Luhana Santos Gonzales Garcia

EFEITO DA ADIÇÃO DE NANOPARTÍCULAS DE HEXAMETAFOSFATO DE SÓDIO EM DENTIFRÍCIOS FLUORETADOS SOBRE O PROCESSO DE REMINERALIZAÇÃO, DESMINERALIZAÇÃO E BIOFILME DENTÁRIO: ESTUDOS *IN SITU*

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Orientadora: Profª. Drª. Marcelle Danelon
Coorientador: Profº. Titular. Alberto Carlos Botazzo Delbem

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Dedico este trabalho,

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"Não é sobre ter todas as pessoas do mundo para si

É sobre saber que em algum lugar alguém zela por ti

É sobre desde cedo aprender a reconhecer a sua voz

É sobre o amor infinito que sempre existiu entre nós

É saber que vocês estão comigo nos momentos

Que eu mais preciso para me acompanhar

Então fazer valer a pena

Cada verso daquele poema sobre o que é amar"

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"Determinação, coragem e auto-confiança são fatores decisivos para o sucesso. Se estamos possuídos por uma inabalável determinação, conseguiremos superá-los. Independentemente das circunstâncias, devemos ser sempre humildes, recatados e despidos de orgulho. "

Dalai Lama

Resumo Geral

Garcia, LSG. **Efeito da adição de nanopartículas de hexametafosfato de sódio em dentifrícios fluoretados sobre o processo de remineralização, desmineralização e biofilme dentário: estudos *in situ*.** 2018 106f. Tese (Doutorado) - Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba 2018.

O objetivo geral deste estudo foi avaliar o efeito de um dentifrício fluoretado convencional (1100 ppm F), contendo nanopartículas de hexametafosfato de sódio (HMPnano) sobre a remineralização de lesões artificiais de cárie e desmineralização do esmalte *in situ* e biofilme. O estudo de remineralização, foi duplo-cego cruzado, realizado em quatro fases de três dias cada. Voluntários (n=12) usaram dispositivos palatinos contendo quatro blocos de esmalte bovino com lesões artificiais de cárie. A seguir foram distribuídos aleatoriamente nos seguintes grupos de tratamento: sem F/HMP/HMPnano (Placebo); 1100 ppm F (1100F); 1100F associado a 0,5% HMP microparticulado e nanoparticulado (1100F/HMP; 1100F/HMPnano). Os voluntários foram instruídos a escovar seus dentes naturais com os dispositivos palatinos na boca durante 1 min (3x/dia), de modo que os blocos foram tratados com slurry de dentifrícios. Após cada fase, determinou-se a porcentagem de recuperação de dureza de superfície (%SHR), recuperação integrada de dureza de subsuperfície (ΔIHR), recuperação mineral integrada (ΔIMR) e concentração de fluoreto (F) no esmalte. Os resultados foram submetidos a análise de variância de medidas repetidas seguido pelo teste Student-Newman-Keuls ($p < 0,001$). A superfície do esmalte tornou-se 68% mais remineralizada quando tratada com 1100F/HMPnano em comparação com 1100F ($p < 0,001$). O tratamento com 1100F/HMP e 1100F/HMPnano promoveu um aumento em ~ 23% e ~ 87% da ΔIHR quando comparado ao 1100F ($p < 0,001$). Além disso, ΔIMR foi de ~ 75% e ~ 33% maior para 1100F/HMPnano quando comparado a 1100F e 1100F/HMP, respectivamente ($p < 0,001$). O estudo de desmineralização foi duplo-cego cruzado, e consistiu em quatro fases (7 dias cada). Voluntários (n=12) usaram aparelhos palatinos contendo quatro blocos de esmaltes bovinos. O desafio cariogênico foi realizado pela solução de sacarose a 30% (6x/dia). Os tratamentos com os dentífricios (3x/dia) foram os seguintes: sem F/ HMP/HMPnano (Placebo); 1100 ppm F (1100F); 1100F associado a 0,5% HMP microparticulado e nanoparticulado (1100F/HMP; 1100F/HMPnano). Após sete dias determinou-se a porcentagem de perda de dureza de superfície (%SH), perda integrada de dureza subsuperfície (ΔKHN), cálcio (Ca), fósforo (P) e fluoreto (F) no

esmalte. Além disso, no biofilme formado sobre os blocos analisou-se as concentrações de polissacarídeos extracelulares (EPS), F, Ca, P. Os resultados foram submetidos a análise de variância de medidas repetidas seguido pelo teste Student-Newman-Keuls ($p < 0,001$). Resultados: 1100F/HMPnano promoveu menor %SH e ΔKHN quando comparado aos demais grupos ($p < 0,001$). A adição de HMPnano a 1100F não aumentou a absorção de F e P no esmalte, mas aumentou significativamente as concentrações de Ca ($p < 0,001$). O grupo 1100F/HMPnano apresentou valores mais baixos de concentração de EPS quando comparado com 1100F (~ 70%) ($p < 0,001$). Todos os grupos foram supersaturados em relação a hidroxiapatita (HA). Somente, o grupo 1100F/HMPnano foi supersaturado em relação ao fluoreto de cálcio (CaF_2) ($p < 0,05$). As atividades iônicas de íon fluoreto de cálcio (CaF^+) e fluoreto de hidrogênio neutro (HF^0) para o grupo 1100F/HMPnano foram significativamente maiores quando comparadas aos demais grupos ($p < 0,001$). Conclui-se que a adição de HMPnano a um dentifrício convencional promoveu um efeito remineralizador significantemente maior em lesões artificiais de cárie e demonstrou um maior efeito protetor contra a desmineralização e variáveis do biofilme *in situ*.

Palavras-chave: Esmalte dentário. Flúor. Fosfato. Nanopartícula. Desmineralização. Remineralização. Dentifrício.

General Abstract

Garcia, LSG. **Effect of the addition of nanoparticles of sodium hexametaphosphate in fluoridated toothpaste on the process of remineralization, demineralization and dental biofilm: *in situ* studies.** 2018 106f. Tese (Doutorado) - Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba 2018.

General Abstract

The aim of this study was to evaluate the effect of a conventional fluoride toothpaste (1100 ppm F) containing nano-sizeds of sodium hexametaphosphate (HMPnano) on the remineralization of artificial caries lesions and enamel demineralization and biofilm *in situ*. The remineralization study was double-blinded crossed, performed in 4 phases of 3 days each. Volunteers (n = 12) used palatal devices containing four blocks of bovine enamel with artificial lesions of caries. They were then randomly assigned to the following treatment groups: without F/ HMP/HMPnano (Placebo); 1100 ppm F (1100F); 1100F associated with 0.5% HMP microparticulate and nano-sized (1100F/HMP; 1100F/ HMPnano). The volunteers were instructed to brush their natural teeth with the palatine devices in the mouth for 1 min (3x/day), so that the blocks were treated with slurry of toothpastes. After each phase, the percentage of surface hardness recovery (%SHR), integrated recovery of subsurface hardness (ΔIHR), integrated mineral recovery (ΔIMR) and fluoride (F) concentration in the enamel were determined. The results were subjected to analysis of variance of repeated measures followed by Student-Newman-Keuls test ($p < 0.001$). The enamel surface became 68% more remineralized when treated with 1100F/ HMPnano compared to 1100F ($p < 0.001$). Treatment with 1100F/HMP and 1100F/ HMPnano promoted an increase in ~ 23% and ~ 87% of ΔIHR when compared to 1100F ($p < 0.001$). In addition, ΔIMR was ~ 75% and ~ 33% higher for 1100F/HMPnano when compared to 1100F and 1100F/HMP, respectively ($p < 0.001$). The study of demineralization was double-blinded crossed, and consisted of four phases (7 days each). Volunteers (n = 12) used palatal appliances containing four blocks of bovine enamel. The cariogenic challenge was accomplished by the solution of sucrose 30% (6x/day). Treatments with the toothpaste (3x/day) were as follows: without F/HMP/HMPnano (Placebo); 1100 ppm F (1100F); 1100F associated with 0.5% HMP microparticulate and nano-sized (1100F/HMP; 1100F/HMPnano). After 7 days the percentage of surface hardness loss (%SH), integrated loss of subsurface hardness (ΔKHN), calcium (Ca), phosphorus (P) and fluoride (F) in the

enamel were determined. The results were submitted to analysis of variance of repeated measurements followed by the Student-Newman-Keuls test ($p < 0.001$). The results were analyzed using the Student-Newman-Keuls test ($p < 0.001$). Results: 1100F/HMPnano promoted lower %SH and ΔKHN when compared to the other groups ($p < 0.001$). Addition of HMPnano to 1100F did not increase the absorption of enamel F, but significantly increased enamel Ca concentrations ($p < 0.001$). The 1100F/HMPnano group had lower values of EPS concentration when compared to 1100F (~ 70%) ($p < 0.001$). All groups were supersaturated with respect to hydroxyapatite (HA). Only, the 1100F/HMPnano group was supersaturated relative to calcium fluoride (CaF_2) ($p < 0.05$). The ionic activities of calcium fluoride ion (CaF^+) and neutral hydrogen fluoride (HF^0) for the 1100F/HMPnano group were significantly higher when compared to the other groups ($p < 0.001$). It was concluded that the addition of HMPnano to a conventional toothpaste promoted a significantly greater remineralizing effect on artificial caries lesions and demonstrated a greater protective effect against demineralization and biofilm *in situ*.

