Giovanna Dalpasquale

EFEITO DA ADIÇÃO DE NANOPARTÍCULAS DE HEXAMETAFOSFATO DE SÓDIO EM DENTIFRÍCIOS FLUORETADOS SOBRE A DESMINERALIZAÇÃO DENTÁRIA: ESTUDO *IN VITRO*

Araçatuba – SP
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Orientadora: Profª. Drª. Marcelle Danelon
Coorientador: Prof. Titular. Alberto Carlos Botazzo Delbem

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"Me chamaste para caminhar na vida contigo,
Decidi para sempre seguir-te, não voltar atrás
Me puseste uma brasa no peito e uma flecha na alma
É difícil agora viver sem lembrar-me de ti...
Te amarei, Senhor, te amarei, Senhor
Eu só encontro a paz e a alegria
Bem perto de ti..." (Pe. Zezinho)

Aos meus irmãos Otávio e Gustavo,

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"Acredite no seu sonho, lute por ele, e quando tiver a oportunidade aproveite como se fosse um momento único em sua vida, e acredite ele pode não ser único, mas vai ser inesquecível." (Heitor Levinski)

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Epígrafe

"Peçam, e lhes será dado; busquem, e encontrarão; batam, e a porta lhes será aberta.

Pois todo o que pede, recebe; o que busca, encontra; e àquele que bate, a porta será aberta. "

Mateus 7:7-8.

Resumo

DALPASQUALE, G. **Efeito da adição de nanopartículas de hexametafosfato de sódio em dentifrícios fluoretados sobre a desmineralização dentária: estudo *in vitro*.** 2016 80f. Dissertação (Mestrado em Ciência Odontológica, área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2016.

Objetivo: Este estudo avaliou o efeito de dentifrícios contendo 1100 ppm F associado a nanopartículas de hexametafosfato de sódio (HMPnano) sobre a desmineralização do esmalte *in vitro*, utilizando um modelo de ciclagem de pH. Desenho: Blocos de esmalte bovino (4 mm x 4 mm, n=72) selecionados pela dureza de superfície inicial (SHi), foram alocados em seis grupos (n=12), de acordo com os dentifrícios teste: sem flúor ou HMPnano (Placebo), 550 ppm F (550F), 1100 ppm F (1100F), 1100F acrescido de HMPnano nas concentrações de 0,25% (1100F/0,25%HMPnano), 0,5% (1100F/0,5%HMPnano), e 1,0% (1100F/1,0%HMPnano). Blocos foram tratados 2x/dia com suspensões de dentífricios e submetidos a cinco ciclagens de pH (soluções desmineralizante/remineralizante) a 37 °C. A seguir, dureza de superfície final (SHF), perda integrada de dureza de subsuperfície (ΔKHN), concentração mineral ($g_{HAp} \times cm^{-3}$) e concentração de fluoreto (F) no esmalte foram determinadas. Os dados foram submetidos à ANOVA seguido pelo teste Student-Newman-Keuls ($p < 0,001$). Resultados: Dentifrício com 1100F/0,5%HMPnano levou à menor perda mineral e maior concentração mineral em relação aos demais grupos ($p < 0,001$), que foram de 26% (SHF) e 21% (ΔKHN) inferior e ~ 58% maior ($g_{HAp} \times cm^{-3}$) quando comparado ao 1100F ($p < 0,001$). Foram observados valores similares de F no esmalte para todos os dentifrícios

fluoretados (p > 0,001). Conclusão: A adição de 0,5%HMPnano ao dentifrício 1100F aumenta significativamente os seus efeitos anticárie quando comparado com o seu equivalente sem HMPnano.

Palavras-chave: Desmineralização, Esmalte dental, Dentifrício, Fosfatos, Dentifrícios, Nanopartícula.

Abstract

DALPASQUALE, G. **Effect on the addition of nano-sized sodium hexametaphosphate in fluoride toothpastes on tooth demineralization: an *in vitro* study.** 2016 80f. Dissertação (Mestrado em Ciência Odontológica, área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2016.

Objective: This study evaluated the effect of toothpastes containing 1100 ppm F associated with nano-sized sodium hexametaphosphate (HMPnano) on enamel demineralization *in vitro*, using a pH-cycling model. Design: Bovine enamel blocks (4 mm x 4 mm, n=72) selected by the initial surface hardness (SHi) were allocated into six groups (n=12), according to the test toothpastes: without fluoride or HMPnano (Placebo), 550 ppm F (550F), 1100 ppm F (1100F), 1100F plus HMPnano at concentrations of 0.25% (1100F/0.25%HMPnano), 0.5% (1100F/0.5%HMPnano), and 1.0% (1100F/1.0%HMPnano). Blocks were treated 2x/day with slurries of toothpastes and submitted to five pH cycles (demineralizing/remineralizing solutions) at 37 °C. Next, final surface hardness (SHf), integrated loss subsurface hardness (ΔKHN), enamel mineral concentration ($g_{HA}p{\times}cm^{-3}$) and enamel fluoride (F) concentration were determined. Data were analyzed by ANOVA and Student-Newman-Keuls' test ($p < 0.001$). Results: Toothpaste with 1100F/0.5%HMPnano led to the lowest mineral loss and the highest mineral concentration among all groups, which were 26% (SHf) and 21% (ΔKHN) lower and ~58% higher ($g_{HA}p{\times}cm^{-3}$) when compared to 1100F ($p < 0.001$). Similar values of enamel F were observed for all fluoridated toothpastes ($p > 0.001$). Conclusion: The addition of 0.5%HMPnano to a 1100F

toothpaste significantly enhances its anticaries effects when compared to its counterpart without HMPnano.

Keywords: Demineralization; Dental enamel; Phosphates; Toothpastes; Nano-size.

Lista de Figuras

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Table 1: Mean (SD) values of hardness and fluoride analysis according to the groups

Lista de Abreviatruras

LISTA DE ABREVIATURAS

ANOVA	Análise de variância
ºC	Graus Celsius
Ca	Cálcio
Ca^{+2}	Íon cálcio
CaF^{+}	Íon Fluoreto de cálcio
$CaHPO_4^0$	Fosfato de cálcio neutro
SD	Desvio padrão
F	Fluoreto
g	Grama
$g_{HAp} \times cm^{-3}$	Concentração mineral
h	Hora
HCl	Ácido clorídrico
HF^0	Fluoreto de hidrogênio neutro
HMP	Hexametafosfato de sódio
HMPnano	Hexametafosfato de sódio nanoparticulado
ie	Ou seja
IF	Fluoreto iônico
IML	Perda mineral integrada
KCl	Cloreto de potássio
kV	Quilovoltagem
KHN	Unidade de dureza Knoop
L	Litro
mA	Miliamper
MEV	Microscopia eletrônica de varredura
MicroCT	Microtomografia computadorizada
mL	Mililitro
mL/mm^2	Mililitro por milímetro ao quadrado
mm	Milímetro
mm^2	Milímetro quadrado
mol L^{-1}	Mol por litro
mmol L^{-1}	Milimol por litro

n	Número de amostra
Na^+	Íon sódio
NaF	Fluoreto de sódio
NaOH	Hidróxido de Sódio
nm	nanométrico
P	Fósforo
pH	Potencial de Hidrogênio
ppm F	Partes por milhão de fluoreto
s	Segundo
SEM	Microscopia eletrônica de varredura
SHi	Dureza de superfície inicial
SHf	Dureza de superfície final
TISAB II	Tampão ajustador de força iônica total
TMP	Trimetafosfato de sódio
SD	Desvio padrão
µg	Micrograma
µg/mm^3	Micrograma por milímetro cúbico
µg F/mL	Micrograma de fluoreto por mililitro
XRD	X-ray diffraction/Difração de raio-x
µm	Micrômetro
∆KHN	Perda integrada de dureza de subsuperfície

Sumário

SUMÁRIO

Capítulo 1

Effect on the addition of nano-sized sodium hexametaphosphate in fluoride toothpastes on tooth demineralization: an *in vitro* study

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Short title: Fluoride toothpaste with nano-sized hexametaphosphate

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***De acordo com as instruções aos autores do periódico Caries Research. (Anexo A)**

ABSTRACT

Objective: This study evaluated the effect of toothpastes containing 1100 ppm F associated with nano-sized sodium hexametaphosphate (HMPnano) on enamel demineralization *in vitro*, using a pH-cycling model. Design: Bovine enamel blocks (4 mm x 4 mm, n=72) selected by the initial surface hardness (SHi), were allocated into six groups (n=12), according to the test toothpastes: without fluoride or HMPnano (Placebo), 550 ppm F (550F), 1100 ppm F (1100F), 1100F plus HMPnano at concentrations of 0.25% (1100F/0.25%HMPnano), 0.5% (1100F/0.5%HMPnano), and 1.0% (1100F/1.0%HMPnano). Blocks were treated 2x/day with slurries of toothpastes and submitted to five pH cycles (demineralizing/remineralizing solutions) at 37 °C. Next, final surface hardness (SHf), integrated loss subsurface hardness (ΔKHN), enamel mineral concentration ($g_{HA}p \times cm^{-3}$) and enamel fluoride (F) concentration were determined. Data were analyzed by ANOVA and Student-Newman-Keuls' test ($p < 0.001$). Results: Toothpaste with 1100F/0.5%HMPnano led to the lowest mineral loss and the highest mineral concentration among all groups, which were 26% (SHf) and 21% (ΔKHN) lower and ~58% higher ($g_{HA}p \times cm^{-3}$) when compared to 1100F ($p < 0.001$). Similar values of enamel F were observed for all fluoridated toothpastes ($p > 0.001$). Conclusion: The addition of 0.5%HMPnano to a 1100F toothpaste significantly enhances its anticaries effects when compared to its counterpart without HMPnano.

