesions with ICDAS code ≥ 2 in the PP-population.

In almost all cases, the categories "absence of inflammation" (= 0) or "change in color of any portion but not the entire marginal or papillary unit" (= 1) were reported. In two cases, "change in color which involves the entire marginal or papillary unit" was reported and in no case "distinctly red". The results on the GI are shown in Fig. 5.

Inter-examiner reliability (ICDAS). To test the inter-examiner reliability, linear and squared weighted Kappa coefficients were calculated (Table 7).

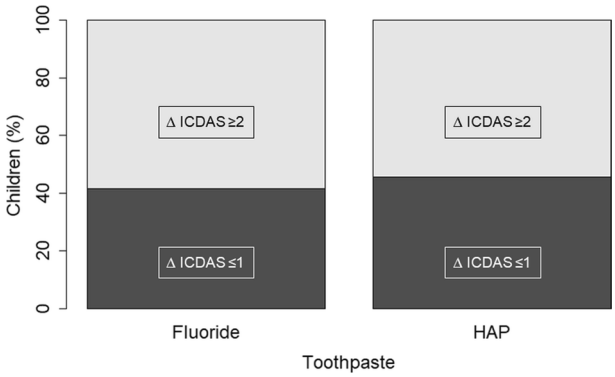

Figure 3. Increase in ICDAS ≥ Δ2 in the fluoride, and HAP-group. In both groups, the proportion of children who developed at least on one tooth a caries lesion of ICDAS ≥ Δ2 is not significantly different (more details are shown in Table 4).

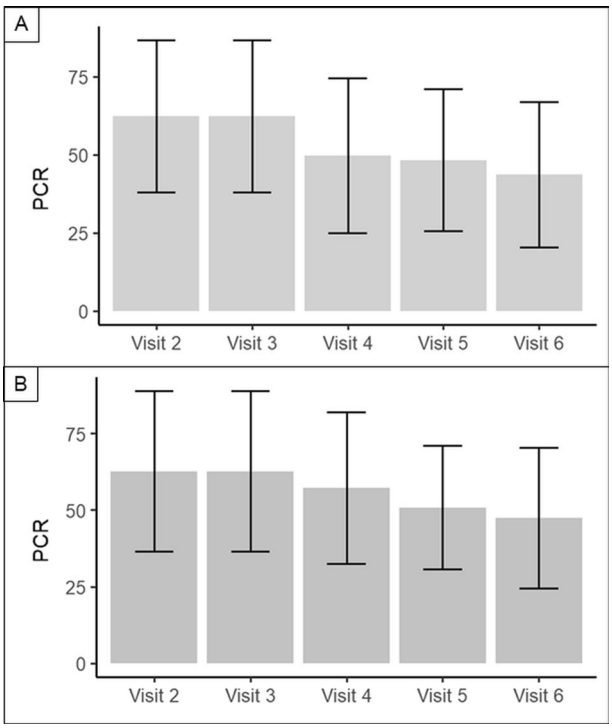

Figure 4. PCR-values from visit 2 to visit 6. A represents the HAP-group, while B shows the fluoride-group. In both groups the PCR-values decreased significantly (Friedman-test, $p < 0.001$). There was no difference between the HAP and the fluoride-group.

Safety. No serious and no severe adverse events (AEs) were reported in course of the study. In total, 79 AEs were reported in 48 subjects:

- 48 AEs in 28 subjects who applied HAP (test toothpaste) and
- 31 AEs in 23 subjects who applied fluoride (control toothpaste).

Table 8 summarizes severity, causality, and outcome of AEs. There were no statistically significant differences between the toothpastes ($p > 0.05$, two-sided exact Fisher Test).