Keywords: Dental Enamel. Fluoride. Phosphate. Nano-sized. Demineralization. Remineralization. Toothpaste.

Lista de Abreviaturas

LISTA DE ABREVIATURAS

Al^{+} ion aluminum

Am Ante Meridiem

ANOVA Analysis of Variance

ºC Degrees Celsius

Ca Calcium

Ca^{+2} Calcium ion

CaF^{+} Calcium fluoride ion

CaF_2 Calcium fluoride

$CaPO_4^{-}$ Calcium phosphate ion

$CaPOH^{-}$ Phosphate hydrogenated calcium ion

$CaHPO_4^{0}$ Phosphate hydrogenated calcium neutral

$CaH_2PO_4^{+}$ Dihydrogenated calcium phosphate

CT Conventional toothpaste

DS Degree of saturation

EPS Insoluble extracellular polysaccharides

F Fluoride

$Fe3^{+}$ Ferric ion

FI Ionic fluoride

FT Total fluoride

g Gram

g/cm^3 Gram per cubic centimeter

$g_{HAp} \times cm^{-3}$ Mineral concentration

h Hour

HA Hydroxyapatite

HCl Hydrochloric acid

HF^0 Neutral hydrogen fluoride

HMP Sodium Hexametaphosphate

HMPnano Nano-sized of Sodium Hexametaphosphate

HPO_4^{-2} Hydrogenated phosphate ion

$H_2PO_4^-$ Dihydrogen phosphate ion

K^+ Potassium ion

KHN Knoop hardness unit

L Liter

LAC Linear attenuation coefficient

IA Ionic activities

IF Ionic fluoride

IML Integrated mineral loss

M Molar

MC Mineral concentration

MicroCT Micro-computed tomography

Min Minutes

mg Milligram

Mg^{+2} Magnesium Ion

mg/g Milligram per gram

ml Milliliter

mm Millimeter

mmol/l Milimol per liter

Mol/l Mol per liter

mol L^{-1} Mol per liter

mol/kg Mol per kilogram

moles/kg Moles per kilograms

mV Millivolts

n Volunteers number

Na^{+} Sodium Ion

NaF Sodium Fluoride

NaOH Sodium hydroxide

nm Nanometers

P Phosphor

P_2O_5 Diphosphorus pentoxide

PO_4^{3-} Orthophosphate

pH Hydrogen potential

ppmF Parts per million of fluoride

pm Post Meridiem

s Seconds

SD Standard deviation

SEM Scanning electron microscopy

SHi Initial surface hardness

SHf Final surface hardness

%SH Surface hardness loss

TF Total fluoride

TISAB Total ionic strength adjuster cap

TMP Sodium trimetaphosphate

TMPnano Nano-sized of sodium trimetaphosphate

μg Microgram

μg/mm^3 Microgram per cubic millimeter

μL/mg Microliters/milligram

μm Micrometer

ΔKHN Integrated loss of subsurface hardness

ΔIHR Integrated recovery of surface hardness

ΔIMR Integrated mineral recovery

XRD X-ray diffraction

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Figure 1 – **A** Enamel block preparation. **B** Surface hardness analysis. **C** Enamel demineralization. **D** Surface hardness analysis. **E** Intraoral phase. **F** Surface hardness analysis. **G** Longitudinal section. **H** Embedded acrylic resin. **I** Integrated recovery of subsurface hardness (ΔIHR). **J** Analysis of enamel mineral concentrations. **K** Self-adhesive polishing discs.

Figure 2. X-ray diffraction patterns of HMP before (HMP) and after (HMPnano) grinding of powder for 48 h in ball mill. The possible phases $(NaPO_3)_6$ PDF# 3643 Sodium hexametaphosphate, $NaPO_3$ PDF# 76788 Sodium metaphosphate, $Na_2H_2P_2O_7$ PDF# 10187 Disodium dihydrogen diphosphate, NaH_2PO_4 (H_2O) DF#11651 SodiumDihydrogen Phosphate Monohydrate, NaH_2PO_4 PDF#11657 Sodium dihydrogen phosphate and $Na_5P_3O_{10}$ PDF# 11652 Pentasodium triphosphate.

Figure 3. SEM images of Sodium hexametaphosphate particles, a) HMP and b) HMPnano after grinding of powder by 48h in ball mill.

Figure 4. (A) Cross-sectional profile of mineral concentration ($g_{HAp} \times cm^3$) as function of depth (µm) from according to the groups. (B) Differential profile obtained from values of mineral concentration of the treatments subtracted from the artificial caries values.

Figure 5. Relationship between integrated mineral recovery values (ΔIMR) and integrated mineral loss (ΔIHR). n = 192

FIGURAS CAPÍTULO 2

Figure 1. X-ray diffraction patterns of HMP before (HMP) and after (HMPnano) grinding of powder for 48 h in ball mill. The possible phases $(NaPO_3)_6$ PDF# 3643 Sodium hexametaphosphate, $NaPO_3$ PDF# 76788 Sodium metaphosphate, $Na_2H_2P_2O_7$ PDF# 10187 Disodium dihydrogen diphosphate, NaH_2PO_4 (H_2O) DF#11651 Sodium Dihydrogen Phosphate Monohydrate, NaH_2PO_4 PDF#11657 Sodium dihydrogen phosphate and $Na_5P_3O_{10}$ PDF# 11652 Pentasodium triphosphate.

Figure 2. SEM images of sodium hexametaphosphate particles. a HMP and b HMPnano after grinding of powder for 48 h in ball mill.

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Sumário

SUMÁRIO

Introdução Geral

1 Introdução Geral

A cárie dentária é uma doença que resulta da colonização da superfície do esmalte por microrganismos, especialmente os *Streptococcus mutans*, os quais metabolizando carboidratos fermentáveis, produzem ácidos, responsáveis pela desmineralização dentária [Fejerskov e Kidd, 2011[1]]. Atualmente, o conceito de cárie dentária foi definido como uma "doença biofilme-açúcar dependente" [Cury & Tenuta, 2014[2]; Sheiham e James, 2015[3]]. Assim, para o seu desenvolvimento, faz-se necessária a presença de um biofilme dental colonizado por microrganismos cariogênicos, além da ingestão de uma dieta rica em sacarose, que pode variar em frequência e intensidade [Neves et al., 2016[4]], sendo este processo influenciado por características individuais, comportamentais e biopsicosociais [Fisher-Owens, 2007[5]]. Apesar da existência de inúmeras fontes de fluoreto (F), ainda existem populações que concentram uma alta prevalência da doença [Pinto et al., 2016[6]; Zemaitiene et al., 2017[7]]. Isto indica, portanto, que ainda há indivíduos com alto risco de cárie, incluindo crianças, adolescentes, indivíduos sem acesso a água de abastecimento fluoretada e a serviços odontológicos [Dye et al., 2007[8]; Ditmyer et al., 2011[9]].

Sabendo-se que os dentifrícios se destacam dentre as formas de administração tópica mais utilizadas pela população e que contribuem para a redução da cárie dentária [Rølla et al., 1991[10]], seria interessante aumentar a eficácia dos mesmos, proporcionando um aumento na diminuição dos índices da doença. Estudos têm demonstrado que é possível aumentar a efetividade de um dentifrício sem aumentar a concentração de F na formulação [de Castro et al., 2015[11]; da Camara et al., 2015[12]; Danelon et al., 2015[13]]. A adição de fosfatos inorgânicos e orgânicos a dentifrícios com concentração reduzida (500 ppm F) e convencional (1100 ppm F) de F mostrou aumentar o efeito anticárie [Takeshita et al., 2009[14]; de Castro et al., 2015[11]]. Estes fosfatos apresentam afinidade pelo esmalte reduzindo a perda mineral e auxiliando na remineralização de lesões iniciais de cárie [Takeshita et al., 2009[14]; Danelon et al., 2013[15]; da Camara et al., 2014[16]; da Camara et al., 2016[17]]. Dentre os sais de fosfatos com atividade anticariogênica, o hexametafosfato de sódio microparticulado (HMP) tem-se destacado na literatura [da Camara et al., 2014[16]; da Camara et al., 2015[12]; da Camara et al., 2016[17]].

[1]Fejerskov O; Kidd E. Cárie dentária: a doença e seu tratamento clínico. São Paulo: Santos, 2ª ed., 2011.
[2]Cury JA, Tenuta LM. Evidence-based recommendation on toothpaste use. Braz Oral Res 2014;28 Spec No:1-7.
[3]Sheiham A, James WP. Diet and Dental Caries: The Pivotal Role of Free Sugars Reemphasized. J Dent Res 2015;94:1341-1347.
[4]Neves PA, Ribeiro CC, Tenuta LM, Leitão TJ, Monteiro-Neto V, Nunes AM, Cury JA. Breastfeeding, Dental Biofilm Acidogenicity, and Early Childhood Caries. Caries Res 2016;50:319-324.