Keywords: Demineralization; Dental enamel; Phosphates; Toothpastes; Nano-size.

INTRODUCTION

Dental caries is still a very common preventable chronic oral disease worldwide. Despite the widespread use of fluorides (F) in different vehicles and modes of application, dental caries is still a very common chronic disease during childhood [Melinda et al., 2014], affecting approximately a quarter of all children and negatively influencing their oral and general health and their quality of life [Ferreira et al., 2007; Tomar & Reeves, 2009]. This significant increase has been followed by the polarization phenomenon, according to which a high prevalence of the disease is observed in a small segment of the population [Ferreira et al., 2007]. This indicates an imbalance of preventive strategies, therefore resulting in individuals with high caries risk [Dye et al., 2007; Ditmyer et al., 2011]. This phenomenon is frequently connected to social-economic factors and to high consumption of sucrose [Ferreira et al., 2007; Nunes et al., 2014; Hennessy et al., 2015].

Giving the benefits and widespread use of F toothpastes [RØlla et al., 1991; Vanichvatana & Auychai, 2013], the increase of their efficacy would be extremely advantageous, especially for individuals with high caries experience [Danelon et al., 2015; Takeshita et al., 2015]. Among the strategies available, the supplementation of toothpastes with organic and inorganic phosphates has been intensively investigated over recent years. Takeshita et al. [2009, 2015] evaluated the protective effect of a low-fluoride toothpaste supplemented with sodium trimetaphosphate (TMP) on enamel demineralization *in vitro* and *in situ*, verifying a similar benefit when compared to conventional toothpastes (1100 ppm F). The synergistic effect of F and TMP was also verified for a conventional toothpaste *in vitro* when adding 3% TMP to a 1100 ppm F formulation [Castro et al., 2015].

Besides TMP, sodium hexametaphosphate (HMP) has also been shown to enhance the effect of toothpastes containing 250 ppm F [da Camara et al., 2014] or 1100 ppm F [da Camara et al., 2015]. Such effects occur due to specific characteristics of this phosphate, such as its capacity to form complexes with cations and its ability of reducing enamel solubility. This phosphate (HMP) is also highly used in the food industry as an antimicrobial agent for increasing permeability of the exterior of the membrane [van Dijk et al., 1980; Vaara, 1992; Andreola et al., 2004; Castellini et al., 2005].

Currently, nano-sized phosphates emerge as an innovative method, aiming to optimize the effect of the F toothpaste on demineralization and remineralization processes [Karlinsey & Zero, 2006]. The effect of nano- sized is a result of their physical and chemical properties that are superior when compared to the above-mentioned micrometric phosphates [Xu et al., 2010]. In addition, nanocomposites of several phosphates were shown to have effect on the metabolic activity of the dental biofilm and, consequently, in reducing acid production [Cheng et al., 2012]. In this sense, it was recently shown that the addition of 3% of nano-sized TMP was more effective to promote enamel remineralization *in situ* when compared to formulations supplemented with micrometric phosphates or not supplemented with any phosphate [Danelon et al., 2015].

Given the positive results obtained by the addition of nano-sized TMP on enamel remineralization and considering the absence of studies assessing the effects of nano-sized sodium hexametaphosphate (HMPnano), the aim of this study was evaluated the effect of toothpastes containing 1100 ppm F associated with HMPnano on enamel demineralization *in vitro*, using a pH cycling model.

The study's null hypothesis was that the effect of the toothpastes on enamel demineralization would not be influenced by the addition of HMPnano.

MATERIALS AND METHODS

Experimental Design

Enamel blocks (4 mm × 4 mm, n=72) were obtained from bovine incisors kept in formaldehyde 2% pH 7.0 for 30 days prior to experimental procedures [Delbem & Cury, 2002] (Anexo B). Blocks were sequentially polished, selected by initial surface hardness test (SHi; 320.0 to 380.0 KHN) and randomly divided in six experimental toothpastes (n=12 each) They were submitted to a pH-cycling regimen (5 days) and to treatments (twice a day) with the following toothpastes: without fluoride or HMPnano (Placebo), 550 ppm F (550F), 1100 ppm F (1100F), 1100F plus HMPnano at concentrations of 0.25% (1100F/0.25%HMPnano), 0.5% (1100F/0.5%HMPnano), and 1.0% (1100F/1.0%HMPnano). Next, final surface harness (SHf), integrated loss subsurface hardness (ΔKHN), enamel mineral concentration ($g_{HAp} \times cm^{-3}$) and enamel fluoride (F) concentration were determined.

Synthesis and characterization of nano-sized sodium hexametaphosphate

The synthesis and characterization of nano-sized HMP was based on study of Danelon et al. [2015]. In short, 70 g of pure sodium hexametaphosphate ($((NaPO_3)_6$), Aldrich, purity ≥ 95% CAS 7785-84-4, United Kingdom) was ball milled using 500 g of zirconia spheres (diameter of 2 mm) in 1 L of hexane. After 48 h, the material was filtered and sealed with aluminum foil, and the vials were dried at 75°C to evaporate the hexane (Anexo C).

X-ray diffraction (XRD) was used to identify the crystalline structure and estimating crystallographic coherency domain HMP and milled for 48h (HMPnano). The X-ray diffractograms were obtained from samples in powder form, using Shimadzu XRD 6000 equipment with CuK radiation source (λ = 1.54056 Å), voltage 30 kV and current of 30 mA. Measurements were made continuously, in the range of 10 ° ≤ 2θ ≤ 80 °, a 2 ° sweep speed/min. The structural identification of the samples was done by comparing the diffraction patterns obtained with tabulated patterns available in databases "Joint Committee on Powder Diffraction Standards - Powder Difraction File (JCPDS - PDF)". The particle morphology of HMP and HMP milled for 48h (HMPnano) was analyzed by scanning electron microscopy (SEM). The SEM images were collected using a Philips XL-30 FEG.

Toothpaste formulation and fluoride and pH assessment

Toothpastes were produced in the laboratory of Pediatric Dentistry Araçatuba Dental School (UNESP, Brazil) with the following components: titanium dioxide, carboxymethyl cellulose, sodium methyl-p-hydroxybenzoate, sucrose, mint oil, glycerine, abrasive silica, sodium lauryl sulfate, sodium sulfate and water [da Camara et al., 2015]. Toothpastes containing 1100 ppm F (as NaF, Merck, CAS 7681-49-4, Germany) and nano-sized HMP at concentrations of 0.25%, 0.5% and 1.0% were prepared. Also, a Placebo toothpaste (without F or HMPnano), and toothpastes with 550 ppm F (550F) and 1100 ppm F (1100F) were prepared with the same as the other toothpastes. F concentrations [Delbem et al., 2009] and pH [Moretto et al., 2010] of the all toothpastes were checked prior to the beginning of the study (Anexo D).

pH-cycling and treatment with toothpastes

The blocks were submitted, in individual vials, to five pH cycles during five days (one cycle/day), and immersed in a fresh remineralizing solution for two additional days [Vieira et al., 2005]. They were immersed under constant stirring in suspensions of toothpastes and deionized water (1:3-weight:weight) when removed from the demineralizing (6 hours – Ca and P 2,0 mmol L^{-1} in acetate buffer 0,075 mol L^{-1}, 0,04 µg F/mL in pH 4,7 – 2,2 mL/mm^2) and from the remineralizing solutions (18 hours – Ca 1.5 mmol L^{-1}, P 0.9 mmol L^{-1}, KCl 0.15 mol L^{-1} in sodium – buffer 0.02 mol L^{-1}, 0. 05 µg F/mL in pH 7,0 – 1,1 mL/mm^2). The blocks were washed with jets of deionized water for 30 seconds whenever blocks were removed from pH-cycling solutions or from toothpaste suspensions (Anexo E).

Analysis of enamel hardness

Surface hardness was determined with Micromet 5114 hardness tester (Buehler, Lake Bluff, USA) and Buehler Omni Met software (Buehler, Lake Bluff, USA) with a Knoop diamond indenter under a 25 g load for 10 s. Five indentations, separated by a distance of 100 µm, were made in the center of each block to analyze initial surface hardness (SHi). After the pH-cycling, final surface hardness (SHf) was calculated by producing five other indentations (100 µm from the baseline indentations). For cross-sectional hardness measurements, blocks were sectioned at the center and one of the halves was included in acrylic resin and gradually polished until the enamel was totally exposed. One sequence of 14

indentations were created at different distances (5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220, and 330 µm) from the surface of the enamel, in the central region of the blocks, using a Micromet 5114 hardness tester (Buehler Lake Bluff, IL, USA) with a Knoop diamond indenter under a 5-g load for 10 s. Integrated hardness (KHN × µm) for the lesion into sound enamel was calculated by the trapezoidal rule (GraphPad Prism, version 3.02) and subtracted from the integrated hardness for sound enamel to obtain the integrated area of the subsurface regions in enamel, which was named integrated loss of subsurface hardness (ΔKHN; KHN × µm) [Spiguel et al., 2009] (Anexo F).