Discussion

The persistent prevalence of ECC at a high level around the globe, despite the proven effectiveness of fluoride, justifies the need for other caries preventive materials that can be used by children of all ages at any concentration. The effectiveness of HAP to prevent caries development and promote caries remineralization has been

		Toothpaste	
		Hap	Fluoride
(V2) PCR index	Mean	62.4	62.7
	SD	24.3	26.2
	N	88	89
(V3) PCR index	Mean	62.4	62.7
	SD	24.3	26.2
	N	88	89
(V4) PCR index	Mean	49.8	57.2
	SD	24.8	24.7
	N	88	89
(V5) PCR index	Mean	48.3	50.8
	SD	22.8	20.2
	N	88	89
(V6) PCR index	Mean	43.7	47.5
	SD	23.3	23.0
	N	88	89

Table 5. Plaque Control Record (PCR) by toothpaste at visit 2 (baseline) to visit 6. *SD* standard deviation

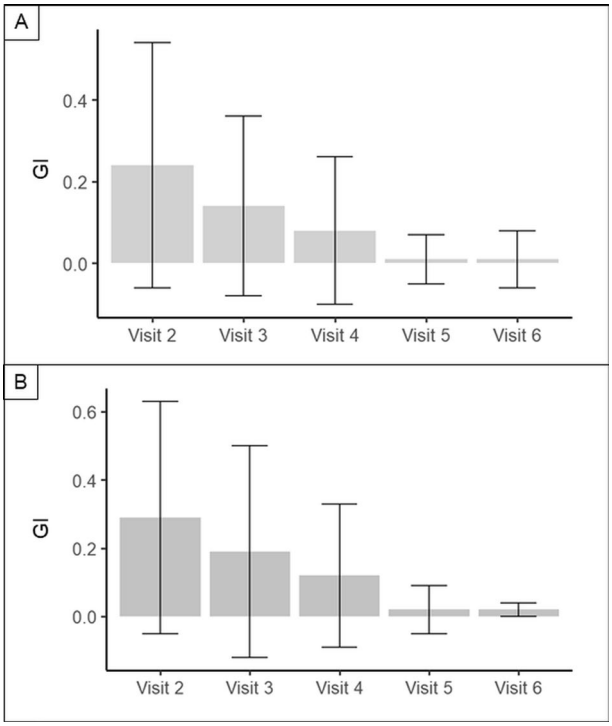

Figure 5. GI from visit 2 to visit 6. A represents the HAP-group, while B shows the fluoride-group. In both groups the GI decreased significantly. There was no difference between the HAP and the fluoride-group.

demonstrated in several clinical studies[31,32,38,42] This clinical trial was conducted to explore future proper clinical application of hydroxyapatite toothpastes. Thus, the present study investigated the non-inferiority of HAP toothpaste to fluoride toothpaste in children. As children swallow, intended or unintended, most of their toothpastes while brushing, this leads to an increased risk of developing (dental) fluorosis or other side effects. Therefore, an active ingredient is needed that is safe if swallowed and as effective as fluorides in caries prevention.

This is the second clinical trial on enamel caries development showing the non-inferiority of a fluoride-free HAP toothpaste compared to fluoride toothpastes with clinically proven caries-preventing effect[14,16,17]. Schlagenhauf et al. observed in a 6-month study in orthodontic patients an increase in enamel caries ICDAS code ≥ 1 in 56.8% (ITT) and 54.7% (PP) of the HAP group subjects compared with 60.9% (ITT) and 61.6% (PP) of the fluoride control group (with 1400 ppm fluoride)[31]. The non-inferiority of HAP compared to fluoride as confirmed

	Toothpaste	
	HAP	Fluoride
(V2) Modified GI		
Mean	.24	.29
SD	.30	.34
N	88	89
(V3) Modified GI		
Mean	.14	.19
SD	.22	.31
N	88	89
(V4) Modified GI		
Mean	.08	.12
SD	.18	.21
N	88	89
(V5) Modified GI		
Mean	.01	.02
SD	.06	.07
N	88	89
(V6) Modified GI		
Mean	.01	.02
SD	.07	.08
N	88	89

Table 6. Modified Gingival Index (GI) by toothpaste at visit 2 (baseline) to visit 6. SD, Standard deviation.