O HMP é caracterizado como um fosfato inorgânico, utilizado na indústria alimentícia como agente antimicrobiano devido a sua capacidade de aumentar a permeabilidade da membrana externa bacteriana [Vaara, 1992[18]], uma vez que se liga ao íon magnésio (Mg^{+2}), presente na célula bacteriana promovendo a lise da mesma, através do aumento da permeabilidade, demonstrando assim, ter efeito sobre a atividade metabólica do biofilme dental e, consequentemente, na redução da produção de ácido [Cheng et al., 2012[19]; Melo et al., 2013[20]; Zhang et al., 2015[21]]. Além da sua afinidade pelos íons Mg, é capaz de se ligar a outros íons metálicos (Ca^{2+}, K^{+}, Al^{+}, Fe^{3+}), favorecendo a formação de complexo na superfície do esmalte, que reduz a solubilidade da hidroxiapatita [Changgen, 1983[22]; Choi, 1993[23]]. O efeito da associação HMP/F foi avaliado por da Camara et al. [2015[12] e 2016[17]] em um estudo *in situ* e *in vitro*, mostrando que a adição de 1% HMP a um dentifrício convencional (1100 ppm F) reduziu significantemente a desmineralização do esmalte quando comparado a 1100 ppm F. Entretanto para que esses bons resultados ocorram (ou seja um efeito sinérgico entre ambos), é necessário uma proporção molar adequada entre o fosfato e o F, para que não haja competição, pois a absorção do polifostafo ocorre rapidamente e pode competir com a absorção do F [Souza et al., 2013[24]].

A nanotecnologia tem sido aplicada na Odontologia como um conceito inovador para o desenvolvimento de produtos com melhores propriedades e potencial anticárie, os quais possuem a escala métrica de 1-100 nm [Hanning e Hanning, 2010[25]; Melo et al., 2013[26]]. As nanopartículas possuem uma elevada relação entre área superficial e seu volume, e apresentam uma porcentagem consideravelmente mais alta de átomos em sua superfície quando comparadas com partículas maiores, o que pode torná-las mais reativas [Melo et al., 2013[26]]. Dalpasquale et al. [2017[27]] em um estudo *in vitro*, avaliou o efeito de dentifrícios com 1100 ppm F associado ao HMP nanoparticulado (HMPnano) em diferentes concentrações (0,25%, 0,5% e 1,0%), sobre a desmineralização do esmalte, obtendo uma melhora significante contra a desmineralização em comparação a um dentifrício convencional, comprovando o melhor desempenho desse sal na forma nanoparticulada quando associado ao dentifrício com 1100 ppm F.

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[5]Fisher-Owens SA, Gansky SA, Platt LJ, Weintraub JA, Soobader MJ, Bramlett MD, Newacheck PW. Influences on children's oral health: a conceptual model. Pediatrics 2007;120:510-520.

[6]Pinto GS, Hartwing AD, Elias R, Azevedo MS, Goettems ML, Correa MB, Demarco FF. Maternal care influence on children's caries prevalence in southern Brazil. Braz Oral Res 2016;30:e70.

[7]Zemaitiene M, Grigalauskiene R, Andruskeviciene V, Matulaitiene ZK, Zubiene J, Narbutaite J, Slabsinskiene E. Dental caries risk indicators in early childhood and their association with caries polarization in adolescence: a crosssectional study. BMC Oral Health 2017;17:2-6.

[8]Dye BA, Tan S, Smith V, Lewis BG, Barker LK, Thornton-Evans G, et al. Trends in oral health status: United States, 1988-1994 and 1999-2004. Vital Health Stat 2007;248:1-92.

Sabendo-se de todas as propriedades do HMP, bem como a ação das nanopartículas de fosfatos, e a ausência de estudos *in situ* avaliando os efeitos do HMPnano, seria interessante a realização de estudos que avaliem novas formulações dentifrícias contendo 1100 ppm F suplementadas com HMP na forma nanoparticulada sobre remineralização de lesões de cárie artificiais, bem como sobre a desmineralização do esmalte sob alto desafio cariogênico e variáveis do biofilme. Assim, o objetivo geral deste estudo foi avaliar o efeito de um dentifrício fluoretado convencional (1100 ppm F), contendo nanopartículas de hexametafosfato de sódio (HMPnano) sobre a remineralização de lesões artificiais de cárie e desmineralização do esmalte *in situ* e biofilme. A hipótese nula do estudo foi a de que o dentifrício convencional (1100 ppm F) associado ao HMPnano, levaria a um efeito anticárie similar quando comparado a um dentifrício contendo 1100 ppm F.

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[9]Ditmyer M, Dounis G, Mobley C, Schawarz E. Inequalities of caries experience in Nevada da youth expressed by DMFT index vs. Significant Caries Index (Sic) overtime. BMC Oral Health 2011;5:11:12.
[10]Rølla G, Ogaard B, Cruz RA. Clinical effect and mechanism cariostact action of fluoride-containing toothpastes: a review. Int Dent J 1991;41:171-174.
[11]de Castro LP, Delbem AC, Danelon M, Passarinho A, Percinoto C. In vitro effect of sodium trimetaphosphate additives to conventional toothpastes on enamel demineralization. Clin Oral Investig 2015;19:1683-1687.
[12]da Camara DM, Pessan JP, Francati TM, Santos Souza JA, Danelon M, Delbem AC. Synergistic effect of fluoride and sodium hexametaphosphate in toothpaste on enamel demineralization in situ. J Dent 2015;43:1249-1254.
[13]Danelon M, Pessan JP, Neto FN, de Camargo ER, Delbem AC. Effect of toothpaste with nano-sized trimetaphosphate on dental caries: In situ study. J Dent 2015;43:806-813.
[14]Takeshita EM, Castro LP, Sassaki KT, Delbem, ACB. In vitro evaluation of dentifrice with low fluoride content supplemented with trimetaphosphate. Caries Res 2009;43:50-56.
[15]Danelon, M, Takeshita, EM, Sassaki, KT, Delbem, ACB. In situ evaluation of a low fluoride concentration gel with sodium trimetaphosphate in enamel remineralization. Am J Dent 2013;26:15-20.
[16]da Camara DM, Miyasaki ML, Danelon M, Sassaki KT, Delbem ACB. Effect of low-fluoride toothpastes combined with hexametaphosphate on in vitro enamel demineralization. J Dent 2014;42:256-262.
[17]da Camara DM, Pessan JP, Francati TM, Souza JA, Danelon M, Delbem AC. Fluoride toothpaste supplemented with sodium hexametaphosphate reduces enamel demineralization in vitro. Clin Oral Investig 2016;20:1981-1985.
[18]Vaara M. Agents that increase the permeability of the outer membrane. Microbiol Rev 1992;56:395-411.
[19]Cheng L, Weir MD, Xu HHK, Kraigsley AM, Lin NJ, Lin-Gibson S, Zhou X. Antibacterial and physical properties of calcium-phosphate and calcium-fluoride nanocomposites with chlorhexidine. Dent Mater 2012;28:573-583.
[20]Melo, MAS, Guedes SFF, Xu HHK, Rodrigues LKA. Nanotechnology-based restorative materials for dental caries management. Trends Biotechnol 2013;31:1-18.
[21]Zhang JQ, Hou XH, Song XY, Ma XB, Zhao YX, Zhang SY. ClpP affects biofilm formation of Streptococcus mutans differently in the presence of cariogenic carbohydrates through regulating gtfBC and ftf. Curr Microbiol 2015;70:716-723.
[22]Changgen LI, Yongxin LÜ. Selective flotation of scheelite from calcium minerals with sodium oleate as a collector and phosphates as modifiers. ii. the mechanism of the interaction between phosphate modifiers and minerals. Int J Miner Process 1983;10:219-235.
[23]Choi IK, Wen WW, Smith RW. Technical note the effect of a long chain phosphate on the adsorption of collectors on kaolinite. Miner Eng 1993;6:1191-1197.
[24]Souza JAS, Amaral JG, Moraes JCS, Sassaki KT, Delbem ACB. Effect of Sodium Trimetaphosphate on Hydroxyapatite Solubility: An in vitro study. Braz Dent J 2013;24:235-240.
[25]Hannig M, Hannig C. Nanomaterials in preventive dentistry. Nature Nanotechnology 2010;5:565-569.
[26]Melo, MAS, Guedes SFF, Xu HHK, Rodrigues LKA. Nanotechnology-based restorative materials for dental caries management. Trends Biotechnol 2013;31:1-18.
[27]Dalpasquale G, Delbem ACB, Pessan JP, Nunes GP, Gorup LF, Souza-Neto FN, Camargo ER, Danelon M: Effect of the addition of nano-sized sodium hexametaphosphate to fluoride toothpastes on tooth demineralization: an in vitro study. Clin Oral Investig 2017;21:1821-1827.

Capítulo 1

2. Effect of fluoride toothpaste containing nano-sized sodium hexametaphosphate on enamel remineralization: an *in situ* study

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Short title: F-toothpaste with nano-sized HMP enhances enamel remineralization

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***De acordo com as instruções aos autores do periódico Caries Research.**

Conflict of Interest Form

The authors Marcelle Danelon, Alberto Carlos Botazzo Delbem, Juliano Pelim Pessan and Emerson Rodrigues de Camargo hold a patent request for a product used in the study, by the National Institute of Industrial Property - INPI/SP, on 10/17/2014 under number BR 10 2014 025902 3. All authors approved the publishing of the manuscript.