Analysis of enamel mineral concentrations

Samples (n=10/per group, 1 mm x 1 mm) of each group were analyzed by micro computed tomography (MicroCT) operated at 70 kV, 142 mA, aluminum filter of 0.5 mm, at 1.5 mm of spatial resolution, rotation step at 0.400º and random moviment at 10. The projections of the images were rebuilt using the NRecon software (version 1.6.10.2, Skyscan1272, Bruker Micro-CT) and smoothing at 5, ring artifact correction at 7, beam hardening correction at 50%. Following image reconstruction, two-dimension virtual slices in the sagittal and coronal plane were acquired using the Data Viewer software (Skyscan1272). The stacked 2D was imported into ImageJ software to produce an overall mineral concentration ($g_{HAp} \times cm^{-3}$) profile in function of the depth (µm) based in the mass attenuation coefficients of inorganic (hydroxyapatite) [Dowker et al., 2003; Dowker et al., 2004].

The integrated area above the curve (cross-sectional profiles of mineral concentration into the enamel), using the mineral concentration values ($g_{HAp} \times cm^{-}$

^{3}), was calculated by trapezoidal rule (GraphPad Prism, version 3.02) in each depth (µm) from the lesion up to sound enamel. This value was subtracted from integrated area of sound enamel, to obtain the integrated area of the subsurface regions in enamel, which was named integrated mineral loss (IML) (Anexo G).

Analysis of F concentration in the enamel

Blocks (n=12/per group, 2 mm × 2 mm) were obtained from the halves of the original 4 mm × 4 mm specimens not embedded, and fixed with adhesive glue on a mandrel for straight. Self-adhesive polishing discs (diameter, 13 mm) and 400-grit silicon carbide (Buehler) were fixed to the bottom of polystyrene crystal tube (J-10; Injeplast, Sao Paulo, SP, Brazil). One layer of 50.0 ± 0.03 µm each enamel block was removed. The vials, after the addition of 0.5 ml HCl 1.0 mol L^{-1}, were kept under constant stirring for 1 hour [Weatherell et al., 1985, modified by Alves et al., 2007]. For F analysis, specific electrode 9409BN (Thermo Scientific, Beverly, MA, USA) and microelectrode reference (Analyser, São Paulo, Brazil) coupled to an ion analyzer (Orion 720A+, Thermo Scientific, Beverly, MA, USA) was used. The electrodes were calibrated with standards containing from 0.25 to 4.00 µg F/mL, under the same conditions as the samples. The readings were going to be conducted at rates of 0.25 ml of the biopsy of the solution with the same volume of TISAB II modified NaOH 1.0 mol L^{-1}. The results were expressed in µg/mm^3 (Anexo H).

Statistical analysis

For statistical analysis, SigmaPlot software version 12.0 (SigmaPlot, Systat Software Incorporation, San Jose, CA, USA) was used, and the significance limit

was set at 5%. The data presented normal (Shapiro-Wilk test) and homogenous (Cochran test) distribution. Data from SHf, ΔKHN and F (log transformation) and $g_{HAp} \times cm^{-3}$ (square root transformation) were submitted to 1-way analysis of variance followed by Student–Newman–Keuls test. Pearson's correlation coefficients between ΔKHN and $g_{HAp} \times cm^{-3}$ were also calculated.

RESULTS

The X-ray diffraction (XRD) pattern of nano-sized HMP 48h (HMPnano) after milling shows broader peaks due to the smaller crystallites (Figure 1). Grinding altered HMP, causing particles to fragment and by reducing their specific surface area and size distribution, but also by inducing structural changes such as crystallization of the material, as can be seen in Scanning electron micrographs of samples in Figure 2a shows the SEM images of HMP with large aggregates and particles of smaller sizes (average size of 31 ± 33 μm). Figure 2b shows MEV images of HMPnano particles with low size distribution and average size of 91 ± 34 nm.

Mean (SD) total (TF) and ionic (IF) F concentrations in the toothpastes were 10.0 (1.23) and 10.5 (0.91); 557.3 (11.41) and 546.1 (6.47); 1102,4 (28.54) and 1186,0 (33.19); 1136,5 (42.61) and 1168,3 (5.88); 1100,9 (27.16) and 1156,6 (19.74); and 1100F 1127,3 (19.71) and 1131,5 (8.41), respectively for Placebo, 550F, 1100F, 1100F/0.25%HMPnano, 1100F/0.5%HMPnano and 1100F/1.0%HMPnano. Mean pH of toothpastes was 7.3 (0.3), ranging from 6.8 to 7.7. Mean (SD) SHi considering all blocks was 374.8 (1.2) KHN. No significant differences were observed among the groups after random allocation (p = 0.474).

SHf of blocks treated with 550F and 1100F were approximately 182% and 306% higher compared to Placebo ($p<0.001$); The addition of 0.5% HMPnano to 1100F further increased SHf by ~26% when compared with1100F ($p < 0.001$), while concentrations of 0.25% and 1.0% promoted similar ($p = 0.542$) or lower ($p < 0.001$) SHf values, respectively, compared to 1100F (Table 1).

The integrated loss subsurface hardness (ΔKHN) was approximately 17% and 60% lower in the presence of F (Placebo × 550F toothpastes; Placebo × 1100F toothpastes, respectively). The association 0.5%HMPnano reduced the ΔKHN when compared with fluoride toothpaste without HMPnano (1100F) ($p < 0.001$). HMPnano at concentrations of 0.25% and 1.0% further increased subsurface hardness loss by approximately 13% and 34% compared to 1100F ($p < 0.001$), while the addition of 0.5%HMPnano reduced mineral loss by ~ 21% compared to 1100F ($p < 0.001$) (Table 1).

Table 1 and Figure 3 show the mean mineral concentrations ($g_{HAp} \times cm^{-3}$) for the different treatments. Significantly higher $g_{HAp} \times cm^{-3}$ (~58%) was observed in enamel treated with 1100F/0.5%HMPnano compared to 1100F ($p < 0.001$); no significant differences were observed among groups treated with 550F, 1100F/0.25%HMPnano and 1100F/1.0%HMPnano ($p > 0.001$). Positive and significant correlations were observed between ΔKHN and $g_{HAp} \times cm^{-3}$ (Pearson's $r = 0.874$; $p < 0.0001$) (Figure 4).

A dose-response pattern was observed between F concentrations in the enamel in toothpastes not supplemented with HMPnano and enamel fluoride uptake. The supplementation of 1100F with HMPnano did not significantly alter enamel fluoride levels ($p > 0.001$) (Table 1).

DISCUSSION

Fluoride toothpastes have been used as an auxiliary method for the control of dental caries, as the incorporation of F into the enamel crystals leads to the formation of fluohydroxirapatite, which is more resistant to acid attacks [Browne et al., 2005; Meyer-Lueckel et al., 2010], besides the ability to form CaF_2 reservoirs [Buzalaf et al., 2011]. The greatest advantage of using F toothpastes when compared to other forms of topical application is the regular delivery of F [Mellberg et al., 1985], along with removal or disorganization of the dental biofilm [Pessan et al., 2006].

Due to the limited effect of these products in caries control, new strategies have been considered to enhance their effects in reducing caries in the most affected population groups [Clifton et al., 2014]. To optimize conventional F toothpastes (1100 ppm F), this study evaluated the efficacy of a 1100 ppm F toothpaste, associated with different concentrations of HMP administered as nano-sizeds, in reducing tooth enamel demineralization. The results showed that HMPnano added at concentration of 0.5% promoted a superior efficacy against enamel demineralization when compared with a conventional toothpaste, leading to the rejection of the study's null hypothesis.

The evaluation of the dose-response relationship is an important step in assessing the effects of any supplement added to toothpastes, as well as to validate the experimental protocol used [ten Cate & Mundorff-Shrestha, 1995]. Such relationship was indeed confirmed, given that data from Placebo, 550F and 1100F were dose-dependent for all variables assessed (Table 1). Anbar et al. [1979] explained that the adsorption of a polyphosphate by the enamel surface is highly fast and can compete with the absorption of IF, which would, in turn,

decrease F diffusion into the enamel, thus leading to greater mineral loss. However, the addition of HMPnano at concentrations of 0.25% to 1.0% did not affect the concentration of IF in 1100F toothpaste, suggesting no changes in the availability of F and the effect of caries was maintained, in opposition to the findings of Anbar et al. [1979].

