UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE ODONTOLOGIA

NICOLE MARCHIORO DOS SANTOS

CARIES-PREVENTIVE EFFECT OF ANTI-EROSIVE FLUORIDE-FREE NANOHYDROXYAPATITE-CONTAINING TOOTHPASTE

Porto Alegre

2014

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Trabalho de Conclusão de Curso apresentado ao Curso de Graduação em Odontologia da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do título de Cirurgião-Dentista.

Orientador: Prof. Dr. Jonas de Almeida Rodrigues

Porto Alegre

2014

CIP - Catalogação na Publicação

Santos, Nicole Marchioro dos
Caries-preventive effect of anti-erosive fluoride-free nanohydroxyapatite-containing toothpaste / Nicole Marchioro dos Santos. -- 2014.
28 f.

Orientador: Jonas de Almeida Rodrigues.

Trabalho de conclusão de curso (Graduação) -- Universidade Federal do Rio Grande do Sul, Faculdade de Odontologia, Curso de Odontologia, Porto Alegre, BR-RS, 2014.

1. Dentifrícios. 2. Desmineralização. 3. Remineralização. 4. Microradiografia Transversal. 5. Prevenção de Cárie. I. Rodrigues, Jonas de Almeida , orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

AGRADECIMENTOS

Ao professor Jonas de Almeida Rodrigues, meu orientador, que sempre se mostrou disponível e compreensivo, me transmitindo confiança, me incentivando e me proporcionando oportunidades de crescimento e aprendizado.

À professora Marcella Esteves Oliveira, que além de me acolher em Aachen, me ajudou, me incentivou, confiou em mim; sempre com palavras de otimismo, me transmitindo ensinamentos e pela importante contribuição a este trabalho.

Ao técnico do laboratório de Aachen, Michael Stiebritz, por estar sempre disponível para me ajudar a realizar a parte experimental deste trabalho.

Ao Departamento de Dentística, Periodontia e Odontologia Preventiva da Faculdade de Medicina da Universidade RWTH Aachen, Alemanha, onde pude desenvolver a parte experimental do presente trabalho.

Aos membros da banca avaliadora, professora Juliana Jobim e professor Luciano Casagrande, que se disponibilizaram a avaliar este trabalho e que também contribuíram para o meu crescimento durante a minha formação acadêmica.

Ao Programa Ciências sem Fronteiras, que me proporcionou a oportunidade de ir estudar em Aachen e realizar este trabalho.

Bom mesmo é ir a luta com determinação, abraçar a vida com paixão, perder com classe e vencer com ousadia, porque o mundo pertence a quem se atreve e a vida é muito para ser insignificante.

Charlie Chaplin

RESUMO

SANTOS, Nicole Marchioro dos. **O efeito cárie-preventivo de um dentifício anti-erosivo sem flúor contendo nanohidroxiapatita.** 2014. 27f. Trabalho de Conclusão de Curso (Graduação) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2014.

O objetivo desse estudo foi investigar o efeito cárie-preventivo de pastas dentais contendo flúor, nanohidroxiapatita e livre de flúor desenvolvidas para prevenção de erosão. Uma vez que os ataques ácidos durante os episódios erosivos são muito mais fortes do que nos de lesões de cárie; a hipótese formulada foi que esses produtos poderiam fornecer uma proteção ainda mais elevada do que a promovida pelos produtos fluoretados desenvolvidos para prevenção de lesões de cárie. Noventa amostras de dentes bovinos foram obtidas e armazenadas em solução de timol. Após o polimento da superfície elas foram imersas em solução desmineralizadora de Buskes por 21 dias para criar lesões de cárie artificiais. A perda mineral inicial (ΔZ) e a profundidade de lesão (LD) foram determinadas através da microradiografia transversal (TMR). As amostras foram dividas de maneira randomizada em cinco grupos (n=18) e escovadas diariamente (2x/dia) usando: dentifrício não fluoretado, como controle negativo (NC), AmF (1400 ppm F) anticárie (AC); AmF/NaF (475 ppm F) + $SnCl_2$ (800 ppm Sn), anti-erosão (AE1); NaF (1400ppm F) + KNO_3, anti-erosão (AE2) e dentifrício com nanohidroxiapatita – contendo (0 ppm F) (NH). A escovação foi realizada por 14 dias, durante a ciclagem de pH, usando "slurries" das respectivas pastas e saliva artificial (tempo total de contato para cada amostra: 2 min). Após a ciclagem as amostras que apresentaram perda de superfície (maioria NC e NH) foram descartadas e para as 77 amostras remanescentes novas análises de TMR foram realizadas. As alterações na perda mineral (ΔΔZ; ΔZinicial - ΔZtratamento) e na profundidade de lesão (ΔLD; LDinicial - LDtratamento) foram calculadas e analisadas estatisticamente. Todas as pastas dentais apresentaram ΔΔZ significativamente inferior à NC (-4.557 ± 2.178% em volume × mM, p <0,05, teste de Kruskal-Wallys), exceto a NH (-2.119 ± 1.896% em volume × mM, p = 0,683). Ambos dentifrícios AE's e AC não diferiram significativamente na ΔΔZ (p>0.05). Com relação a ΔLD, apenas AE2 apresentou valores menores do que NC (p=0.033). Entre todos os outros grupos não foi observada diferença estatisticamente significante. Enquanto ambos dentifrícios anti-erosivos e o dentífrico anti-cárie reduziram a perda mineral numa extensão semelhante, a pasta de dental contendo nanohidroxiapatita parece não ser adequada para a inibição da progressão de cárie *in vitro*.

Palavras-chave: Dentifrícios. Desmineralização. Microradiografia transversal. Perda mineral. Prevenção de cárie. Remineralização.

ABSTRACT

SANTOS, Nicole Marchioro dos. **Caries-preventive effect of anti-erosive fluoride-free nanohydroxyapatite-containing toothpaste.** 2014. 27f. Final Paper (Graduation in Dentistry) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2014.