2.1 Abstract

Objective: To evaluate the remineralizing potential of a conventional toothpaste (1,100 ppm F) supplemented with nano-sized sodium hexametaphosphate (HMPnano) in artificial caries lesions *in situ*. Design: This double-blinded crossed study was performed in four phases of three days each. Twelve subjects used palatal appliances containing four bovine enamel blocks with artificial caries lesions. Volunteers were randomly assigned into the following treatment groups: no F/HMP/HMPnano (Placebo); 1,100 ppm F (1100F); 1100F plus 0.5% micrometric HMP (1100F/HMP) and 1100F plus 0.5% nano-sized HMP (1100F/HMPnano). Volunteers were instructed to brush their natural teeth with the palatal appliances in the mouth for 1 min (3 times/day) so that blocks were treated with natural slurries of toothpastes. After each phase, the percentage of surface hardness recovery (%SHR), integrated recovery of subsurface hardness (ΔIHR), integrated mineral recovery (ΔIMR) and enamel F concentration were determined. Results: The results were submitted to analysis of variance and Student-Newman-Keuls' test ($p < 0.001$). Enamel surface became 68% harder when treated with 1100F/HMPnano in comparison with 1100F ($p < 0.001$). Treatment with 1100F/HMP and 1100F/HMPnano promoted an increase of ~23% and ~87% in ΔIHR when compared to 1100F ($p < 0.001$). In addition, ΔIMR for the 1100F/HMPnano was ~75% and ~33% higher when compared to 1100F and 1100F/HMP, respectively ($p < 0.001$). Enamel F uptake was similar among all groups except for the Placebo ($p < 0.001$). Conclusion: The addition of 0.5% HMPnano to a conventional toothpaste was able to promote an additional remineralizing effect of artificial caries lesions.

Keywords: Dental enamel, Phosphate, Remineralization, Toothpaste, Nano-sized.

2.2 Introduction

Fluoride toothpastes have made an important contribution through reducing dental caries prevalence in many industrialized countries [Rølla et al., 1991], and can be regarded as the best topical method as it combines the disorganization of dental plaque with the therapeutic effects of fluoride (F) [Bratthall et al., 1996]. However, owing to the limited effect of these products on caries control, new strategies have been considered to enhance their efficacy in reducing caries in the most affected population groups [Carey, 2014]. Thus, the development of new toothpaste formulations to enhance anticaries effects have been investigated, which include the addition of inorganic phosphate salts [Danelon et al., 2015; Takeshita et al., 2015; Danelon et al., 2017]. Among these, sodium hexametaphosphate micrometric (HMP) has a strong affinity to the enamel surface because of multiple binding sites, resulting in a reduced mineral loss when associated with fluoride as demonstrated by da Camara et al. [2014; 2015; 2016].

Nano-sized phosphates (nano) have also emerged as an innovative method with the goal of optimizing the effect of fluoride toothpaste on the demineralization and remineralization process [Danelon et al., 2015; Dalpasquale et al., 2017]. Dalpasquale et al. [2017] evaluated the effect of conventional toothpaste plus HMPnano at concentrations of 0.25%, 0.5% and 1.0% HMPnano in reducing enamel demineralization. The addition of 0.5% HMPnano to a conventional toothpaste significantly enhances its impacts against enamel demineralization. This improved performance is mainly because of physicochemical properties that make them more reactive when compared to micrometric particles [Xu et al., 2010].

Given the positive results obtained by the addition of HMPnano with regards to enamel demineralization and considering the absence of studies assessing the effects of remineralization initial caries lesion, the aim of this study was to evaluate the effect of toothpastes containing 1,100 ppm F associated with HMPnano on enamel remineralization *in situ*. The study's null hypothesis was that the effect of toothpaste on enamel remineralization would not be influenced by the addition of HMPnano.

2.3 Material and Methods

Experimental Design

This study was approved by the Human Ethical Committee of São Paulo State University (UNESP), School of Dentistry, Araçatuba, Brazil (Protocol: 45716715.0.0000.5420). This was a double-blinded crossed *in situ* study performed in four phases of three days each [Danelon et al., 2015]. A sample size of 12 volunteers was based on previous studies [do Amaral et al., 2013; Danelon et al., 2015] considering primary outcomes from surface and cross-sectional hardness analysis, and the mean difference among groups (30 and 1300, respectively), standard deviation (20 and 900, respectively), an α-error of 5% and a β-error of 20%. Volunteers aged 20-30 years, who were in good general and oral health [Delbem et al., 2005] presented normal salivary flow [Rios et al., 2006] and did not violate the exclusion criteria (use of any form of medication likely to interfere with salivary secretion, use of fixed or removable orthodontic appliances, being an active smoker or having systemic illness) were included in the study. No restrictions were imposed regarding the volunteer's diet. All participants read and signed informed consent statements prior to study initiation. Enamel blocks (4 mm × 4 mm, n = 192) (Figure 1A) from bovine incisor teeth were sequentially polished and selected by surface hardness test (SH) (Figure 1B). Blocks were demineralized (Figure 1C) and submitted to post-demineralization surface hardness (SH1) testing (Figure 1D). Surface hardness measurements (SH and SH1) were used to eliminate blocks with anomalous properties prior to further testing. Based on the percentage of SH loss (post-demineralization), blocks were divided into four treatment groups: no F/HMP/HMPnano (Placebo); 1,100 ppm F (1100F); 1100F plus 0.5% micrometric HMP (1100F/HMP); and 1100F plus 0.5% nano-sized HMP (1100F/HMPnano). After three days of the remineralization period (Figure 1E), surface hardness (SH2) (Figure 1F) was again applied to calculate the percentage of surface hardness recovery (%SHR), integrated recovery of surface hardness (ΔIHR) (Figure 1I) and integrated mineral recovery (ΔIMR) (Figure 1J), and enamel fluoride (F) (Figure 1K) concentration was also determined.

Processing and characterization of nano-sized HMP

The processing and characterization of nano-sized HMP was based on the study by Dalpasquale et al. [2017]. Initially, 70 g of pure HMP ($Na_6P_6O_{18}H_6$, CAS 68915-31-1, average size of 31 ± 33 μm, purity ≥ 95%, Aldrich Chemistry, CAS 68915-31-1, 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. X-ray diffraction (XRD) was used to identify the crystalline structure and estimate the crystallographic coherency domain of HMP, thereafter milled for 48 h (HMPnano). The X-ray diffractograms were obtained from samples in powder form using Shimadzu XRD 6000 equipment with a CuK radiation source (λ = 1.54056 Å), voltage of 30 kV and current of 30 mA. Measurements were made continuously in the range of $10\ ^\circ \leq 2\theta \leq 80\ ^\circ$ with a 2 ° scan speed/min. The structural identification of the samples was carried out by comparing the diffraction patterns obtained with tabulated patterns available in the databases, BJoint Committee on Powder Diffraction Standards - Powder Difraction File (JCPDS - PDF). The particle morphology of HMP and HMP milled for 48 h (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

The toothpastes were produced with the following components: titanium dioxide, carboxymethyl cellulose, methyl p-hydroxybenzoate sodium, saccharin, mint oil, glycerin, abrasive silica, sodium lauryl sulfate and deionized water. Toothpastes containing micrometric or nano-sized HMP were prepared (Aldrich Chemistry, CAS 68915-31-1, United Kingdom) at a concentration of 0.5% micrometric HMP (HMP) or nano-sized HMP (HMPnano). To these toothpastes, NaF (Vetec, Duque de Caxias, Rio de Janeiro, Brazil) was added to reach a concentration of 1,100 ppm F. In addition, toothpastes without F/HMP/HMPnano (Placebo) as well as with 1,100 ppm F (without HMP/HMPnano) were prepared. The toothpastes used in this study were stored at room temperature and kept properly closed to prevent any change in the samples.

Total fluoride (TF) and ionic fluoride (IF) amounts were determined [Delbem et al., 2009] using a fluoride-specific electrode (Orion 9609-BN; Orion Research Inc., Beverly, Mass., USA) connected to an ion analyzer (Orion 720 A+; Orion Research Inc.). Three toothpastes per group were analyzed by these methods and the data were presented as micrograms per gram of toothpastes. The pH levels of the toothpastes slurries were determined using a pH electrode (2A09E, Analyser, São Paulo, Brazil) calibrated with standard pH levels of 7.0 and 4.0.

Subsurface enamel demineralization

Enamel blocks were covered with a protective acid-resistant nail varnish (Risqué®, Brazil) were applied on the sides (cut surfaces) and on the bottom of each block excepting the enamel surface. Subsurface enamel demineralization was produced (Figure 1B) by immersing each enamel block in 32 ml of a solution with 1.3 mmol/l Ca, 0.78 mmol/l P in 0.05 mol/l acetate buffer, pH 5.0; 0.03 ppm F; for 16 h at 37°C [Queiroz et al., 2008].