HMPnano concentrations tested in this study were based on HMP/F ratio from previous studies showing that the addition of micrometric particles of HMP at 0.5% and 1.0% significantly increased the anticaries effect of a 250 ppm F toothpaste to levels comparable to those of a 1100 ppm F toothpaste [da Camara et al., 2014; da Camara et al., 2015]. In the present study, the addition of 0.5%HMPnano to a 1100 ppm F toothpaste significantly increased surface hardness (~ 26%) and reduced subsurface hardness (~ 21%) in comparison with 1100F, justifying the use of nano-sized rather than HMP. The most spoken effect of 0.5%HMPnano concentration by ΔKHN suggests that, under clinical conditions, the subsurface lesion would take longer to develop when compared to conventional toothpaste. This is extremely desirable in a clinical perspective, as cavity could take longer to occur or would not even occur when using the formulation tested in this study. The use of nano-sized cyclic phosphate in this study, and the positive results, also agree with Danelon et al. [2015], who evaluated the addition of a nano-sized phosphate, as well cyclic sodium trimetaphosphate (TMP), in promoting remineralization of initial caries lesions *in situ,* by finding optimal and superior outcomes when compared to conventional fluoride toothpaste.

The procedure used to synthesize HMPnano promoted more reactive particles with an increase in adsorption into the enamel, due to the reduction in

size and increase in surface area (in proportion to its volume), which leads to a higher number of atoms on the surface and, therefore, best results (Figure 2). However, hardness data (SHf and ΔKHN) show that, only at concentration of 0.5%HMPnano, this effect is higher than other toothpastes to reduce mineral loss, ie, there must be an adequate molar ratio of F/HMPnano for optimal anticaries effect.

Besides the analysis of enamel by traditional hardness measurements (surface and cross sectional), the use of micro computed tomography after pH-cycling was of great importance to explain how treatments modified the mineralization patterns throughout the subsurface lesions and how these patterns were influenced by treatments with F, especially when associated with HMPnano. In the analysis of mineral concentration ($g_{HAp} \times cm^3$) through MicroCT at different depths, mineral profiles were very different between treatment groups, and the findings showed that, the 1100F 0.5%HMPnano toothpaste had the highest mineral concentration, with approximately 58%, compared to the toothpaste 1100F (Table 1 and Figure 3). The mineral profile of the lesion was also different, confirming previous findings that the F and HMP have a different action mode of the dynamics of caries process (Figure 3) [da Camara et al., 2014; da Camara et al., 2015].

Furthermore, the addition of HMPnano 1100F toothpaste has not increased the incorporation of F into the enamel, therefore, its effect was similar to 1100F toothpaste. The incorporation of F in areas of artificially demineralized lesions may be a positive indication of anticariogenic activity of the toothpaste, but it is not the only reason which a compound may inhibit demineralization or increase remineralization of enamel [Pfarrer et al., 2002]. The analysis of F content in the

enamel showed that, the F levels (Table 1) after the treatment with supplemented 1100F toothpastes, were similar to the 1100F toothpaste without HMPnano. This suggests that the presence of HMPnano does not interfere the incorporation of F, (ie: it can be concluded that the action of fluoride toothpastes with the HMPnano mechanism is somewhat different from what it is described for toothpastes containing F as the only active anticaries ingredient).

This study's findings can also be explained by the characteristics of the studied salt. The studied HMP is considered a non-hydrolyzable and cyclic phosphate [Choi et al., 1993; Castellini et al., 2005]. This type of cyclic phosphate form strong complexes with metal ions [Andreola et al., 2004; Cochrane et al., 2008] in the oral environment. These ions are absorbed into the enamel surface and retain the charged ions CaF^{+} and Ca^{2+} through the replacement of Na^{+} in the cyclic structure, leading to reticular formation [van Wazer & Campanella, 1950] through the binding of Ca^{2+} with one or more HMP molecules. As a result of these multiple connections, HMP molecules form a layer of condensed phosphates changing the selective permeability of the enamel [van Dijk et al., 1980] and, in this case, increasing the cations selectivity. These data are in agreement with findings of da Camara et al. [2015], where the ionic activity of species such as CaF^{+} and Ca^{2+}, as well as neutral species of HF^{0} and $CaHPO_4^{0}$ levels, significantly increased when compared to the 1100 ppm F toothpaste. There will be an increased calcium flow within the lesions, adding the mineral content into deeper layers of the lesion. According to van Dijk et al. [1980], compounds which alter the selective permeability of enamel can interfere in two processes that determine the progression of caries: the longitudinal diffusion through the enamel

pores due to their influence on ion selectivity and, somehow, in the dissolution of crystals of apatite.

Although it is known that the pH-cycling models are appropriate for evaluating the impact of new active ingredients in fluoride toothpastes as well as their association with other anticaries treatments, *in vitro* protocols have limitations: 1) as they do not address the impact of saliva/biofilm on the interaction of HMPnano with the enamel surfaces, 2) not just reproducing the intraoral conditions, 3) there is not the presence of saliva and plaque as found *in vivo*, 4) periods of des/re are faster than *in vivo* and 5) the reactivity of fluoride is lower than *in vivo*, which may result in incorrect evaluations or conclusions on the anticaries potential of toothpastes [Buzalaf et al., 2010]. Therefore, the results should be evaluated with caution, and, *in situ* study models of dental caries and clinical studies, in order to elucidate the effect on des/remineralization process, are recommended.

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Table legend

Table 1: Mean (SD) values of hardness and fluoride analysis according to the groups

Figure legends

Figure 1. X-ray patterns of the HMP and of the HMPnano after milling for 48h.

Figure 2. SEM images of Sodium hexametaphosphate particles, a) HMP and b) HMPnano after grinding of powder by 48h in ball mill.

Figure 3. Depth profiles of mineral concentration ($g_{HAp} \times cm^{-3}$) in lesions for each treatment.

Figure 4. Graphic representation of correlation coefficient between: ΔKHN and $g_{HAp} \times cm^{3}$.

Table 1: Mean (SD) values of hardness and fluoride analysis according to the toothpastes

Toothpastes	Variables			
	[1]SHf (KHN)	[2]ΔKHN (KHN x µm)	[3]Min. conc. (g_{HAp}×cm^{-3})	[4]F (µg/mm^3)
Placebo	61.2 (8.8)[a]	7373.4 (958.3)[a]	45.5 (10.3)[a]	0.6 (0.2)[a]
550F	172.6 (8.1)[b]	6096.1 (669.7)[b]	22.2 (3.6)[b]	0.9 (0.4)[b]
1100F	248.6 (9.1)[c]	3003.3 (403.4)[c]	8.7 (3.4)[c]	1.4 (0.8)[c]
1100F/0.25%HMPnano	240.5 (7.7)[d]	3390.6 (308.8)[d]	21.4 (4.0)[b]	1.1 (0.5)[b,c]
1100F/0.5%HMPnano	312.9 (7.3)[e]	2383.6 (282.9)[e]	3.7 (2.2)[d]	1.3 (0.4)[c]
1100F/1.0%HMPnano	238.7 (9.4)[d]	4024.5 (592.0)[f]	18.4 (7.2)[b]	0.9 (0.3)[b,c]

[1]SHf: final surface hardness - KHN

[2]ΔKHN: integrated loss of subsurface hardness - KHN x µm

[3]Min. conc: Mineral concentration in lesion - g_{HAp}×cm^{-3}

[4]F: Fluoride concentration in enamel - µg/mm^3

Distinct superscript lowercase letters indicate statistical significance among groups in each variable (1- way ANOVA, Student-Newman-Keuls test, $p < 0.001$).

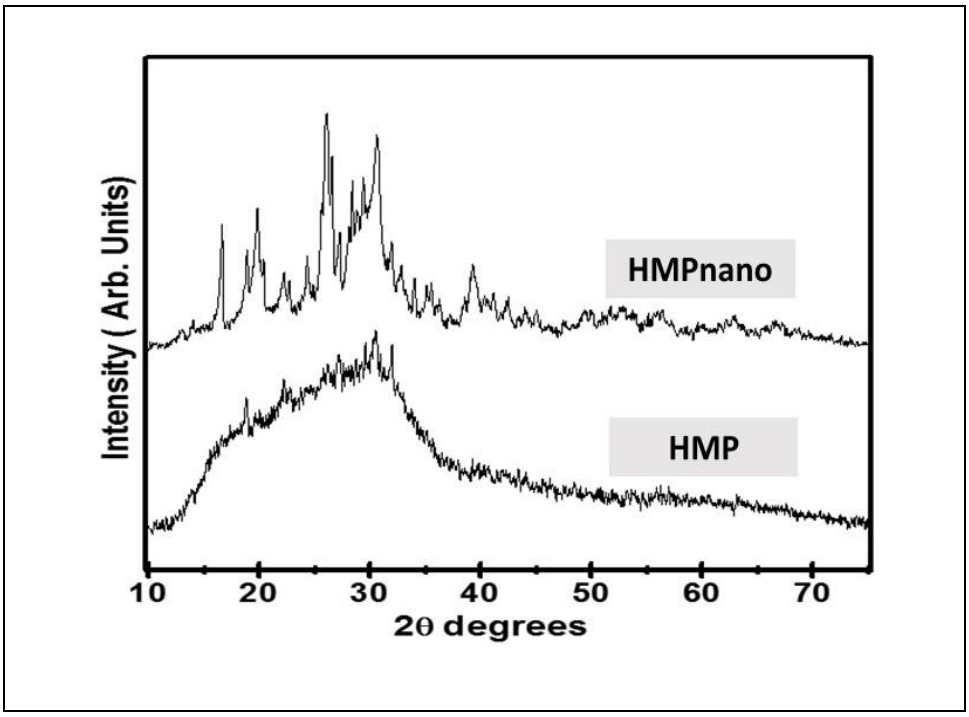

Figure 1. X-ray patterns of the HMP and of the HMPnano after milling for 48h.