The aim of the study was to investigate the caries protective effect of fluoride and nanohydroxyapatite-containing fluoride-free toothpastes developed for erosion prevention. Since the acid challenges during erosive episodes are much stronger than in caries process, the hypothesis was that these products might show even superior caries-inhibiting effect than regular fluoride toothpaste. Ninety bovine enamel samples were obtained and stored in thymol solution. After surface polishing they were immersed in Buskes's demineralizing solution for 21 days to create artificial caries lesions. Baseline mineral loss (ΔZ) and lesion depth (LD) were determined by transversal microradiography (TMR). The samples were then randomly divided into five groups (n=18) and brushed 2x daily using: fluoride-free toothpaste, as negative control (NC); AmF (1400 ppm F) anti-caries (AC); AmF/NaF (475 ppm F) + $SnCl_2$ (800 ppm Sn), anti-erosion (AE1); NaF (1400ppm F) + KNO_3, anti-erosion (AE2) and nanohydroxyapatite-containing (0 ppm F) (NH) toothpastes. Brushing was performed during pH-cycling for 14 days using slurries of the respective toothpastes with artificial saliva (1:3 wt/wt, total contact time for each sample: 2 min). After cycling samples presenting lesion surface loss (mainly by NC and NH) were discarded and for the remaining 77 samples new TMR analyses were performed. The changes in mineral loss (ΔΔZ; ΔZbaseline - ΔZtreatment) and in lesion depth (ΔLD; LDbaseline - LDtreatment) were calculated and statistically analysed. All toothpastes caused significantly lower ΔΔZ than NC (-4557 ± 2178 vol%×µm, $p<0.05$; Kruskal-Wallys test), except for NH (-2119±1896 vol%×µm, $p=0.683$). Both AE's and AC toothpaste did not differ significantly in ΔΔZ ($p>0.05$). In respect to lesion depth, only AE2 showed statistically significantly lower ΔLD means than NC ($p=0.033$). Between all other groups no statistically significant differences were detected. While both anti-erosive and the anti-caries toothpastes reduced mineral loss to a similar extent, the nanohydroxyapatite-containing toothpaste seemed not to be suitable for inhibition of caries progression *in vitro*.

Keywords: Caries prevention. Demineralization. Dentifrices. Mineral loss. Remineralization. Transversal microradiography.

LISTA DE ABREVIATURAS

ΔZ	perda mineral
ΔΔZ	mudança na perda mineral (ΔZinicial – ΔZfinal)
LD	profundidade de lesão
ΔLD	mudança na profundidade de lesão (LDinicial – LDfinal)
TMR	microradiografia transversal
NC	grupo controle negativo
AC	grupo anti-cárie
AE1	grupo anti-erosão 1
AE2	grupo anti-erosão 2
NH	grupo nanohidroxiapatita

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1 INTRODUÇÃO

A cárie dentária é um doença que causa a destruição localizada dos tecidos duros suscetíveis do dente, através dos subprodutos ácidos da fermentação bacteriana dos carboidratos da dieta.[1] É uma doença bacteriana, geralmente crônica, específica do local e multifatorial. É um processo dinâmico, que resulta do desequilíbrio fisiológico entre o mineral dos dentes e do fluido da placa; isto é, quando há queda de pH resulta em perda mineral.[2] Lesões iniciais sofrem muitos ciclos de des- e remineralização antes de serem detectadas clinicamente e este processo pode ser interrompido a qualquer momento. [3,4]

Lesões de cárie de esmalte são caracterizadas pela perda mineral no corpo da lesão, enquanto a sua superfície continua altamente mineralizada. Em estágios iniciais, essas lesões podem ser paralisadas ou até mesmo remineralizadas.[4] Nesse estágio o tratamento mais frequente é a aplicação tópica de flúor, orientações de higiene bucal e dieta apropriada. [4,5]

Sabe-se que o flúor permanece como o mais eficaz agente anticárie para prevenir as lesões iniciais de cárie ou para controlar o avanço dessas lesões.[6] No presente momento novos dentifrícios têm sido lançados, afirmando serem eficazes contra erosão dentária, pois contém substâncias que prometem ser especificamente eficazes contra desafios ácidos mais agressivos (ex: SnF_2, nanohidroxiapatita ou biopolímeros), ou então, afirmam serem formulados para proporcionar a melhor disponibilidade de íon fluoreto. Contudo, há muito pouca informação sobre a eficácia destes produtos, particularmente em relação ao creme dental com flúor convencional.[7]

Fluoretos de estanho formam uma camada protetora na superfície do esmalte, que protege a partir de desafio de ácido, mas a eficácia da remineralização na presença dessa barreira ainda não é clara.[8] A hidroxiapatita age como um reservatório de cálcio e fosfato, ajudando a manter um estado de supersaturação em relação aos minerais do esmalte, assim diminui a desmineralização do esmalte e aumentando a remineralização.[9] As nanopartículas são semelhantes com os cristais de apatita do esmalte dentário tanto na sua morfologia quanto na estrutura do cristal e além disso, alguns estudos descrevem potencial para reparar o esmalte dental.[10]

Uma vez que os ataques ácidos durante os episódios erosivos são muito mais fortes do que nos de lesões de cárie; a hipótese formulada foi que os produtos fluoretados desenvolvidos para proteger o dente contra a desmineralização erosiva poderiam fornecer uma proteção mais forte do que a proteção dos produtos fluoretados desenvolvidos para prevenção de lesões de cárie. Com isso, o objetivo desse estudo *in vitro* foi investigar o efeito cárie-preventivo de pastas dentais contendo flúor, nanohidroxiapatita e livre de flúor desenvolvidas para prevenção de erosão.