No. of training	Mean of linear weighted Kappa coefficients	Mean of square weighted Kappa coefficients
1 (Initial training)	0.82	0.91
2 (Retraining 1)	0.84	0.93
3 (Retraining 2)	0.83	0.92

Table 7. Overview of linear and squared weighted Kappa coefficients as measures of interrater reliability (re-training 1 and 2 was performed during the course of the study).

in our study (both for the primary and secondary endpoints regarding ICDAS; Tables 2, 3 and 4) goes in line with the results of a recently published in situ study by Amaechi et al. that analyzed the remineralization efficacy of a fluoride-fee HAP toothpaste on initial caries lesions in human primary teeth[32]. Moreover, a placebo-controlled trial published in 1989 demonstrated a caries preventing effect of a hydroxyapatite containing toothpaste in Japanese school children[14,38]. The non-inferiority of the HAP toothpaste compared to a fluoride toothpaste with antibacterial counter-ions on PCR and GI as shown in Tables 5 and 6 was already reported in other clinical trials[35,36].

Our results show that the development of new enamel caries lesion of ICDAS ≥ code 1 severity was observed in 73.4% of the subjects (Tables 2 and 3). This number seems very high but can be explained by the high prevalence of ECC in Poland and also by the fact that we used ICDAS to assess the caries status, i.e. non-cavitated caries lesions were included in the assessment. If perhaps we had used dmft index this number would be much lower. However, ICDAS was used in order to capture any developed lesion as early as possible. On the other hand, as expected, the proportion of subjects with development of at least one new enamel caries lesion with ICDAS ≥ code 2 up to visit 6 was lower (56.5%) than proportion of subjects with development of enamel caries lesions with ICDAS ≥ code 1 or the progression of an existing enamel caries lesion by at least ICDAS ≥ code 1. These findings are supported by data of Milsom et al.[59]. In that study, it was found that caries-active children have a 5–6 times higher incidence of new cavities compared to caries-free children. In our study we also chose caries-active children (the presence of a caries restoration (filling) on a minimum of 1 primary molar was one of the inclusion criteria) because of the very high prevalence of ECC in developed countries and especially developing countries[1,3,8–11]. Consequently, the caries risk of our study populations (i.e. a caries restoration on a minimum of 1 primary molar) represented the average caries risk within children. Thus, the HAP toothpaste was shown to be effective in a general population as well as in high caries-risk group such as orthodontic patients[31]. The PP and ITT analyses of the primary and secondary endpoint (ICDAS) of our study indicated slightly less, but not statistically different caries development in children that used HAP toothpaste compared to children that used fluoride toothpaste (Tables 2, 3 and 4). These results clearly confirmed the clinical non-inferiority of the HAP toothpaste compared to the fluoride toothpaste. The PP analysis showed that 27.3% children of the HAP group

	Treatment					
	HAP		Fluoride		Total	
	n	%	n	%	n	%
Severity						
Mild	40	83.3%	28	90.3%	68	86.1%
Moderate	8	16.7%	3	9.7%	11	13.9%
Severe	0	0.0%	0	0.0%	0	0.0%
Severe AE						
No	48	100.0%	31	100.0%	79	100.0%
Causality						
Probable/likely	1	2.1%	3	9.7%	4	5.1%
Possible	5	10.4%	0	0.0%	5	6.3%
Unlikely	41	85.4%	28	90.3%	69	87.3%
Unrateable	1	2.1%	0	0.0%	1	1.3%
Outcome						
Ongoing	0	0.0%	2	6.5%	2	2.5%
Resolved	47	97.9%	28	90.3%	75	94.9%
Resolved with sequelae	1	2.1%	1	3.2%	2	2.5%
Death	0	0.0%	0	0.0%	0	0.0%
Lost to follow-up	0	0.0%	0	0.0%	0	0.0%

Table 8. Overview of severity, causality, and outcome of adverse events.

and 25.8% of the children in the fluoride group did not show any change in caries status on the evaluated primary molars during the course of the study.