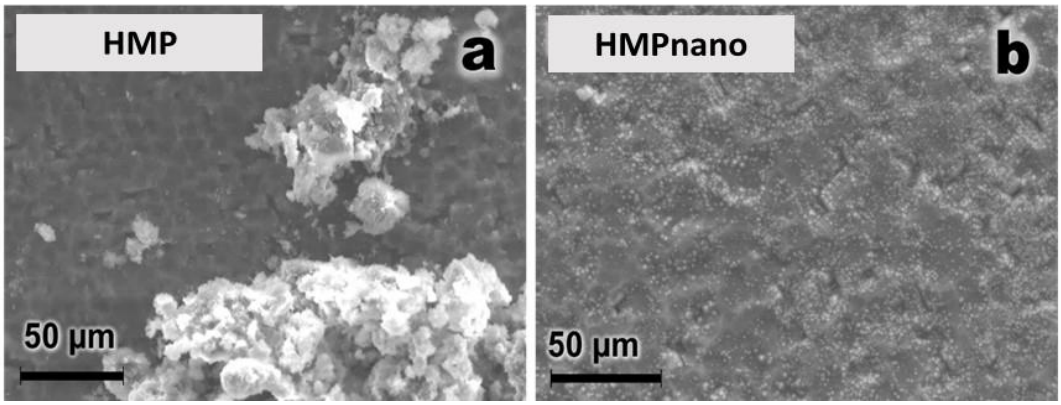

Figure 2. SEM images of Sodium hexametaphosphate particles, a) HMP and b) HMPnano after grinding of powder by 48h in ball mill.

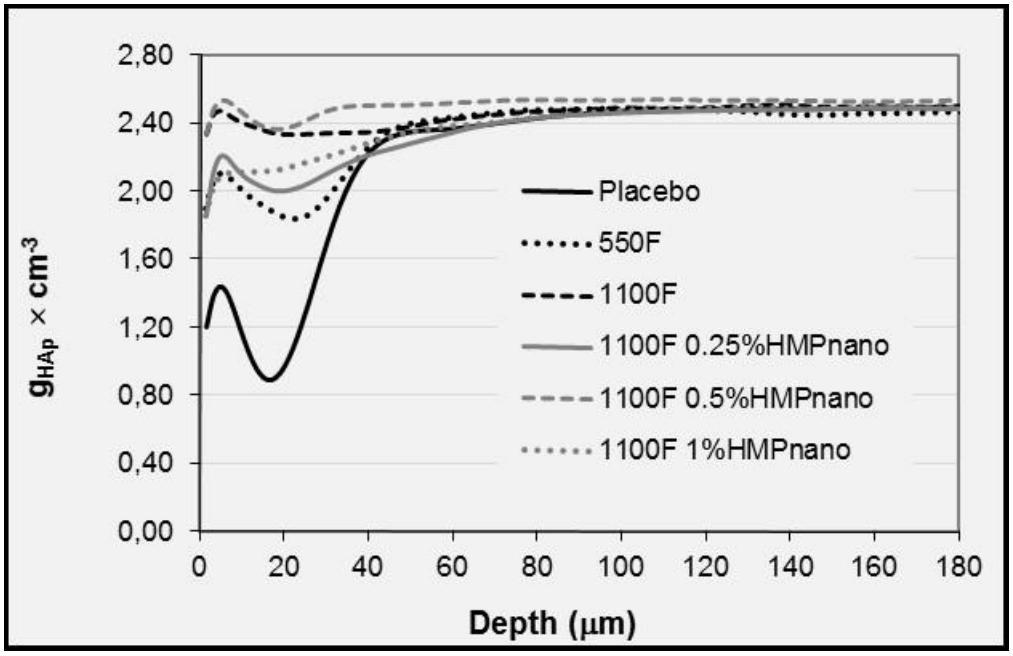

Figure 3. Depth profiles of mineral concentration ($g_{HAp} \times cm^{-3}$) in lesions for each toothpaste.

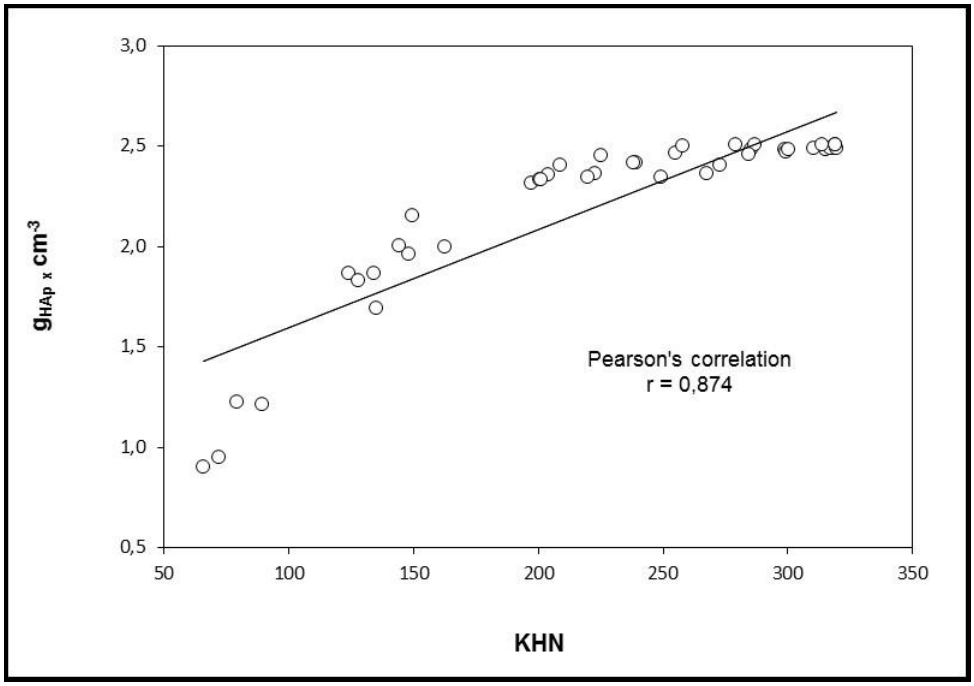

Figure 4. Graphic representation of correlation coefficient between: ΔKHN and $g_{HAp} \times cm^{-3}$.

Anexo

ANEXO A

INSTRUÇÕES AOS AUTORES

Caries Research

Guidelines for Authors
www.karger.com/cre_guidelines

Aims and Scope
'Caries Research' is an international journal, the aim of which is to promote research in dental caries and related fields through publication of original research and critical evaluation of research findings. The journal will publish papers on the aetiology, pathogenesis, prevention and clinical control or management of dental caries. Papers on health outcomes related to dental caries are also of interest, as are papers on other disorders of dental hard tissues, such as dental erosion. Aspects of caries beyond the stage where the pulp ceases to be vital are outside the scope of the journal. The journal reviews papers dealing with natural products and other bacterial inhibitors against specific criteria, details of which are available from the Editor.

Submission
Manuscripts written in English should be submitted online:
Should you experience problems with your submission, please contact:
Prof. David Beighton
(Editor-in-Chef, Caries Research)
Department of Microbiology
The Henry Wellcome Laboratories for Microbiology and Salivary Research
KCL Dental Institute, Floor 17, Guys Tower
London Bridge SE1 9RT (UK)
Tel. +44 2071887465
Fax +44 2071887466

david.beighton@kcl.ac.uk

During the online submission you will be asked to list complete mailing addresses, including e-mail addresses of three potential reviewers for your manuscript. Copies of any 'in press' papers cited in the manuscript must accompany the submission. Manuscripts reporting on clinical trials must be accompanied by the CONSORT checklist (see below).

Plagiarism Policy

Whether intentional or not, plagiarism is a serious violation. We define plagiarism as a case in which a paper reproduces another work with at least 25% similarity and without citation. If evidence of plagiarism is found before/after acceptance or after publication of the paper, the author will be offered a chance for rebuttal. If the arguments are not found to be satisfactory, the manuscript will be retracted and the author sanctioned from publishing papers for a period to be determined by the responsible Editor(s).

Conditions

All manuscripts are subject to editorial review. Manuscripts are received with the explicit understanding that the data they contain have not previously been published (in any language) and that they are not under simultaneous consideration by any other publication. Submission of an article for publication implies the transfer of the copyright from the author to the publisher upon acceptance. Accepted papers become the property of Caries Research and may not be reproduced by any means, in whole or in part, without the written consent of the publisher. It is the author's responsibility to obtain permission to reproduce illustrations, tables, etc., from other publications. Authors of papers describing research on human subjects are required to state that they have adhered to the Declaration of Helsinki.

Types of Papers

Original papers or Short Communications are reports of original work (including systematic reviews and meta-analyses). Both have the structure outlined below but for Short Communications the abstract should be less than 100 words and

the manuscript should not exceed 3 printed pages, equivalent to about 9 manuscript pages (including tables, illustrations and references).
Reviews can have a freer format but should nevertheless commence with a Title page, an Abstract and an Introduction defining the scope. Current topics are concise articles that present critical discussion of a topic of current interest, or a fresh look at a problem, and should aim to stimulate discussion.