Palatal appliance preparation and treatments

This was a blind and cross-over *in situ* study previously approved by the Human Ethical Committee of Araçatuba Dental School, São Paulo State University, Brazil (Protocol: 45716715.0.0000.5420). Palatal appliances were prepared with acrylic resin (Jet-Articles Classic Odontológico, São Paulo, Brazil) as described by Danelon et al. [2015]. Twelve volunteers wore acrylic palatal appliances (Figure 1E) with four demineralized enamel bovine blocks each that were subjected to four phases of three days each with a seven-day washout period among experimental phases [Danelon et al., 2015]. The treatments with the toothpastes were performed three times per day with the palatal devices inside the volunteers' mouths during their habitual oral hygiene routine. They were instructed to initially brush their natural teeth and conduct three brushing strokes in each row of enamel blocks on the oral appliance with the natural slurry (saliva/toothpaste) formed. Palatal appliances were employed at all times during each experimental phase (including during sleep) and were to be removed only during the main meals. During the seven-day pre-experimental period and washout periods, the volunteers brushed their teeth with a F-free toothpaste. The volunteers received verbal and written instructions prior to the beginning of the study [Danelon et al., 2015].

Hardness Analysis

The enamel SH was determined before (Fig. 1A) and after induction of subsurface lesions (SH1) (Fig. 1D) as well as after each experimental phase (SH2) (E) using a Shimadzu HMV-2000 microhardness tester (Shimadzu Corp., Kyoto, Japan) under a 25g load for 10s [Danelon et al., 2015]. Five indentations, spaced 100 μm from each other, were made at the center of the enamel surface (SH). Indentations for post-demineralization surface hardness (SH1) and for post-experimental surface hardness

(SH2) (Fig. 1F) spaced 100 µm from each other and from the baseline. Moreover, the percentage of SHR (%SHR = ((SH2 – SH1)/ (SH – SH1)) x 100) was calculated. For the cross-sectional hardness measurements, the enamel blocks were longitudinally sectioned through their center (Figure 1G) and embedded in acrylic resin (Figure 1H) with the cut face exposed and gradually polished. One sequence of 14 indentations was created 100 µm apart at different distances (5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220 and 330 µm) from the outer enamel surface using a Micromet 5114 hardness tester (Buehler, Lake Bluff, USA) and the software Buehler OmniMet (Buehler) with a Knoop diamond indenter under a 5 g load for 10 s [Danelon et al., 2013]. The integrated area above the curve (cross-sectional profiles of hardness into the enamel), using the hardness values (KHN), was calculated by trapezoidal rule (GraphPad Prism, version 3.02) in each depth (µm) from the lesion up to sound enamel and subtracted from the integrated area of the sound enamel. The values obtained were subtracted from the integrated hardness area of the post-demineralized enamel, resulting in the integrated recovery of subsurface hardness (ΔIHR) (Fig. 1I).

Analysis of enamel mineral concentrations

Enamel blocks (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, with 1.5 mm of spatial resolution, a rotation step at 0.600º and random moviment at 15. The projections of the images were rebuilt using NRecon software (version 1.6.10.2, Skyscan1272, Bruker MicroCT, Kontich, Belgium) and smoothing at 5, ring artifact correction at 7 and beam hardening correction at 52%. Following image reconstruction, two-dimensional (2D) virtual slices in the sagittal and coronal plane were acquired using Data Viewer software (Skyscan1272). The stacked 2D was imported into ImageJ software to produce an overall mineral concentration ($g_{Hap} \times cm^{-3}$) profile as a function of the depth (µm) (Figure 3A). The mineral concentrations (MC) were calculated from the linear attenuation coefficient (LAC) and expressed as the mass of pure hydroxyapatite ($\rho = 3.15$ $g \times cm^{-3}$) per unit volume of tissue ($g_{Hap} \times cm^{-3}$) [Dowker et al., 2003; Dowker et al., 2004; Dalpasquale et al., 2017].

To analyze the patterns of remineralization, differential mineral concentration profiles were calculated by subtracting the mineral concentration values ($g_{Hap} \times cm^{-3}$) of the artificial caries enamel from those of the treated groups (i.e., artificial caries values minus the Placebo, 1100F, 1100F/HMP and 1100F/HMPnano group values) at each depth

(Figure 4B). The integrated area above the curve (differential cross-sectional profiles of mineral concentration into the enamel) was calculated by trapezoidal rule (GraphPad Prism, version 3.02) at each depth (µm) from the mineral recovery area up to sound enamel to yield of integrated mineral recovery values (ΔIMR) (Figure 1J).

Analysis of the F concentration present in enamel

Enamel blocks (2 mm x 2 mm) were obtained from half of the longitudinally sectioned blocks and were fixed to a mandrel. Self-adhesive polishing discs (diameter, 13 mm) and 400-grit silicon carbide (Buehler) were fixed to the bottom of polystyrene crystal tubes (J-10; Injeplast, Sao Paulo, Brazil) and attached to a handpiece (N 270; Dabi Atlante, Ribeirão Preto, Sao Paulo, Brazil) fixed to the top of a modified microscope with a micrometer (Pantec, Sao Paulo, Brazil). One layer of enamel (51.3 ± 2.1 µm) was removed from each block (Figure 1K) [Weatherell et al., 1985; Takeshita et al., 2009]. 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, Mass., USA) and microelectrode reference (Analyser, Sao Paulo, Brazil) coupled to an ion analyzer (Orion 720A+, Thermo Scientific, Beverly, Mass., USA) was used. The electrodes were calibrated with standards containing from 0.25 to 4.00 µg F/ml (100 ppm F, Orion 940907) under the same conditions as the samples. The readings were conducted using 0.25 ml of the biopsy solution buffered with the same volume of TISAB II modified by NaOH. The results were expressed in $\mu g/mm^3$ [Akabane et al., 2018].

Statistical analysis

The analysis was performed using SigmaPlot software (version 12.0, Systat Software Inc., San Jose, Calif., USA) at a significance level of 5%. The variables %SHR, ΔIHR, ΔIMR and F exhibited normal (Shapiro-Wilk test) and homogeneous (Cochran test) distributions and submitted to one-way repeated measures analysis of variance (ANOVA) followed by Student-Newman-Keuls' testing. Pearson's correlation coefficients between ΔKHN and $g_{Hap} \times cm^{-3}$ were also calculated.

2.4 Results

The X-ray diffraction (XRD) pattern of 48 h HMPnano after milling shows broader peaks owing to the smaller crystallites (Figure 2). Figure 3a depicts the SEM images of HMP with large aggregates and particles of smaller sizes (average size of 31 ± 33 µm). Figure 3b portrays the SEM images of HMPnano particles with low size distribution and an average size of 91 ± 34 nm.

Mean (SD) TF and IF (n = 3) were: 10.5 (0.1) and 10.0 (1.2) for the Placebo, 1186.0 (33.2) and 1102.4 (28.5) for 1100F, 1168.3 (5.9) and 1136.5 (42.6) for 1100F/HMP and 1156.6 (19.7) and 1100.9 (27.1) for 1100F/HMPnano. Mean (SD) pH value from the groups was 7.2 (0.3), ranging from 6.8 to 7.7.

Mean (SD) initial SH for all blocks was 374.0 (1.0), and the means varied between 371.0 (1.6) up 375.2 (2.0) KHN. No significant differences were observed among the groups after random allocation ($p = 0.974$). Mean (SD) of SH after demineralization (SH1) was 57.0 KHN (3.7) and the means varied between 42.5 and 72.4 ($p = 0.441$). The addition of micrometric HMP to fluoride toothpaste increased the %SHR to approximately 68% when compared with the Placebo group ($p < 0.001$) and similar to 1100F ($p > 0.001$). With 1100F/HMPnano, increased remineralization was ~ 66% and ~ 68% when compared to 1100F/HMP and 1100F toothpastes ($p < 0.001$), respectively. In addition, the capacity to reduce the lesion body (ΔIHR) was ~ 87% higher with 1100F/HMPnano and ~ 23% 1100F/HMP ($p < 0.001$) when compared to 1100F (Table 1).

The ΔIMR ($g_{Hap} \times cm^{-3}$ x µm) was higher (~74%) for the 1100F/HMPnano when compared to 1100F ($p < 0.001$) and 33% was higher compared to 1100F/HMPnano x 1100F/HMP ($p < 0.001$) (Table 1). Figure 4A correlated the mineral concentration ($g_{HAp} \times cm^{-3}$) profile as a function of depth (µm), indicating a different profile for all treatments to a depth of 40 µm. Figure 4B shows the patterns of mineral concentration ($g_{HAp} \times cm^{-3}$) according to the treatments groups. The increased mineral content in the subsurface lesion was 1100F/HMPnano>1100F/HMP>1100F>Placebo groups ($p < 0.001$). Positive and significant correlations were observed between ΔIMR and ΔIHR (Pearson's $r = 0.720$; $p < 0.001$) (Figure 5).

The addition of HMP and HMPnano to the F toothpaste did not influence enamel F concentration, so its effect was similar to 1100F except for the Placebo that featured the lowest concentration ($p < 0.001$) (Table 1).