It is well-known that the concentration of fluorides in toothpastes for children (especially for infants and toddlers) has always been balanced against the potential fluorosis-risk[16,21,22]. Hellwig et al. demonstrated in an in situ study that 500 ppm fluoride significantly remineralized initial caries lesions in deciduous enamel, and this remineralization effect could not be improved by higher fluoride concentrations (i.e. 1,000 and 1,500 ppm fluoride)[17]. These findings are supported by in vivo data by Biesbrock et al.[56]. Consequently, we chose as control a toothpaste with 500 ppm fluoride because this represents a good compromise between caries preventing efficacy and fluorosis risk for children of the control group. Furthermore, teeth were brushed three times a day in our study, thus three fluoride impulses (not only 2 × daily toothbrushing like recommended by most dentists) were ensured in our study. Finally, we chose amine fluoride (control toothpaste), a more efficient fluoride compound than e.g. sodium fluoride. Amine fluoride shows (in contrast to sodium fluoride or sodium monofluorophosphate) antibacterial properties due to the ammonium salt (i.e. the counterion of the fluoride)[52,54]. Taken together, this proves that the use of the control toothpastes with 500 ppm fluoride provided as amine fluoride has caries preventing effects on the primary dentition. Consequently, the results of our study demonstrated the caries preventive effects of a HAP toothpaste which is non-inferior to a fluoride toothpaste.

The amount of toothpaste used in our study was 'pea-sized' because of regulatory reasons on fluoride in toothpastes[62]. In contrast to fluoride toothpastes, the amount of HAP toothpastes does not have to be 'pea-sized', because of the high biocompatibility of HAP[48]. However, due to the double-blind study design, the HAP toothpaste could not be dosed higher than the fluoride toothpaste in our study. In principle, the caries preventing effect of the tested HAP toothpaste may be further increased by using higher amounts of this toothpaste. Fabritius-Vilpoux et al., for example, demonstrated in an in vitro scanning electron study that the quantitative adhesion of HAP-particles to enamel surfaces can be increased by higher HAP concentrations in mouthwash formulations[34].

The clinical non-inferiority of HAP compared to fluorides as shown both in the study of Schlagenhauf et al.[31], and in our present study can be explained by different modes of action of HAP in the oral cavity. The modes of action of HAP are based on physical, bio-chemical, and biological principles (for details see e.g. Ref.[29]). HAP interacts with tooth surfaces and dental plaque[30,33,34,43,63,64]. Several in situ and in vitro studies have analyzed the modes of action of hydroxyapatite with respect to cavity protection. In situ and in vitro studies were able to show a remineralizing effect of HAP on dentin and enamel[32,39,45]. Here, TMR (transverse microradiography) was used to measure not only the mineral gain, but also a reduction in lesion depth when using a HAP-toothpaste[32,39,42,45]. Moreover, HAP significantly reduces bacterial colonization to the tooth surface *in situ*[30]. The effect of bacterial reduction to tooth-surfaces is comparable to chlorhexidine[30,40]. Furthermore, HAP is organized in microclusters, when formulated in toothpastes[34]. Those microclusters act as soft toothpaste abrasives[14,65]. Besides that, HAP has been shown to function as calcium- and phosphate-ion releasing active ingredient[33]. The same study shows its pH-buffering properties when present in acid-producing cariogenic biofilms[33,64,66]. Recent SEM (scanning electron microscopy) studies clearly confirm the attachment of the active ingredient HAP to the enamel surface as well as to dental materials[34,43]. Interestingly, Shaw et al., for example, reported significantly higher calcium and phosphorus levels in dental plaque of caries-free children compared to caries-active children[67]. Consequently, the use of biomimetic HAP as a calcium-phosphate-reservoir in dental plaque of caries-active children seems to be a promising approach to improve tooth remineralization, to decrease the level of tooth demineralization, and

thus to reduce the overall caries risk. The simplified chemical equations of HAP acting as a calcium-phosphate-reservoir under acidic conditions (cariogenic biofilms, erosive conditions due to dietary habits etc.) is[33]:

$$Ca_5(PO_4)_3(OH) + 7\ H^+ \rightarrow 5\ Ca^{2+} + 3\ H_2PO_4^- + H_2O.$$

Limiting factors of our study are as follows. In this study teeth were brushed with electric toothbrushes in the morning and in the evening by the parents in our study. Electric toothbrushes are known to be more efficient in plaque removal compared to manual toothbrushes[68]. On the other hand, the percentage of users of electric toothbrushes has increased within the last years, especially in developed countries. Since parents brushed the teeth of their child twice a day (the third brushing was performed by the children themselves under supervision of an adult), an influence of the age of the children (i.e. a possible improvement of motor skills within the study course) can be excluded.