Letters to the Editor, commenting on recent papers in the journal, are published occasionally, together with a response from the authors of the paper concerned.

Preparation of Manuscripts
Text should be one-and-a-half-spaced, with wide margins. All pages and all lines must be numbered, starting from the title page. A conventional font, such as Times New Roman or Arial, should be used, with a font size of 11 or 12. Avoid using italics except for Linnaean names of organisms and names of genes.

Manuscripts should be prepared as a text file plus separate files for illustrations. The text file should contain the following sequence of sections: Title page; Declaration of interests; Abstract; Introduction; Materials and Methods; Results; Discussion; Acknowledgements; References; Legends; Tables. Each section should start on a new page, except for the body of the paper (Introduction to Acknowledgements), which should be continuous. Lines in the manuscript must be numbered consecutively from the title page until the last page. Submissions which do not conform to these simple guidelines will be returned to the author.
Title page: The first page of each manuscript should show, in order:
_ the title, which should be informative but concise;
_ the authors' names and initials, without degrees or professional status, followed by their institutes;
_ a short title, maximum length 60 characters and spaces, for use as a running head;
_ a list of 3-10 key words;
_ the name of the corresponding author and full contact details (postal address, telephone and fax numbers, and e-mail address).

Declaration of Interests: Potential conflicts of interest should be identified for each author or, if there are no such conflicts, this should be stated explicitly. Conflict of interest exists where an author has a personal or financial relationship that might introduce bias or affect their judgement. Examples of situations where conflicts of interest might arise are restrictive conditions in the funding of the research, or if an author or their employer holds patente (s) on a product used in the study, or payment to an investigator from organisations with an interest in the study (including employment, consultancies, honoraria, ownership of shares, travel grant). Investigators should disclose potential conflicts to study participants and should state whether they have done so. The possible existence of a conflict of interest does not preclude consideration of a manuscript for publication, but the Editor might consider it appropriate to publish the disclosed information along with the paper.

Abstract: The abstract should summarise the contents of the paper in a single paragraph of no more than 250 words (to ensure that the abstract is published in full by on-line services such as PubMed). No attempt should be made to give numerical results in detail. References are not allowed in the abstract.

Introduction: This section should provide a concise summary of the background to the relevant field of research, introduce the specific problem addressed by the study and state the hypotheses to be tested.

Materials and Methods (or Subjects and Methods): All relevant attributes of the material (e.g. tissue, patients or population sample) forming the subject of the research should be provided. Experimental, analytical and statistical methods should be described concisely but in enough detail that others can repeat the work. The name and brief address of the manufacturer or supplier of major equipment should be given.

Statistical methods should be described with enough detail to enable a knowledgeable reader with access to the original data to verify the reported results. When possible, findings should be quantified and appropriate measures

of error or uncertainty (such as confidence intervals) given. Sole reliance on statistical hypothesis testing, such as the use of P values, should be avoided. Details about eligibility criteria for subjects, randomization and the number of observations should be included. The computer software and the statistical methods used should be specified. See Altman et al.: Statistical guidelines for contributors to medical journals [Br Med J 1983;286:1489–93] for further information.

Manuscripts reporting studies on human subjects should include evidence that he research was ethically conducted in accordance with the Declaration of Helsinki (World Medical Association). In particular, there must be a statement in Materials and Methods that the consent of an appropriate ethical committee was obtained prior to the start of the study, and that subjects were volunteers who had given informed, written consent. Information detailing the power and sample size calculations must be included in the manuscript.

Randomized clinical trials should be reported according to the standardised protocol of the CONSORT Statement. The CONSORT checklist must be submitted together with papers reporting clinical trials. Randomized clinical trials must be registered at clinicaltrials.gov or similar national authority and the trial number included in the manuscript. Trials beginning after 1 July 2012 must be registered before recruitment of the first patient. Caries Research will accept 'retrospective registration' of trials that began before 1 July 2012 (retrospective meaning registration occurs after patient enrolment begins). When submitting a paper on a clinical trial, the trial registration number should be stated at the end of the abstract in the following format: Trial registration: [name of the trial registry, the registry URL and the trial registration number].

In studies on laboratory animals, the experimental procedures should conform to the principles laid down in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and/or the National Research Council Guide for the Care and Use of Laboratory Animals. Unless the purpose of a paper is to compare specific systems or

products, commercial names of clinical and scientific equipment or techniques should only be cited, as appropriate, in the 'Materials and Methods' or 'Acknowledgements' sections. Elsewhere in the manuscript generic terms should be used. In any manuscript involving microradiography, the following information must be included: the radiation source and filters used and the kV used (this determines the wavelength of radiation and hence the validity of using Angmar's equation).

Manuscripts on experimental enamel caries should show that the lesions retain a relatively well-preserved surface layer, i.e. are not surfacesoftened lesions. Proof of surface integrity can be provided either as illustrations in the paper or as supplementary material for the reviewers. Transverse microradiography, polarized light microscopy of a section immersed in water or backscattered scanning electron microscopy of a polished cross-section can be used to provide the necessary proof. To allow the nature of experimental changes to be assessed, microradiographs or micrographs should be provided to show part of the experimental lesion and the adjacent control (e.g. figure 2 of Zaura et al.: Caries Res 2007;41:489–492). Again, these images can be provided as part of the paper or as supplementary material for review purposes. Results: Results should be presented without interpretation. The same data should not be presented in both tables and figures. The text should not repeat numerical data provided in tables or figures but should indicate the most important results and describe relevant trends and patterns. Discussion: This section has the functions of describing any limitations of material or methods, of interpreting the data and of drawing inferences about the contribution of the study to the wider field of research. There should be no repetition of preceding sections, e.g. reiteration of results or the aim of the research. The discussion should end with a few sentences summarising the conclusions of the study. However, there should not be a separate 'Conclusions' section. Acknowledgements: Acknowledge the contribution of colleagues (for technical assistance, statistical advice, critical comment etc.) and provide the position(s) of author(s) employed by commercial firms. This section should describe the source(s) of funding that have supported the work inlcuding relevant grant numbers. Please also include this sentence: "The funders had no role in

study design, data collection and analysis, decision to publish, or preparation of the manuscript." If this statement is not correct, you must describe the role of any sponsors or funders, and amend the sentence as needed. Additionally, the roles of all authors must be described (For example: Conceived and designed the experiments: AA, BB. Performed the clinical examination: AA, CC. Performed the experiments: DD, FF. Analyzed the data: BB, FF. Wrote the paper: AA, CC, FF, EE). Legends: The table headings should be listed first, followed by the legends for the illustrations. Tables: Tables should be numbered in Arabic numerals. Each table should be placed on a separate page. Tables should not be constructed using tabs but by utilising the table facilities of the word-processing software.
Illustrations:
_ Illustrations should be numbered in Arabic numerals in the sequence of citation. Figure numbers must be clearly indicated on the figures themselves, outside the image area.

Black and white half-tone illustrations must have a final resolution of 300 dpi after scaling, line drawings one of 800-1200 dpi.
_ Figures with a screen background should not be submitted.
_ When possible, group several illustrations in one block for reproduction (max. size 180 x 223 mm).

Color Illustrations
Online edition: Color illustrations are reproduced free of charge. In the print version, the illustrations are reproduced in black and white. Please avoid referring to the colors in the text and figure legends.

Print edition: Up to 6 color illustrations per page can be integrated within the text at CHF 800.00 per page.

References
Reference to other publications should give due acknowledgement to previous work; provide the reader with accurate and up-to-date guidance on the field of research under discussion; and provide evidence to support lines of argument.

Authors should select references carefully to fulfil these aims without attempting to be comprehensive. Cited work should already be published or officially accepted for publication. Material submitted for publication but not yet accepted should be cited as 'unpublished results', while unpublished observations communicated to the authors by another should be cited as 'personal communication', with credit in both cases being given to the source of the information. Neither unpublished nor personally communicated material should be included in the list of references. Abstracts more than 2 years old and theses should not be cited without a good reason, which should be explained in the covering letter accompanying the paper.

References should be cited by naming the author(s) and year. Where references are cited in parenthesis, both names and date are enclosed in square brackets. Where the author is the subject or object of the sentence, only the year is enclosed in brackets.
One author: [Frostell, 1984] or Frostell [1984].
Two authors: [Dawes and ten Cate, 1990] or Dawes and ten Cate [1990].
More than two authors: [Trahan et al., 1985] or Trahan et al. [1985].
Several references cited in parenthesis should be in date order and separated by semi-colons: [Frostell, 1984; Trahan et al., 1985; Dawes and ten Cate, 1990].