2.5 Discussion

Studies have shown there to be an additional effect of nano-sized phosphates in preventing enamel demineralization and promoting remineralization when added to fluoride formulations [Danelon et al., 2015; Danelon et al., 2017; Dalpasquale et al., 2017]. The present results showed that the addition of 0.5%HMPnano to 1100F led to superior remineralization effects when compared to conventional toothpaste. Thus, the null hypothesis was rejected. The short-term *in situ* model used was based on the studies by Afonso et al. [2013] and Danelon et al. [2015] and was chosen to compare the formulations regarding their potential to boost remineralization of incipient caries lesions given that longer periods would be more related to the remineralizing effect of salivary ions rather than to the treatments. A dose-response relationship between fluoride content in the toothpastes and their effects on enamel was observed, thus validating the model used.

Determination of the ideal HMPnano concentration was based on the study of da Camara et al. [2015], wherein the authors demonstrated that supplementation of a 1,100 ppm F toothpaste with 1.0%HMP promoted a superior effect on enamel demineralization *in situ* compared to a conventional toothpaste without HMP. However, in our study, the reduction of concentration of HMP/HMPnano from 1.0% to 0.5% was shown to be effective in the remineralization of initial caries lesions as well as increasing SHR (68%) (Table 1) in comparison with 1100F, justifying the use of nano-size HMP instead of micrometric particles. Unlike the previous findings, our results show that it is possible to reduce the particle concentration through obtaining an additional result when compared to 1100F; however, if in nano-sized form, its impact will be greater and more effective. This is in line with Danelon et al. [2015] who observed that the addition of a similar phosphate (sodium trimetaphosphate-TMP) as nano-sized particles to a fluoride toothpaste promoted greater remineralizing than conventional toothpastes.

ΔIHR values observed in this study confirm previous findings that HMP reduces mineral loss in deep regions of enamel [da Camara et al., 2015, 2016]. The capacity to reduce the lesion body (ΔIHR) was higher with 1100F/HMP and 1100F/HMPnano when compared 1100F (Table 1). This effect is greater using the salt in its nano-sized form (~ 87%) as already shown by the study of Dalpasquale et al. [2017]. This interaction may result in a barrier that reduces acid diffusion, reducing enamel demineralization while favoring high incorporation of Ca^{2+} in enamel. HMP is a negatively charged cyclic phosphate as demonstrated by other studies [Choi et al., 1993; da Camara et al., 2015; da

Camara et al., 2016]. Furthermore, HMP retains charged CaF^{+} and Ca^{2+} ions by replacement of Na^{+} in the cyclic structure, leading to a reticular formation via Ca^{2+} binding to one or more HMP molecules [van Wazer & Campanella, 1950] and is retained with more intensity in its nano-sized form (HMPnano). A similar finding was arrived at by Danelon et al. [2015] - TMPnano increased mineral gains by 44% in relation to microparticle TMP, mainly in terms of depth, concluding that the use of nano-sized particles is a strategy that promotes effective remineralization of caries lesions.

In this study, the analysis of mineral concentration (ΔIMR) after the remineralization period, at different depths, was of great importance as it was possible to observe that mineral profiles are very different between treatments (Figure 4A), and the results showed that the 1100F/HMP and 1100F/HMPnano groups had higher mineral concentration than the other groups tested with approximately 31% and 78% when compared to the 1100F toothpaste (Table 1 and Figure 4B). Similar findings were observed in the study of Dalpasquale et al. [2017], demonstrating a 58% decrease in integrated mineral loss (IML). This occurrence is mainly because of the mechanism of action of HMP, which increases $CaHPO_4^0$ and HF^0. Thus, the HMP fosters for the formation of the aforementioned species as well as their diffusion in the lesion body, increasing the mineral content of the deeper lesion layers. The use of microCT was of great importance for explaining how the treatments modified the mineralization patterns throughout the subsurface lesions, and how these patterns were influenced by treatments with F, especially when associated with HMPnano, allowing dynamic detection of mineral change after the de- and remineralization of incipient carious lesions and comparing favorably with the current gold standard of transverse microradiography [Lo et al., 2010]. In addition to determining lesion volume, microCT offered a profile that indicated the amount of deposited mineral at each lesion depth examined [Songsiripradubboon et al., 2014].

Furthermore, the addition of HMP/HMPnano to 1100F toothpaste did not increase the incorporation of F into enamel, therefore its effect was similar in all groups except for the Placebo. 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 why a compound may inhibit demineralization or increase remineralization of enamel [Pfarrer et al., 2002]. In addition, these findings are in line with previous studies [da Camara et al., 2015; da Camara et al., 2016; Dalpasquale et al., 2017]. Given that the anticaries effect of conventional toothpastes (i.e., without HMP/HMPnano) is usually

related to the ability to increase enamel fluoride concentrations, it can be concluded that the mechanism of action of HMP/HMPnano-containing fluoride toothpastes is somehow different from that described for toothpastes containing fluoride as the only anticaries active ingredient, as discussed earlier.

When a remineralizing agent is used in the clinical setting, its action is expected to take place within the shortest length of time possible. However, as *in vivo* demineralization and remineralization processes depend on multiple factors, accurate determination of the length of time for *in situ* protocols becomes a difficult task. Therefore, a number of important variables should be considered before determining the experimental period of an *in situ* study. The type of substrate and depth of the artificial caries lesion seem to be the most important. Bovine enamel has a higher reactivity and porosity, leading to faster remineralization when compared to human enamel [Lynch et al., 2006]. As for the substrate, the depth of enamel demineralization may also interfere with the amount of remineralization time; however, few investigations have considered the depth of the demineralized area in their protocols. It is known that the remineralization process is slower in deep lesions (±100 µm) based on a longer distance for ion diffusion when compared to that seen in the present study [Mellberg, 1991]. Another factor allowing fast remineralization of the three-day protocol, after using the toothpastes, was the type of lesion. This type of lesion presents faster remineralization rates based on the higher number and diameter of lesion pores, being suitable to compare the efficacy of different remineralizing regimens [Lynch et al., 2006].

It is worth noting that this *in situ* study presents certain limitations, such as: 1) lack of dental biofilm; 2) bovine teeth substrates; 3) the lesions are larger and easily remineralized, leading to faster remineralization when compared to human enamel; 4) we may not have completely controlled for the environmental factors; and 5) we did not evaluate inorganic and organic composition of the biofilm and biofilm fluid, so the experimental toothpaste may yield benefits in terms of prevention of new lesions or as a remineralization therapy, mainly for young children and with high caries experience. It is concluded that the addition of HMPnano in conventional toothpaste promoted a significantly higher remineralizing effect compared with conventional toothpaste. Thus, this toothpaste could be an alternative for patients at high caries risk and activity.

Acknowledgments

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2.6 References

Afonso RL, Pessan JP, Igreja BB, Cantagallo CF, Danelon M, Delbem ACB. In situ protocol for the determination of dose response effect of low-fluoride dentifrices on enamel remineralization. J App Oral Sci 2013;21:525-532.

Akabane S, Delbem AC, Pessan J, Garcia L, Emerenciano N, Gonçalves DF, Danelon M. In situ effect of the combination of fluoridated toothpaste and fluoridated gel containing sodium trimetaphosphate on enamel demineralization. J Dent 2018;68:59-65.

Alves KMRP, Pessan JP, Brighenti FL, Franco KS, Oliveira FAL, Buzalaf MAR, Sassaki KT, Delbem ACB. In vitro evaluation of the effectiveness of acidic fluoride dentifricies Caries Res 2007;41:263-267.

Carey CM. Focus on fluorides: update on the use of fluoride for the prevention of dental caries. J Evid Based Dent Pract 2014;14:95-102.

Choi IK, Wen WW, Smith RW (1993) Technical note the effect of a long chain phosphate on the adsorption of collectors on kaolinite. Miner Eng 6:1191-1197.

Bratthal D, Hansel-Petersson G, Sundberg H. Reasons for the Caries Decline: What Do the Experts Believe? Eur J Oral Sci 1996;104:416-422.

da Camara DM, Miyasaki ML, Danelon M, Sassaki KT, Delbem AC. Effect of low-fluoride toothpastes combined with hexametaphosphate on in vitro enamel demineralization. J Dent 2014;42:256-262.

da Camara DM, Pessan JP, Francati TM, Santos Souza JA, Danelon M, Delbem AC. Synergistic effect of fluoride and sodium hexametaphosphate in toothpaste on enamel demineralization in situ. J Dent 2015;43:1249-1254.

da Camara DM, Pessan JP, Francati TM, Souza JA, Danelon M, Delbem AC. Fluoride toothpaste supplemented with sodium hexametaphosphate reduces enamel demineralization in vitro. Clin Oral Investig 2016;20:1981-1985.

Dalpaquale G, Delbem AC, Pessan JP, Nunes GP, Gorup LF, Neto FN, de Camargo ER, Danelon M. Effect of the addition of nano-sized sodium hexametaphosphate to fluoride toothpastes on tooth demineralization: an in vitro study. Clin Oral Invest 2017;21:1821-1827.