A further limitation of our study is the unknown influence of the diet. It is well-known that sugar consumption and its frequency have a significant influence on caries development[13]. Here, we did not monitor the diet of each subject as the aim was to test two different toothpastes, but not the influence of the diet on the caries progress. Nevertheless, one may argue that we tested both, the HAP and the fluoride toothpaste, under real-life conditions due to the absence of any specific inclusion or exclusion criteria concerning the diet. However, we can assume that subjects had comparable dietary habits as both study centers are located in urban areas of Poland. A recently published meta-analysis on the nutrient intake by Polish pre-school children showed that the carbohydrate-intake is 24% higher than recommended[69].

A strength of the study is the fact that the influence of other preventive measures (e.g. the use of antiseptics like e.g. chlorhexidine or professional tooth cleaning) were excluded in our study, i.e. the study focused exclusively on toothbrushing with toothpaste (HAP vs. fluoride).

Caries lesions were assessed with ICDAS, a state-of-the-art system for measuring caries, which was developed by an international team of caries researchers[50,51,70]. In contrast to the frequently used dmft index, ICDAS offers the advantage to evaluate also initial enamel caries lesions (i.e. caries at a non-cavitated stage)[50]; thus, compared to dmft index, more caries lesions can be assessed using ICDAS. Investigation of initial caries is of great importance, because these early caries lesions can be remineralized by dental care products for home use either with HAP or fluoride[17,32,42]. Moreover, in a combined in vivo-in vitro study the use of ICDAS was shown to be more suitable than DIAGNOdent Pen and CarieScan PRO in detecting and assessing occlusal caries in primary molars[70]. The calculation of interrater reliability in our study indicated a high interrater reliability of the ICDAS scoring throughout the course of our study. This was shown both by the linear weighted and in particular the squared weighted Kappa coefficients between the raters and the benchmark rater (an experienced cariologist) and among the raters themselves (Table 7). Nevertheless, further clinical studies might analyze the impact of HAP toothpastes on DMFS/DMFT [dmfs/dmft] indices, thus making the results also comparable to previously performed studies which focus on fluoride toothpastes[16].

In general, due to the high risk for young children to develop caries in the primary dentition, as demonstrated in our study as well as previous studies, using a comprehensive caries-preventive approach, i.e. a combination of regular dental visits (at least every 6 months), thorough plaque removal by tooth brushing with electric toothbrush and toothpaste coupled with low sugar consumption[13], and when necessary, the application of fissure sealants[71–73], is well-suited for prevention or at least to significantly reduce the ECC-risk. These preventive measures are very important considering that it was been demonstrated that caries in the primary dentition of 7-year-old Polish children increased the risk for the development of caries in the permanent dentition for more than 5 times[8].

Conclusions

In children, the impact of the daily use of a toothpaste with microcrystalline HAP on enamel caries progression in the primary dentition is not inferior to that of a fluoride control toothpaste. Thus, the active ingredient hydroxyapatite is a biomimetic alternative to fluorides in toothpastes for children. Unlike fluorides (e.g. risk of dental fluorosis), HAP has a high biocompatibility and is safe if accidentally swallowed.

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Author contributions

*These authors E.P. and M.P. contributed equally to this work. E.P. and M.P. wrote the main manuscript. U. S., T.M. B.A. participated in the writing and revision, including review and editing of the manuscript. All other authors: M.G., I.K., J. O-S, G. M–K, S.R., K.S., A.O. and E. L-C had substantial contribution to the study, including conceptualization, methodology, investigation, supervision. All authors reviewed the manuscript.

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Competing interests

The authors declare no competing interest.

Additional information

Correspondence and requests for materials should be addressed to E.P.

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Introduction to Hydroxyapatite

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