2 ARTIGO CIENTÍFICO

Caries-preventive effect of anti-erosive and nanohydroxyapatite-containing toothpastes

M. Esteves-Oliveira[1], *N.M. Santos*[1,2], *J.A. Rodrigues*[2], *H. Meyer-Lückel*[1]
[1]Department of Operative Dentistry, Periodontology and Preventive Dentistry, RWTH Aachen University, Aachen, Germany; [2]Department of Pediatric Dentistry, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Brazil

Abstract: The aim of the study was to investigate the caries-preventive effect of fluoride and nanohydroxyapatite-containing fluoride-free toothpastes developed for erosion prevention. The hypothesis was that these products might show superior caries-inhibiting effect than the regular fluoride toothpaste. Ninety bovine enamel samples were obtained. After surface polishing they were immersed in Buskes's demineralizing solution for 21 days to create artificial caries lesions. Baseline mineral loss (ΔZ) and lesion depth (LD) were determined by transversal microradiography (TMR). The samples were then randomly divided into five groups (n=18) and brushed 2x daily using: fluoride-free toothpaste, as negative control (NC); AmF (1400 ppm F) anti-caries (AC); AmF/NaF (475 ppm F) + $SnCl_2$ (800 ppm Sn), anti-erosion (AE1); NaF (1400ppm F) + KNO_3, anti-erosion (AE2) and nanohydroxyapatite-containing (0 ppm F) (NH) toothpastes. Brushing was performed during pH-cycling for 14 days using slurries of the respective toothpastes with artificial saliva. After cycling samples presenting lesion surface loss (mainly by NC and NH) were discarded and for the remaining 77 samples new TMR analyses were performed. The changes in mineral loss (ΔΔZ; ΔZbaseline - ΔZtreatment) and in lesion depth (ΔLD; LDbaseline - LDtreatment) were calculated and statistically analysed. All toothpastes caused significantly lower ΔΔZ than NC (-4557 ± 2178 vol%×µm, $p<0.05$; Kruskal-Wallys test), except for NH (-2119±1896 vol%×µm, $p=0.683$). Both AE's and AC toothpaste did not differ significantly in ΔΔZ. In respect to lesion depth, only AE2 showed statistically significantly lower ΔLD means than NC ($p=0.033$). Between all other groups no statistically significant differences were detected. While both anti-erosive and the anti-caries toothpastes reduced mineral loss to a similar extent, the nanohydroxyapatite-containing toothpaste seemed not to be suitable for inhibition of caries progression *in vitro*.

Introduction

Dental caries is a localized destruction of susceptible dental hard tissue by acidic by-products from bacterial fermentation of dietary carbohydrates.[1] It is a bacterial driven disease, usually chronic, site-specific, multifactorial and a dynamic process that results from the imbalance in the physiologic equilibrium between the tooth mineral and the plaque fluid; that is, when the pH drop results in net mineral loss over time.[2] In which early lesions undergo many cycles of de- and itany point in time.[3,4]

Enamel caries lesions are characterized by a loss of mineral in the lesion body, whereas the surface remains comparably highly mineralized. In an early stage, these lesions can be arrested or even remineralized.[4] At this stage the most frequent treatment is the fluoride application, oral hygiene education and proper diet.[4,5]

It is known that fluoride remains one of the most clinically effective anticaries agent to inhibit white spot lesions formation or arrestment of early caries lesions.[6] At the present time new toothpastes have been marketed claiming to be specifically effective against erosion. They contain ingredients promised to exhibit particular efficacy against acid challenges (i.e. SnF_2, nanohydroxyapatite or biopolymers) or affirm to be formulated to provide the best availability of fluoride ion. Nevertheless there is very limited information on the efficacy of these products, particularly in relation to conventional fluoride toothpaste.[7]

Stannous fluorides are claimed to form a barrier layer on top of enamel, which protects it from acid challenge, but the efficiency of remineralization in the presence of this barrier is still not clear.[8] The hydroxyapatite acts as a calcium-phosphate reservoir, helping to maintain a state of supersaturation with respect to enamel minerals, thereby depressing enamel demineralization and enhancing remineralization.[9] Nano-sized particles have similarity to the apatite cristals of the tooth enamel in morphology and cristal structure and also some studies have describe potencial to repair dental enamel. [10]

Since the acid attacks during erosive episodes are much stronger than in the caries process, the hypothesis formulated was that fluoride products developed to protect the tooth against erosive demineralization could provide a stronger caries protection than the caries-preventive fluoride products. The aim of this *in vitro* study

was to investigate the caries-preventive effect of fluoride and fluoride-free nanohydroxyapatite-containing toothpastes developed for erosion prevention.

Materials and Methods

Samples preparation

From 100 bovine incisors, 250 enamel specimens (approximately 5 x 4 mm) were prepared.[5] After embedding in acrylic resin (Technovit 4071; Heraus Kulzer, Hanau, Germany) enamel surfaces were ground flat and polished (Waterproof Silicon Carbide Paper FEPA, 800, 1200, 2400, 4000; Struers) (**fig. 1**). Acid resistant nail varnish (P2 Kosmetik GmbH, made in EU, color 090) was applied on a part of the enamel surface (1-2 mm) of each specimen, leaving the rest of the surface unprotected (4mm). To create artificial enamel caries lesions in the unprotected area, specimens were stored in 5l of a demineralizing solution[15] containing 50 mM acetic acid, 3 mM CaCl2.H2O, 3 mM KH2PO4, 6 µM methylhydroxydiphosphonate and traces of thymol (pH 5,0; 37ºC) for 21days. During that period, pH was daily monitored and, when necessary, it was adjusted with small amounts of either 10% HCl or 10 mM KOH.