Danelon M, Takeshita EM, Sassaki KT, Delbem ACB. In situ evaluation of a low fluoride concentration gel with sodium trimetaphosphate in enamel re-mineralization. Am J Dent 2013;26:15-20.

Danelon M, Pessan JP, Neto FN, de Camargo ER, Delbem AC. Effect of toothpaste with nano-sized trimetaphosphate on dental caries: In situ study. J Dent 2015;43:806-813.

Danelon M, Pessan JP, Souza-Neto FN, Camargo ER, Delbem ACB. Effect of fluoride toothpaste with nano-sized trimetaphosphate on enamel demineralization: An in vitro study. Arch Oral Biol 2017;78:82-87.

Delbem ACB, Carvalho LPR, Morihisa RKU, Cury JA. Effect of rinsing with water immediately after APF gel application on enamel demineralization in situ. Caries Res 2005;39:258-260.

Delbem ACB, Sassaki KT, Vieira AE, Rodrigues E, Bergamaschi M, Stock SR, et al. Comparison of methods for evaluating mineral loss: hardness versus synchrotron microcomputed tomography. Caries Res 2009;43:359-365.

do Amaral JG, Sassaki KT, Martinhon CCR, Delbem ACB. Effect of low-fluoride dentifrices supplemented with calcium glycerophosphate on enamel deminetalization in situ. Am J Dent 2013;26:75-80.

Dowker SE, Elliott JC, Davis GR, Wassif HS. Longitudinal study of the three-dimensional development of subsurface enamel lesions during in vitro demineralization. Caries Res 2003;37:237-245.

Dowker SE, Elliott JC, Davis GR, Wilson RM, Cloetens P. Synchrotron X-ray microtomographic investigation of mineral concentrations at micrometer scale in sound and carious enamel. Caries Res 2004;38:514-522.

Lynch RJM, Mony U, ten Cate JM. Effect of lesion characteristics and mineralizing solution type on enamel remineralization in vitro. Caries Res 2007;41:257-262.

Lo EC, Zhi QH, Itthagarun A. Comparing two quantitative methods for studyng remineralization of artificial caries. J Dent 2010;38:352-359.

Mellberg JR. Relationship of original mineral loss in carieslike lesions to mineral changes in situ. Caries Res 1991;25:459-61.

Pfarrer AM, White DJ, Rapozo-Hilo, Featherstone ID. Anticaries and hard tissue abrasion effects of a “dual action” whitening, sodium hexametaphosphate tartar control dentifrice. J Clin Dent 2002;13:50-54.

Queiroz CS, Hara AT, Leme FP, Cury JA. pH-cycling models to evaluate the effect of low fluoride dentifrice on enamel de- and remineralization. Braz Dent J 2008;19:21-27.

Rios D, Honorio HM, Magalhaes AC, Delbem ACB, Machado MAAM, Silva SMB, Buzalaf MA. Effect of salivary stimulation on erosion of human and bovine enamel

subjected or not to subsequent abrasion: an in situ/ex vivo study. Caries Res 2006;40:218-223.

Rølla G, Ogaard B, Cruz RA. Clinical effect and mechanism cariostact action of fluoride-containing toothpastes: a review. Int Dent J 1991;41:171-174.

Songsiripradubboon S, Hamba H, Trairatvorakul C, Tagami J. Sodium fluoride mouthrinse used twice daily increased incipient caries lesion remineralization in an in situ model. J Dent 2014;42:271-278.

Takeshita EM, Castro LP, Sassaki KT, Delbem ACB. In vitro evaluation of dentifrice with low fluoride content supplemented with trimetaphosphate. Caries Re 2009;43:50-56.

Takeshita EM, Danelon M, Castro LP, Sassaki KT, Delbem AC. Effectiveness of a Toothpaste with low fluoride content combined with trimetaphosphate on dental biofilm and enamel demineralization in situ. Caries Res 2015;49:394-400.

van Wazer JR, Campanella DA. Structure and properties of the condensed phosphates. IV. Complex ion formation in polyphosphate solutions. J Am Chem Soc 1950;72:655-663.

Weatherell JA, Robinson C, Strong M, Nakagaki, H. Micro-sampling by abrasion. Caries Res 1985,19:97-102.

Xu HH, Weir MD, Sun L, Moreau JL, Takagi S, Chow LC, et al. Strong nanocomposites with Ca, PO4 and F release for caries inhibition. J Dent Res 2010;89:19-28.

Contributions made by each author to the paper

Study's idea and design: MD, ACBD and JPP.

Synthesis and characterization of nano-sized HMP: ERC

Accomplishment of experiments: LSGG, MD, AP, JPP and ACBD.

Data analysis: ACBD, MD, LSGG, ERC and JPP.

Manuscript preparation: LSGG, ACBD, JPP, AP, ERC and MD.

Table legend

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

Table figure

Figure 1 – **A** Enamel block preparation. **B** Surface hardness analysis. **C** Enamel demineralization. **D** Surface hardness analysis. **E** Intraoral phase. **F** Surface hardness analysis. **G** Longitudinal section. **H** Embedded acrylic resin. **I** Integrated recovery of subsurface hardness (ΔIHR). **J** Analysis of enamel mineral concentrations. **K** Self-adhesive polishing discs.

Figure 2. X-ray diffraction patterns of HMP before (HMP) and after (HMPnano) grinding of powder for 48 h in ball mill. The possible phases $(NaPO_3)_6$ PDF# 3643 Sodium hexametaphosphate, $NaPO_3$ PDF# 76788 Sodium metaphosphate, $Na_2H_2P_2O_7$ PDF# 10187 Disodium dihydrogen diphosphate, NaH_2PO_4 (H_2O) DF#11651 Sodium dihydrogen phosphate monohydrate, NaH_2PO_4 PDF#11657 Sodium dihydrogen phosphate and $Na_5P_3O_{10}$ PDF# 11652 Pentasodium triphosphate.

Figure 3. SEM images of Sodium hexametaphosphate particles, a) HMP and b) HMPnano after grinding of powder by 48h in ball mill.

Figure 4. (A) Cross-sectional profile of mineral concentration ($g_{HAp} \times cm^3$) as function of depth (µm) from according to the groups. (B) Differential profile obtained from values of mineral concentration of the treatments subtracted from the artificial caries values.

Figure 5. Relationship between integrated mineral recovery values (ΔIMR) and integrated mineral loss (ΔIHR). n = 192

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

	Variables			
Toothpastes	**%SHR (KHN) [1]**	**ΔIHR (KHN x µm) [2]**	**ΔIMR (g_{HAp} x cm^{-3}) [3]**	**F (µg/mm^3) [4]**
Placebo	19.0 (3.5)[a]	1505.2 (557.5)[a]	6.3 (3.0)[a]	0.2 (0.1)[a]
1100F	31.7 (2.0)[b]	2431.9 (227.2)[b]	11.2 (2.0)[b]	0.4 (0.1)[b]
1100F/HMP	32.0 (3.8)[b]	2987.5 (347.9)[c]	14.7 (3.3)[c]	0.4 (0.1)[b]
1100F/HMPnano	53.3 (2.4)[c]	4560.7 (585.0)[d]	19.6 (5.8)[d]	0.4 (0.1)[b]

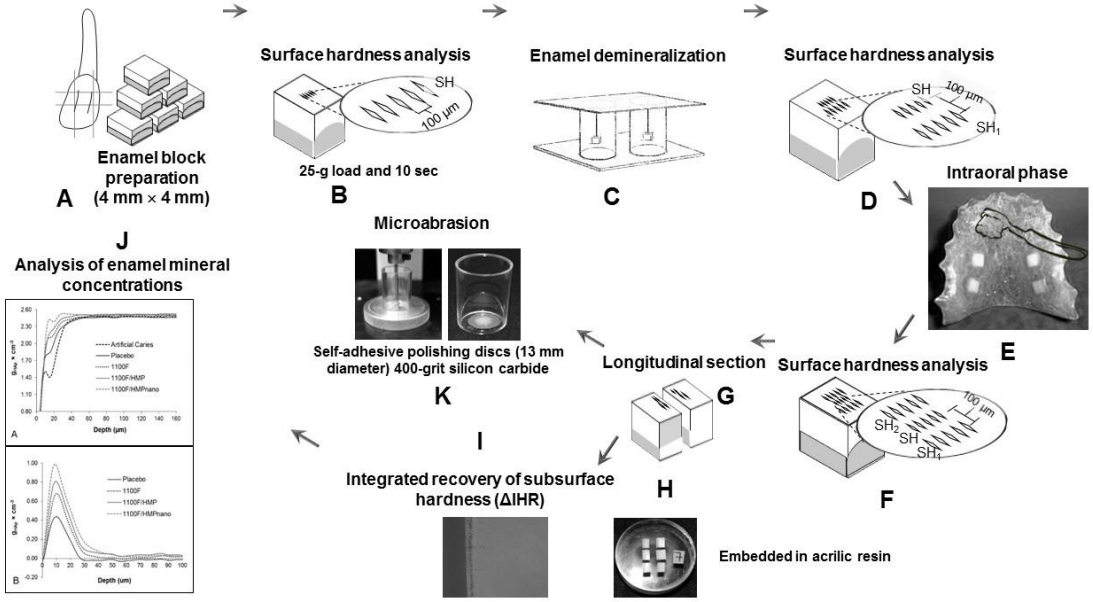
[1]%SHR: percentage of surface hardness recovery - KHN

[2]ΔIHR: integrated loss of subsurface hardness – KHN x µm

[3]ΔIMR: Integrated mineral loss - g_{Hap} x 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, $p < 0.001$).