Material published on the World Wide Web should be cited like a reference to a print publication, and the URL included in the reference list (not in the text), together with the year when it was accessed. The reference list should include all the publications cited in the text, and only those publications. References, formatted as in the examples below, should be arranged in strict alphabetical order. All authors should be listed. For papers by the same authors, references should be listed according to year. Papers published by the same authors in the same year should be distinguished by the letters a, b, c, ... immediately following the year, in both the text citation and the reference list. For abbreviation of journal names, use the Index Medicus system. For journals, provide only the year, volume number and inclusive page numbers.
Examples

(a) Papers published in periodicals: Lussi A, Longbottom C, Gygax M, Braig F: Influence of professional cleaning and drying of occlusal surfaces on laser fluorescence in vivo. Caries Res 2005;39:284-286.
(b) Papers published only with DOI numbers: Theoharides TC, Boucher W, Spear K: Serum interleukin-6 reflects disease severity and osteoporosis in mastocytosis patients. Int Arch Allergy Immunol DOI: 10.1159/000063858.
(c) Monographs: Matthews DE, Farewell VT: Using and Understanding Medical Statistics. Basel, Karger, 1985.
(d) Edited books: DuBois RN: Cyclooxygenase-2 and colorectal cancer; in Dannenberg AJ, DuBois RN (eds): COX-2. Prog Exp Tum Res. Basel, Karger, 2003, vol 37, pp 124-137.
(e) Patents: Diggens AA, Ross JW: Determining ionic species electrochemically. UK Patent Application GB 2 064 131 A, 1980.
(f) World Wide Web: Chaplin M: Water structure and behavior. www.lsbu.ac.uk/water, 2004.

Supplementary Material

Supplementary material is restricted to additional information which is directly pertinent to the contentand conclusion of the paper. Please note that all supplementary files will undergo editorial review and should be submitted together with the original manuscript. The editors reserve the right to reject or limit the scope and length of supplementary material. Supplementary material must meet production quality standards for web publication without the need for[any modification or editing. In general, supplementary files should not exceed 10 MB in size. Acceptable file formats are word or pdf, excel spreadsheets (only if the data cannot be converted properly to a pdf file), video files (.mov, .avi, .mpeg), and audio files (.wav), either free standing or incorporated into html or ppt files in each case to illustrate the sound.

Accepted supplementary material will be published as submitted and no proofs will be provided to the authors.

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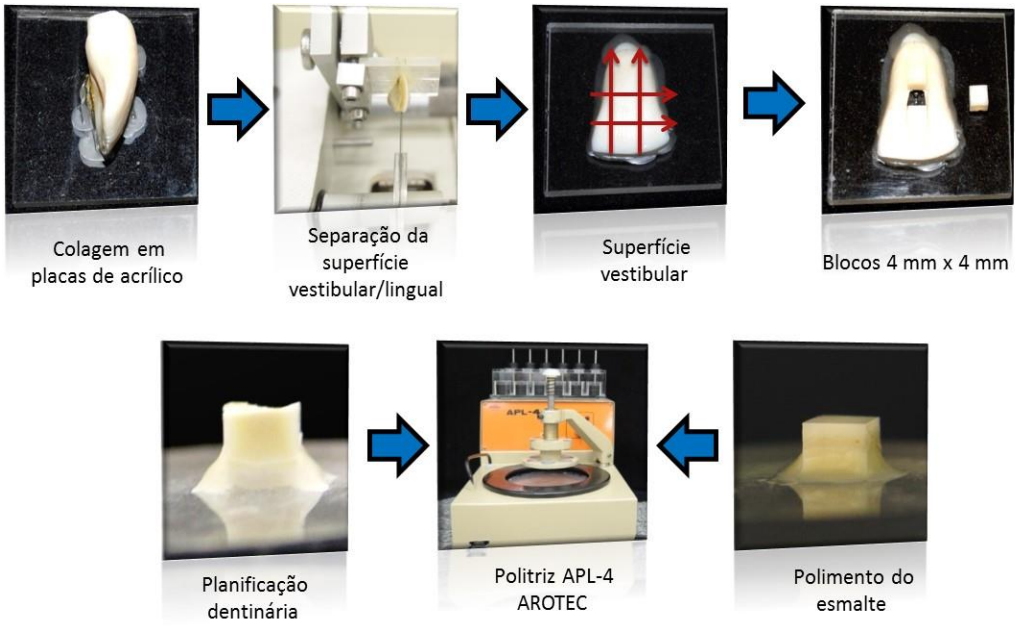
Unless indicated otherwise, proofs are sent to the first-named author and should be returned with the least possible delay. Alterations other than the correction of printer's errors are charged to the author. No page proofs are supplied to the author.

Reprints

Order forms and a price list are sent with the proofs. Orders submitted after this issue is printed are subject to considerably higher prices.