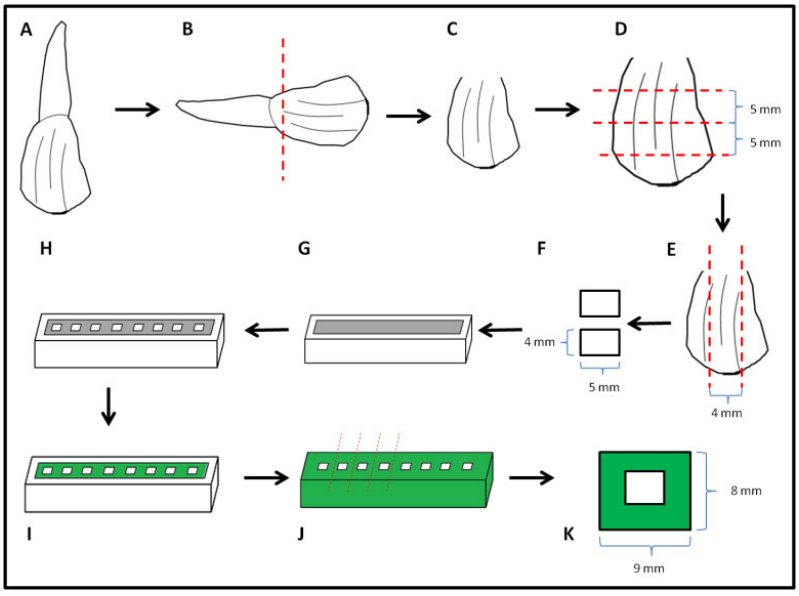

Fig 1- A) Frontal view of a bovine front tooth; B) Separation of crown and root; C) Bovine frontal crown; D) Perpendicular cut to the long axis of the bovine crown; E) Parallel cut to the long axis of the bovine crown; F) Samples after the two cuts; G)

Silicon cast; H) Samples inside the silicon cast; I) Silicon cast after embedding in acrylic resin; J) Acrylic resin with the samples showing the parallel cuts; K) Final sample.

After demineralization, all specimens showed artificial carious lesions. In order to calculate baseline mineral loss and lesion depth one fifth of each partially demineralized enamel specimen were cut perpendicular to the surface. These specimens were prepared for transversal microradiograph analysis as described below to select specimens with comparable and homogeneous demineralization. This way, 90 specimens presenting average integrated mineral loss (ΔZ) within 6027 ±1546 vol% ×µm were chosen from the 250 specimens originally prepared.[12]

Surface Treatment

The obtained specimens were divided into 5 groups, which received different treatments: fluoride-free toothpaste, as negative control (NC); AmF (1400 ppm F) anti-caries (AC); AmF/NaF (475 ppm F) + $SnCl_2$ (800 ppm Sn), anti-erosion (AE1); NaF (1400ppm F) + KNO_3, anti-erosion (AE2) and nanohydroxyapatite-containing (0 ppm F) (NH) toothpastes (Table 1).

Table 1- Enamel surface treatment at the different groups.

Groups		Toothpaste	N	Fluoride Content (F^-)	Active Ingridients*
1. Negative control	NC	Toothpaste Fluoride-Free, (Weleda, Germany)	n=18	0 ppm	--
2. Anti-caries Toothpaste	AC	Toothpaste Elmex Caries (GAGA, Germany)	n=18	1400 ppm	AmF (1400 ppm F)
3. Anti-erosion Toothpaste 1	AE1	Toothpaste Elmex Erosion Protection (GABA, Germany)	n=18	1400 ppm	AmF (700 ppm F) / NaF (700 ppm F) SnCl2 (3500 ppm Sn)
4. Anti-erosion Toothpaste 2	AE2	Toothpaste Pronamel (GlaxoSmithCline, Germany)	n=18	1150 ppm	NaF (1400ppm F) KNO3
5. Nanohydroxyapatite-containing	NH	Toothpaste Biorepair (Dr. Kurt Wolf, Germany)	n=18	0 ppm	Zinc-carbonate-hydroxyapatite (0 ppm F)

*according to the manufacturer

Twice a day all groups were immersed in toothpaste slurry (1 part of toothpaste and 3 parts of mineral solution, by weight) for 1min and 50s and brushed for 5s. The mineral solution was composed of 4.08 mmol H_3PO_4, 11.90 mmol $NaHCO_3$, 20.10 mmol KCl, and 1.98 mmol $CaCl_2$.[16,17] The mineral solution was supersaturated with respect to hydroxyapatite and had a pK-pI of 10.37.[7] The brushing force was 1.5 N (150 grams), standardized using a customized toothbrush holder (**fig. 2**) and a powered toothbrush connected to an oscillating-rotating brush head (Braun Oral B Professional Care 8500 + Brush Head: Precision Clean, Oral B, Procter & Gamble GmbH, Germany) was used.[17] The most external and longer bristles were removed from the brushing heads and only the center rounded-end bristles fitting exactly the exposed enamel area were maintained. In order to simulate the time needed to brush one tooth, the brushing time per sample was calculated dividing the mean brushing time of adults[18] by the total number of teeth (28) excluding the thirds molars.

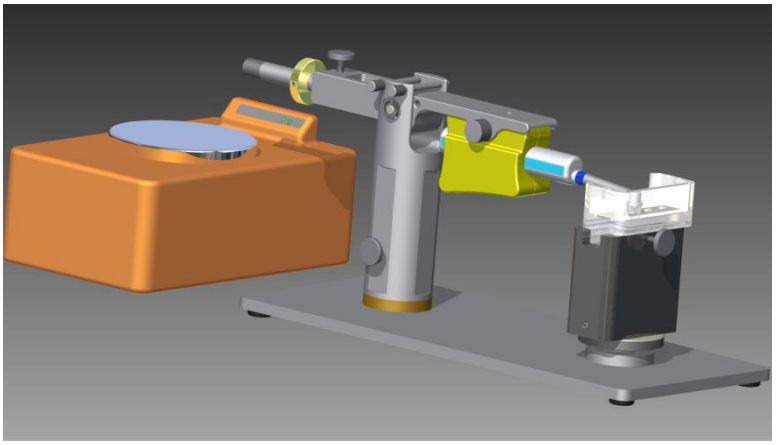

Fig. 2- Specially developed toothbrush holder to assure a standardized of 150 grams force during brushing. Before each 10 measurements brushing force was controlled using a laboratory balance. In order to cover the samples 25 ml of slurries was needed.

Toothpastes

From the toothpastes, slurries were prepared by mixing with the mineral salt solution (1 part toothpaste to 3 parts mineral solution, by weight). The slurries were freshly prepared at the beginning of each experimental day.[7] The pH values of all solutions were monitored with a pH-sensitive electrode.

Determination of Fluoride in the Slurries

For the fluoride-containing toothpastes, fluoride concentrations in the slurries prepared as described above were determined directly (D) and 12h after preparation. The slurries were centrifuged at 10000 g for 10 min and 1 ml of the supernatant was added to 1 ml TISAB II (Thermo Fisher Scientific, Beverly, Mass. USA). The fluoride concentration was determined with a fluoride-sensitive electrode.[11] Analyses were made in triplicate.

pH-cycling

At the surface of the selected samples (ΔZ= 6027 ±1546 vol% ×µm) the nail varnished protecting the control area was substituted for a flow resin (Tetric EvoFlow; A4, Ellwangen, Germany). Since in the next steps samples would also be brushed, a more mechanically resistant protection material was needed.

After surface treatment, samples were submitted to a pH-Cycling for 14 days.[15] The demineralization contained 3 m*M* $CaCl_2$ *2 H20, 3 m*M* KH_2PO_4, 50 m*M* CH_3COOH (latic acid) and 6 µ*M* methylhydroxydiphosphonate (MHDP), pH 5 (37°C) and the demineralization was done 4 times of 1 hour, during the day, in total 4 hours per day. The remineralization solution contained 1.5 m*M* $CaCl_2$ *2 $H_2$0, 0.9 m*M* KH_2PO_4 and 20 m*M* N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES) as buffer, pH 7 (37°C). The remineralization was done three times of 2 hours and another time of 12 hours, in total 18 hours per day (**Table 2**). For both solutions, pH was adjusted with KOH since they contained traces of thymol.[15]

Table 2. Demineralisation, remineralisation and brushing times during pH-cycling.

Duration	Event
1h	Demineralization
40 min	Brushing
2h20min	Remineralization
1h	Demineralization
3h	Remineralization
1h	Demineralization
3h	Remineralization
1h	Demineralization
40 min	Brushing
11h20min	Remineralization

Transversal microradiography analysis

After pH-cycling, samples were cut perpendicular to their dental enamel surfaces. Subsequently, specimens were mounted on a transparent plexiglas microscope slide and sections (200 µm) were cut (Exact 300 CL, Norderstedt, Germany). The slices obtained were grounded (1200, 2400 and 4000 grit, Exakt Mikroschleifsystem 400 CS; EXAKT Apparatebau,Norderstedt, Germany) from the opposite side to obtain a uniform thickness of 100 µm (±10). The parallelism of the samples was tested with a digital micrometer with a precision of 0.001 mm (Mitutoyo, Japan). Contact microradiographs of the enamel specimens were obtained with a nickel-filtered copper (CuKa) X-ray source (PW 1730; Philips, Kassel, Germany) operating at 20 kV and 20 mA. For all microradiographs an aluminium step wedge was used. The radiation source-to-film distance is 34 cm. The exposure time was 10 s, which was determined in preliminary experiments as optimum for the type of section used. A high-resolution film (Motion picture fine grain positive film 71337"; FUJIFILM Corporation Japan) was used and developed under standardized conditions according to the manufacturer's recommendations.

Microradiographs were first digitalized by an image-analysing system (DISKUS; programme for microscope discussion, photos and documentation; version 4.80; Königswinter, Germany) that is interfaced to an universal microscope (Leica DMRX; Germany). A TMR Software (version 5.25 by Joop de Vries, Groningen, The Netherlands) was used to calculate the mineral content depth profiles from the image scans and the reference step wedge data.

Calculation of mineral loss

The mineral content was calculated from the specimen grey levels using the formula of Angmar et al[19], assuming the density of the mineral to be 3.15 kg/l. The average mineral content of sound enamel was assumed to be 87 vol%, as measured by previous studies.[19,20] The lesion depth was calculated using a threshold of 95% of the mineral content of sound enamel (i.e. 82.7%). Integrated mineral loss (ΔZ), the lesion depth and the average mineral loss over the depth of the lesion (R) were also calculated.[21,22]

Mineral losses (ΔZ_{Sound}) and lesion depths (LD_{Sound}) of sound areas were subtracted from the respective values of demineralized surfaces ($\Delta Z_{Baseline}$, $LD_{Baseline}$) as well as from those of the surfaces being exposed to pH-cycling ($\Delta Z_{Treatment}$ $LD_{Treatment}$); these corrected values were then analyzed.[12] Changes in mineral losses ($\Delta\Delta Z = \Delta Z_{Baseline} - \Delta Z_{tTreatment}$) as well as changes in lesion depths ($\Delta LD = LD_{Baseline} - LD_{Treatment}$) were calculated.

Statistical analysis

As the mineral loss and lesion data were not normally distributed (Shapiro-Wilk, $p<0.05$) statistical analysis was performed by means of a Kruskal-Wallys test and post-hoc comparisons (alpha=0.05).

Results

Fluoride in the slurries

The highest amount of total soluble fluoride was detected for AE1 both immediately as 12 hours after slurry preparation. For NC and NH very insignificant amounts of fluoride were detected immediately and no fluoride at all after 12 hours (Table 3).

Table 3. Measurements free fluoride content and pH of the toothpaste slurries, directly after and twelve hours after preparation (12h).

Toothpastes	Slurries Directly				Slurries 12 h	
	pH		Free Fluoride (ppm)		Free Fluoride (ppm)	
Groups	Mean	SD	Mean	SD	Mean	SD
NC	-	-	8,8	±0,9	0	±0,0
AC	5,32	±0,07	2020	±20,0	1464	±6,9
AE1	5,08	±0,03	2680	±40,0	1863	±7,0
AE2	6,88	±0,03	1825	±11,4	1329	±9,2
NH	7,46	±0,02	86	±74,5	0	±0,0

TMR Analysis

Regarding mineral loss, all toothpastes caused significantly lower ΔΔZ than NC (-4557 ± 2178 vol%×µm, $p<0.05$), except for NH (-2119±1896 vol%×µm, $p=0.683$). Both AE's and AC toothpaste did not differ significantly in ΔΔZ ($p>0.05$). (Table 3 and Figure 1).