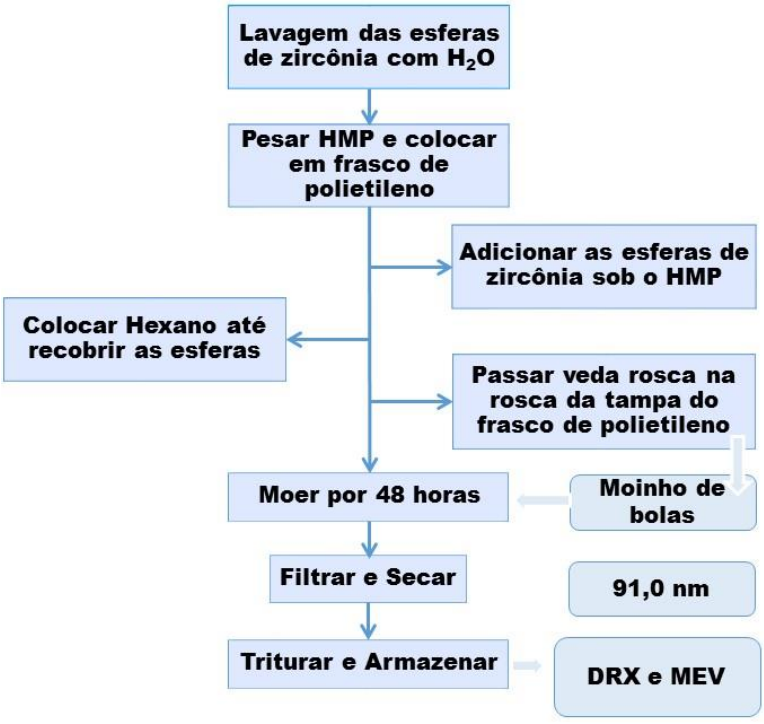
ANEXO B

PREPARO DOS BLOCOS DE ESMALTE



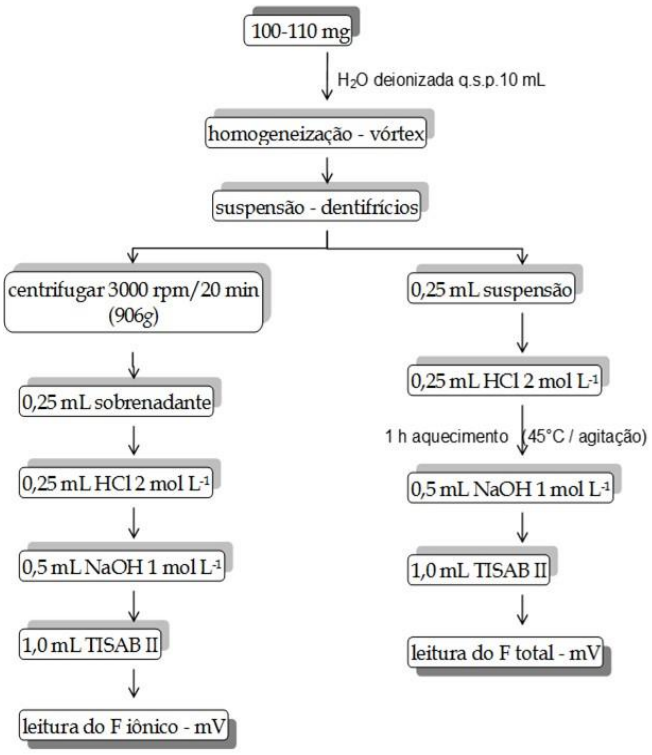
ANEXO C

SÍNTESE DO HEXAMETAFOSFATO DE SÓDIO NANOPARTICULADO



ANEXO D

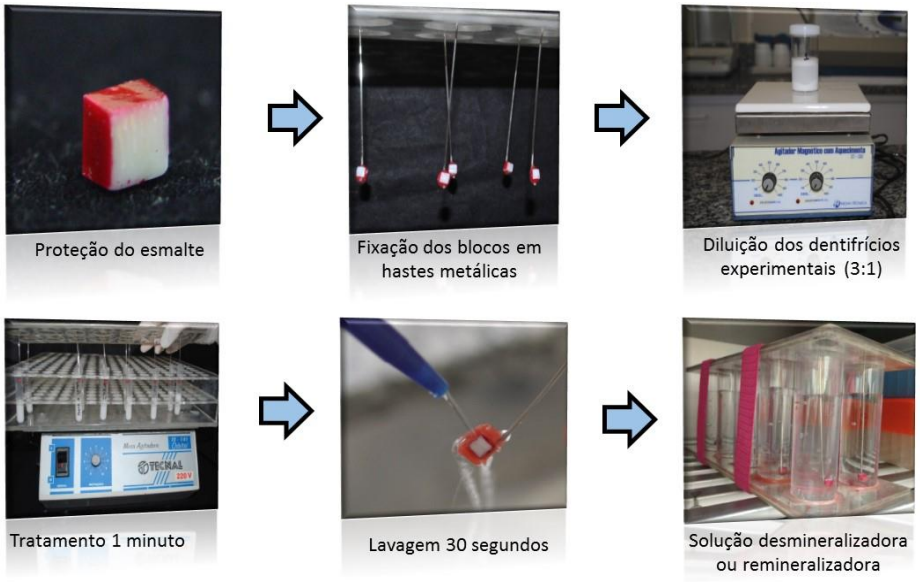
DOSAGEM DE FLUORETO NOS DENTIFRÍCIOS EXPERIMENTAIS

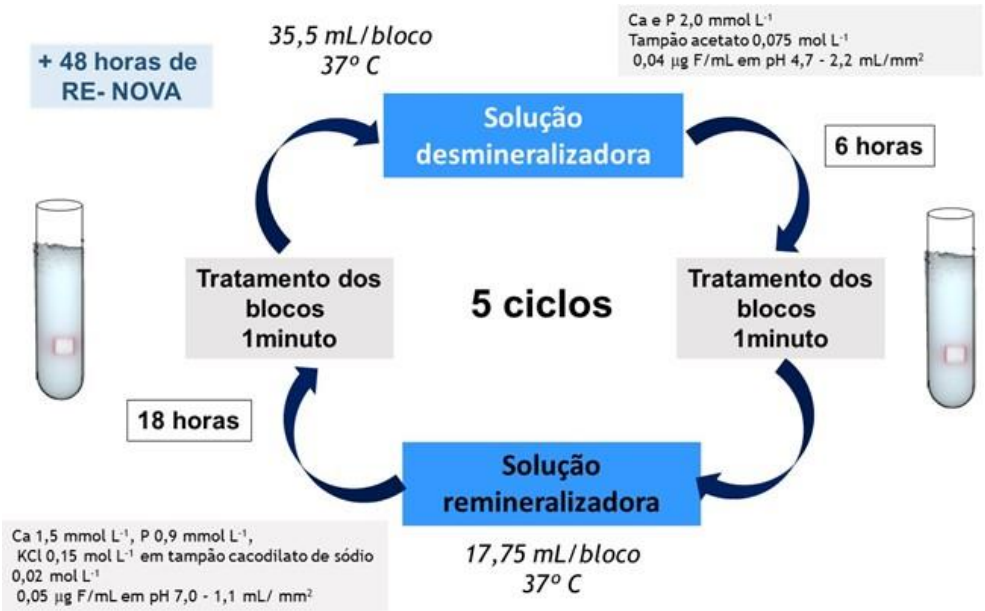


- Eletrodo específico para F; Orion 9409-BN
- Microeletrodo de referência
- Analisador de íons

ANEXO E

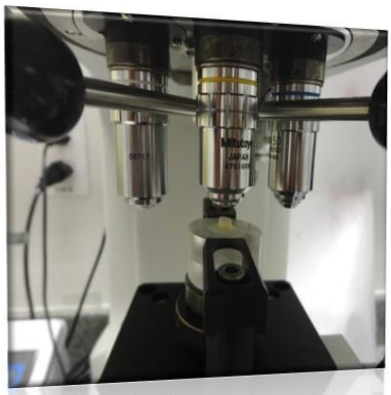
CICLAGEM DE pH



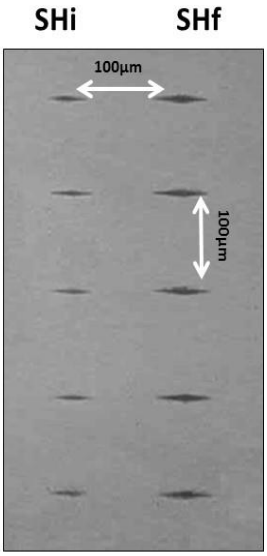


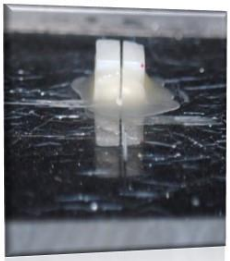
ANEXO F

ANÁLISE DA DUREZA SUPERFICIAL E LONGITUDINAL DO ESMALTE



Microdurômetro Buehler
Carga 25 gramas
Tempo 10 segundos



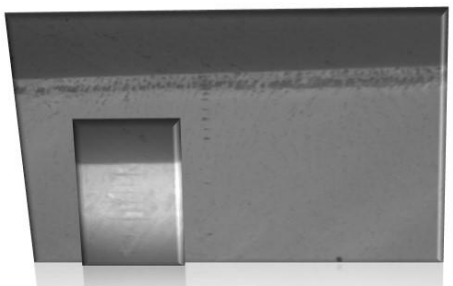


Secção dos blocos de esmalte no sentido longitudinal

Embutimento dos blocos de esmalte com resina acrílica

Aspecto final dos blocos de esmalte

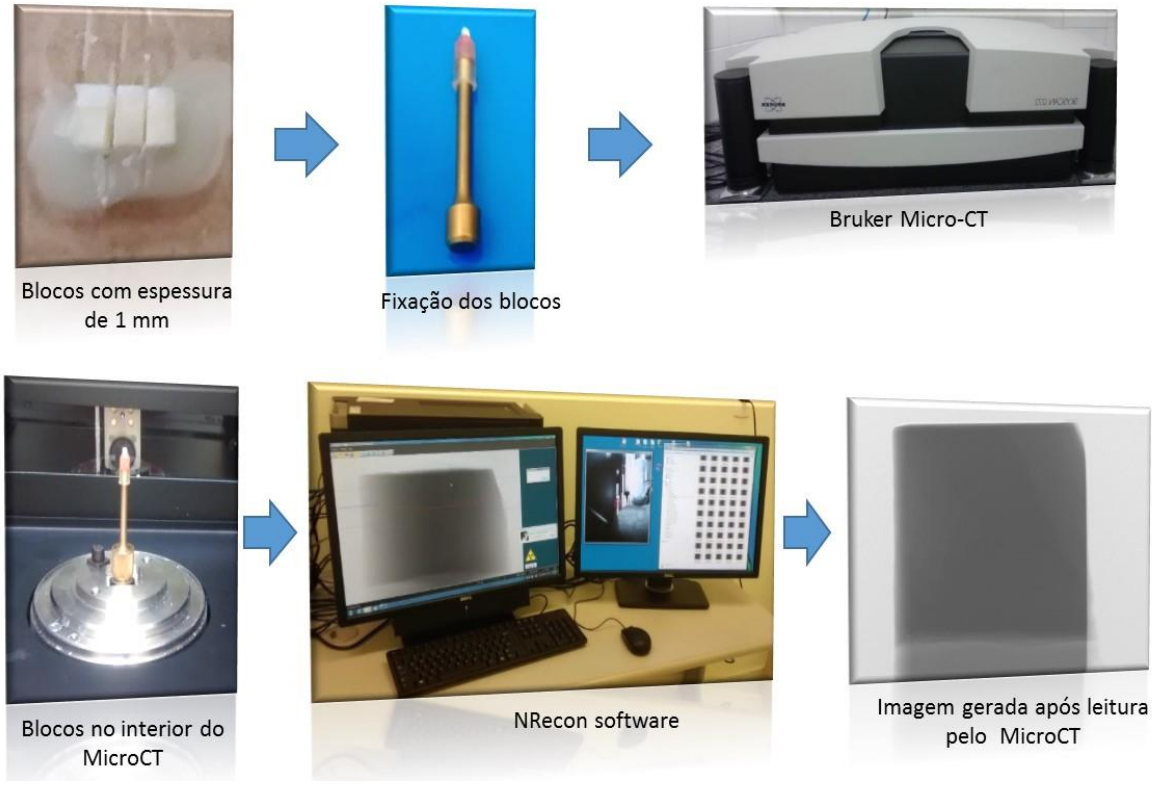
Microduromêtro Buehler
Carga 5g; Tempo 10 segundos



Análise da Lesão em Profundidade

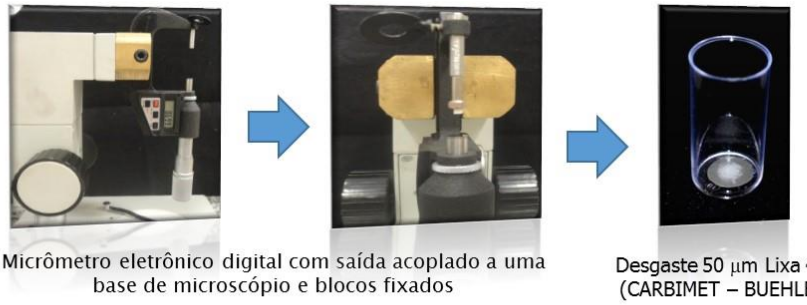
ANEXO G

ANÁLISE DA CONCENTRAÇÃO MINERAL DO ESMALTE PELA MICROTOMOGRAFIA COMPUTADORIZADA



ANEXO H

ANÁLISE DE FLUORETO NO ESMALTE



Micrômetro eletrônico digital com saída acoplado a uma base de microscópio e blocos fixados

Desgaste 50 μm Lixa 400 (CARBIMET – BUEHLER)

0,5 mL de HCl 1,0 mol/L

Agitação por 1 hora

0,25mL da amostra + 0,25 mL TISAB II modificado com NaOH 1,0 mol/L.