In respect to lesion depth, only AE2 showed statistically significantly lower ΔLD means than NC ($p=0.033$). Between all other groups no statistically significant differences were detected (Table 3 and Figure 2).

Table 4. Mean mineral loss (ΔZ) and lesion depth (LD) at baseline and after treatment as well as changes in mineral loss (ΔΔZ) and lesion depth (ΔLD) for all groups.

	Mineral loss						Lesion depth					
	Baseline ΔZ (vol%.µm)		Treatment ΔZ (vol%.µm)		ΔΔZ (vol%.µm)		Baseline LD (µm)		Treatment LD (µm)		ΔLD (µm)	
	Mean	SD	Mean	SD	Mean*	SD	Mean	SD	Mean	SD	Mean*	SD
NC	9868,2	2066,1	5310,4	696,8	-4458,0[a]	2178,0	145,2	19,6	209,6	35,7	-64,4[a,c]	39,9
AC	5968,0	1700,5	6583,4	1743,1	-615,5[b,c]	2542,4	136,4	25,5	196,6	42,4	-60,2[c,d]	53,7
AE 1	6016,1	1264,6	6116,6	1718,6	-100,5[b,c]	2244,7	133,8	27,7	167,4	44,8	-33,7[c,d]	43,2
AE 2	6059,3	1781,7	6524,9	997,5	-465,6[b,c]	2262,0	142,1	24,7	156,3	43,4	-14,3[b,d]	58,3
NH	7090,8	1802,1	9209,9	2206,3	-2119,1[a]	1896,4	174,8	33,7	214,0	42,6	-30,2[c,d]	39,1

*different superscript letters indicate statistically significant differences

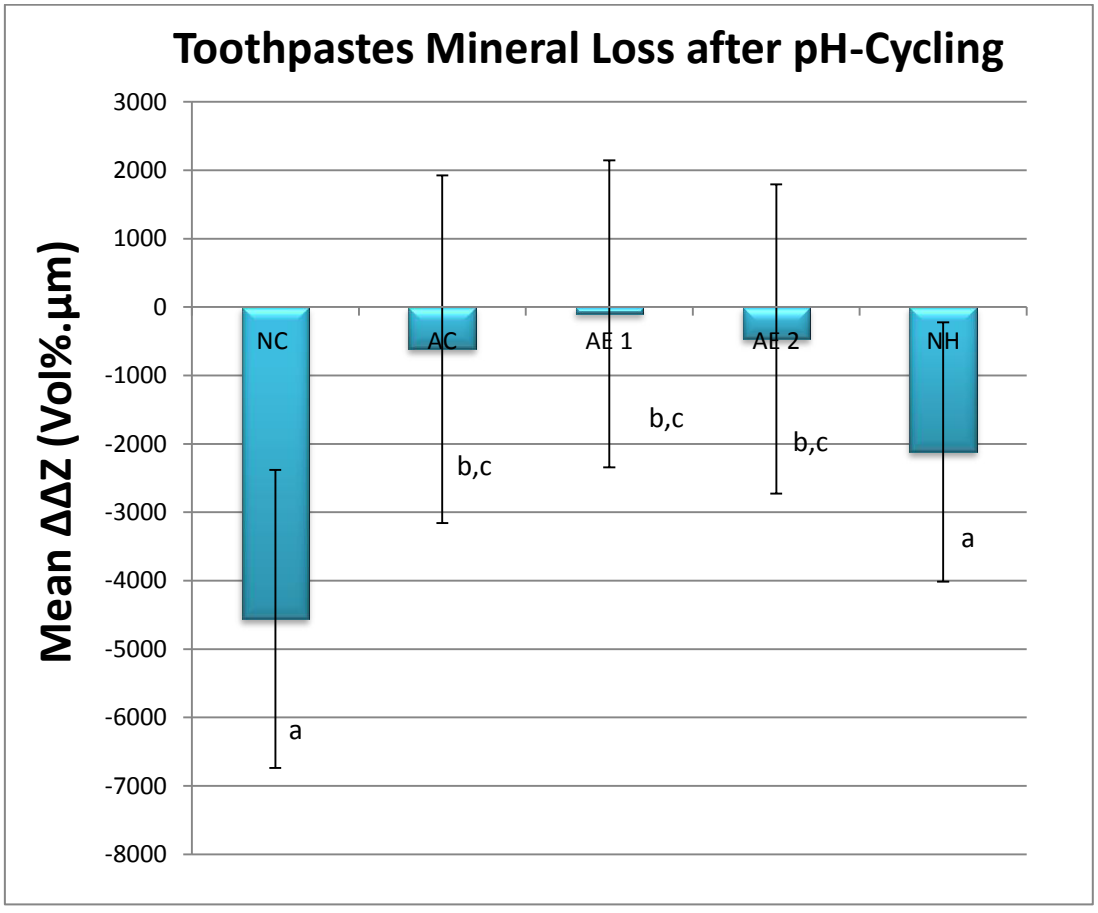

Figure 1. Toothpastes Mineral Loss after pH-Cycling. Different letters show significant differences (Kurskal-Wallis test).

As shown in graphic 1, AC, AE1 and AE2 toothpastes caused statistically significant lower ΔΔZ than NC (-4557 ± 2178 vol%×µm, p<0.05; Kruskal-Wallys test). NH toothpaste showed lower but not significant ΔΔZ values (-2119±1896 vol%×µm, p=0.683). Both AE's and AC toothpaste did not differ significantly in ΔΔZ (p>0.05).

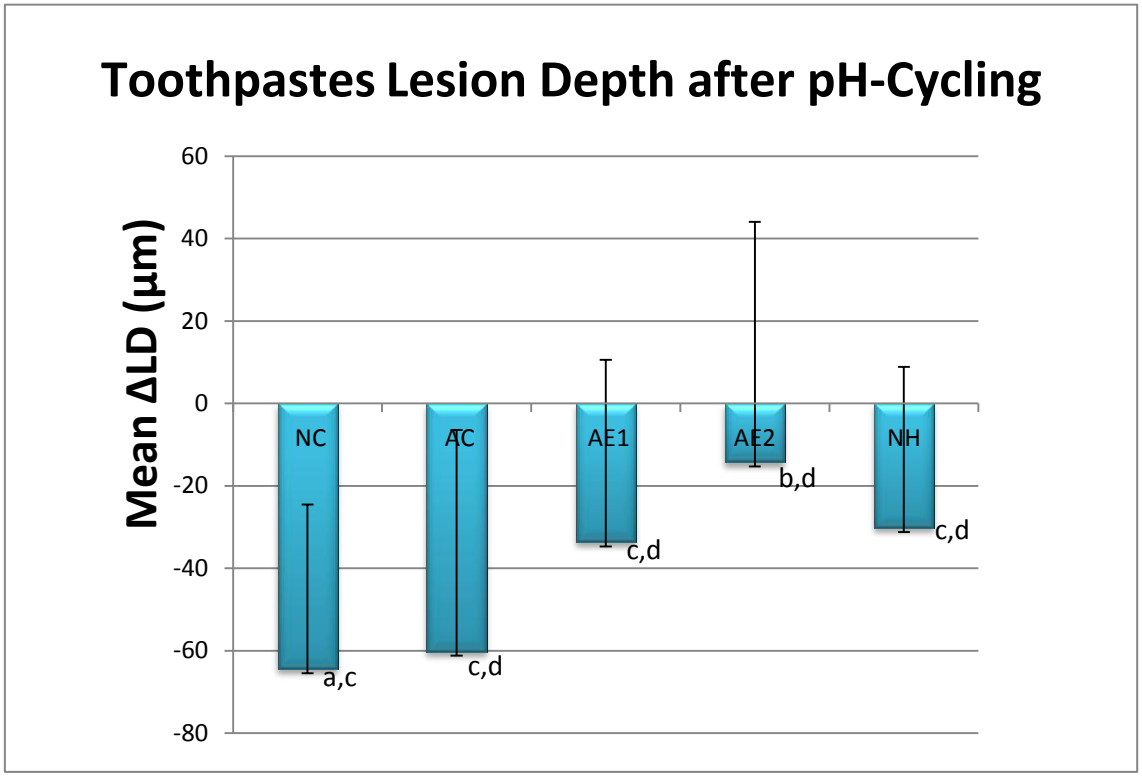

Figure 2. Toothpastes Lesion Depth after pH-Cycling

Figure 3 shows examples of enamel blocks after TMR analysis for each toothpaste used. The graphics show the lesion depth (µm) on x-axis and mineral loss (vol.%) on y-axis. The very superficial layer is more mineralized and in the body of the lesion the graphic drops down, due to the higher mineral loss. At deeper layers an increase of the mineral content can be observed, until the graphic becomes a constant line indicating that sound enamel was reached and the point where lesions end.

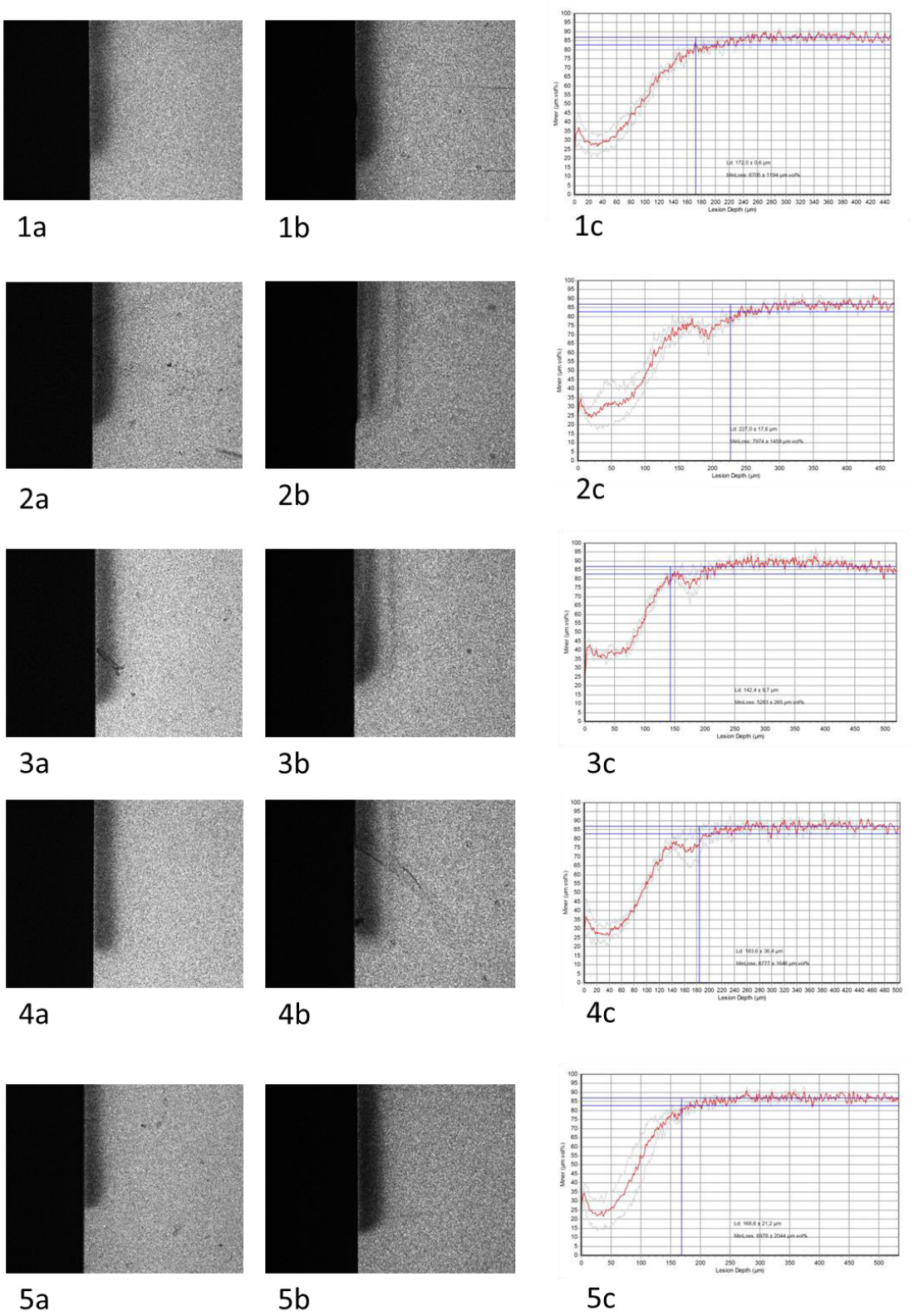

Fig 3. 1a) NC baseline TRM photo, 1b) NC after treatment TMR photo, 1c) NC TMR graphic; 2a) AC1baseline TRM photo, 2b) AC after treatment TMR photo, 2c) AC TMR graphic; 3a) AE1 baseline TRM photo, 3b) AE1 after treatment TMR photo, 3c) AE1 TMR graphic; 4a) AE2 baseline TRM photo, 4b) AE2 after treatment TMR photo, 4c) AE2 TMR graphic; 5a) NH baseline TRM photo, 5b) NH after treatment TMR photo, 5c) NH TMR graphic.

Discussion

This study was performed to test the hypothesis of whether erosion toothpastes containing amine fluoride and sodium fluoride (AE1), sodium fluoride (AE2) or a toothpaste with nanohidroxyapatite (NH) could be as good as or even better to inhibit demineralisation induced in vitro than a toothpaste containing only amine fluoride (AC).

Demineralization protocol was used to create artificial carious lesions and the TMR used to selected initial ΔZ with similar values for all the groups, once it has been demonstrated that, for the same demineralization protocol, lesions with lower initial ΔZ tend to demineralize more than lesions with higher initial ΔZ. Therefore, it was important to standardize the initial mineral loss of the samples, so this influential factor could be excluded from the analysis.[23]

Artificial enamel caries lesions are commonly created to simulated in vivo caries development.[11] The use of artificially created lesions over natural white spot lesions, as a study substrate, for caries research, in general is preferred. While this allows for better control, it is somewhat removed from the in vivo situation, especially since very little is known about the mineral distribution characteristics of naturally occurring white spot lesions.[12] To allow the formation of initial enamel lesion, the specimens are usually exposed to demineralising solutions and gels composed of acetic or lactic acid (pH between 4.5 and 5.0) undersaturated with respect to hydroxyapatite, due to simulate the plaque fluid conditions.

Transverse microradiography (TMR) has been the most used gold standard for diagnostic methods aimed at quantifying small lesions.[13] TMR provides a quantitative measure of the mineral and the lesion depth.[11,14]

The use of a fluoride-containing dentifrice also increased the similarities of the protocol to the real situation. Nowadays, about 95% of the dentifrices on the market in Europe, USA and developing countries are fluoridated, indicating that the majority of the world population brushes their teeth with this kind of products.[17] Toothpaste with the least amount of fluoride (NC, NH) showed the least preventive effect even if they contained another active substances like nanohidroxyapatite. This fact has been also demonstrated by other studies.[10,24] Queiroz et al, have demonstrated that the tested low fluoride dentifrice did not have the same performance to that of the

positive control, which contained 1.100 µg F/g.[24] Tschoppe et al 2011, have showed that treatment of specimens with nanohidroxyapatite and $ZnCO_3$ and nanohidroxyapatite toothpastes did not have superior results when compared to the pure reminaralization solution. From this outcome the authors speculated that nanohidroxyapatite has no influence at all for the remineralization.[10]

In accordance to the results of the present study, Camara et al, compared different experimental toothpastes (no F + hexametaphosphate – HMP –, named as placebo; 250 µgF/g + HMP; 500 µgF/g; 1,100 µgF/g and a commercial toothpaste – Crest with 1100 µgF/g – positive control). When the experimental toothpaste (1100 µgF/g) was compared with the positive control also containing 1100 µgF/g, both showed the same reduction of mineral loss. The experimental toothpaste containing 1100 µgF/g and the positive control a 2.7 times higher reduction of the mineral loss than the others toothpastes, revealing a greater effect of the fluoride on the outer enamel.[25]

As expected, the three toothpastes that had similar content of free fluoride in the slurries (AC, AE1, AE2), caused also similar reduction of mineral loss, with no statistically significant differences between their ΔΔZ values being detected. This indicates that the preventive effect of these toothpastes may be more related to the amount of free fluoride than to the type of fluoride compound (AmF/NaF/SnCl/KNO3).

In agreement with ours results, Toda et al., observed a comparable significant inhibitory effect on lesion formation from both the AmF 1,250 µgF/g dentrifice and the NaF 1,100 µgF/g dentrifice[26] and this result was consistent with that in previous animal[27], in vivo[28,29] and clinical studies.[30]

As it could be observed in the present study, some groups (AC, AE1 and AE2) showed a reduction of mineral loss despite the fact that in some of them (NC, AC, AE1 and NH) also an increase of lesion depth was observed. Only AE2 had a smaller mineral loss and at the same time avoided the lesion progression in depth.

Conclusion

Based on the results, both anti-erosive (AE1, AE2) and the anti-caries (AC) toothpastes reduced mineral loss to a similar extent and seemed to be suitable for caries inhibition *in vitro*. However, the nanohydroxyapatite-containing fluoride-free toothpaste seemed not to be suitable for inhibition of caries progression *in vitro*.

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3 CONCLUSÃO

Baseado nos resultados desse estudo, tanto os dentifrícios anti-erosivos (AE1, AE2), quanto o anti-cárie (AC) reduziram a perda mineral de maneira semelhante e sendo estes capazes reduzir a perda mineral, enquanto que o dentifrício contendo nanohidroxiapatita não pareceu ser capaz de inibir a progressão de lesões de cárie incipientes *in vitro.*

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