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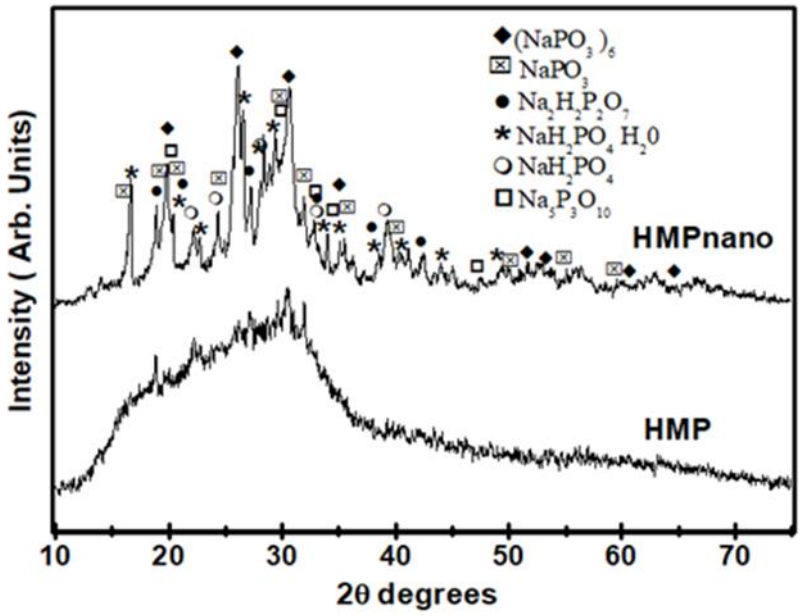

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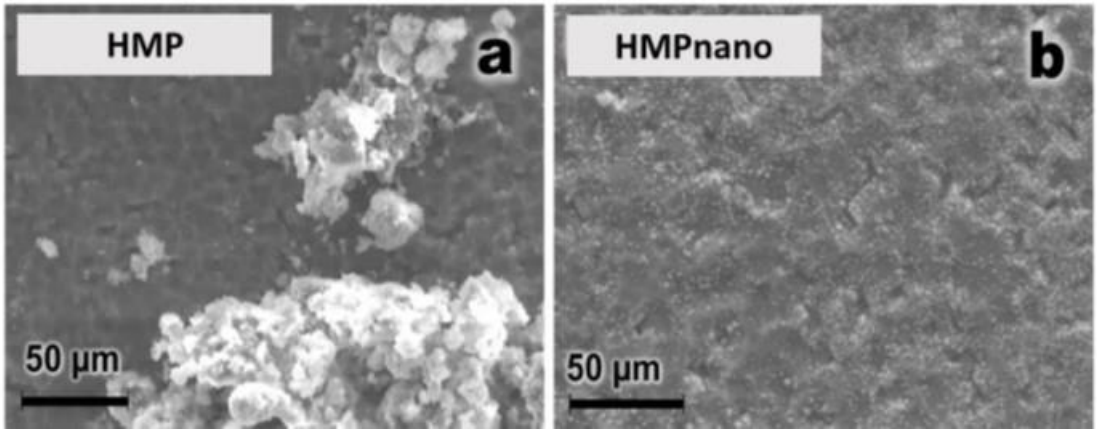

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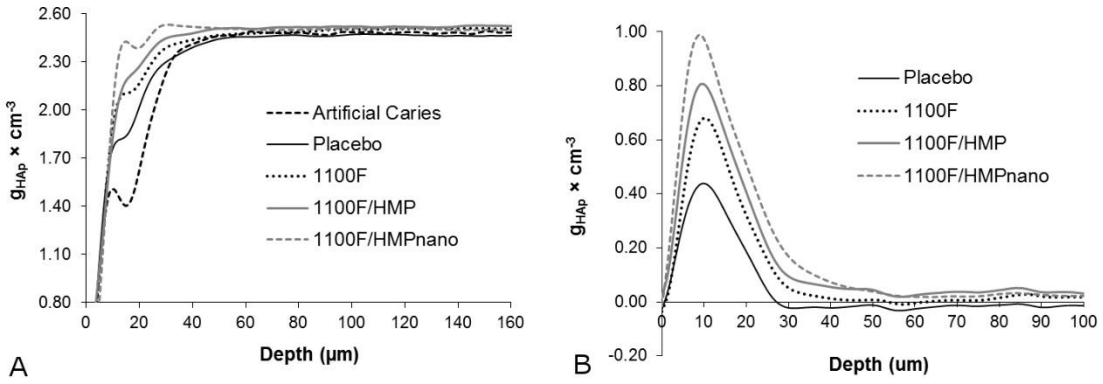

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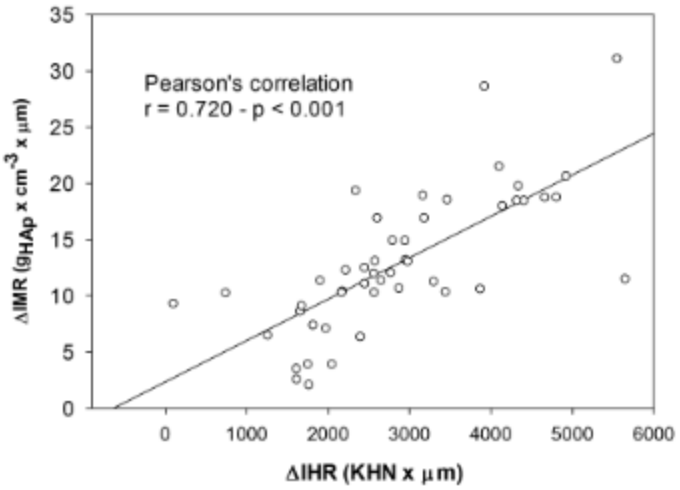

Figure 5. Relationship between integrated mineral recovery values (ΔIMR) and integrated mineral loss (ΔIHR). n = 192

Capítulo 2

3. Anticaries effect of toothpaste with nano-sized sodium hexametaphosphate

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Short title: Fluoride toothpaste with nano-sized hexametaphosphate

Keywords: Caries; Biofilm; Fluoride; Demineralization; Nano-sized.

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***De acordo com as instruções aos autores do periódico Caries Research.**

Conflict of Interest Form

The authors Marcelle Danelon, Alberto Carlos Botazzo Delbem, Juliano Pelim Pessan and Emerson Rodrigues de Camargo hold a patent request for a product used in the study, by the National Institute of Industrial Property - INPI/SP, on 10/17/2014 under number BR 10 2014 025902 3. All authors approved the publishing of the manuscript.

3.1 Abstract

Objective: To evaluate the effect of a fluoride toothpaste containing nano-sized sodium hexametaphosphate (HMPnano) on enamel demineralization and on the composition of the biofilm formed *in situ*. Methods: This crossover double-blind study consisted of four phases (7 days each), in which 12 volunteers wore intraoral appliances containing four enamel bovine blocks. The cariogenic challenge was performed using 30% sucrose solution (6x/day). Blocks were treated 3x/day with the following toothpastes: no F/HMP/HMPnano (Placebo), 1,100 ppm F (1100F), 1100F plus 0.5% micrometric or nano-sized HMP (1100F/HMP and 1100F/HMPnano, respectively). The percentage of surface hardness loss (%SH), integrated loss of subsurface hardness (ΔKHN), and enamel calcium (Ca), phosphorus (P), and fluoride (F) were determined. Moreover, biofilms formed on the blocks were analyzed for F, Ca, P, and insoluble extracellular polysaccharide (EPS) concentrations. Data were analyzed using one-way ANOVA, followed by Student–Newman–Keuls' test ($p < 0.001$). Results: 1100F/HMPnano promoted the lowest %SH and ΔKHN among all groups ($p < 0.001$). The addition of HMPnano to 1100F did not enhance enamel F and P uptake, but significantly increased Ca concentrations ($p < 0.001$). The 1100F/HMPnano promoted lower values of EPS when compared with 1100F (~70%) ($p < 0.001$); and higher values of fluoride and calcium in the biofilms ($p < 0.001$). Conclusion: 1100F/HMPnano demonstrated a greater protective effect against enamel demineralization and on the composition of biofilm *in situ* when compared to 1100F toothpaste.

Keywords: Caries; Biofilm; Fluoride; Demineralization; Nano-sized.

3.2 Introduction

In the last few decades, a decline in dental caries prevalence was observed, mainly assigned to the use of fluoride toothpaste [Browne et al., 2005]. Since dental caries has been shown to be polarized in some groups, several studies have been conducted to evaluate new formulations of conventional toothpastes (CT, i.e., 1,100 ppm F) with enhanced potential in reducing caries lesions, including the association of F with phosphates [Danelon et al., 2015; da Camara et al., 2016; Dalpasquale et al., 2017].

Sodium hexametaphosphate (HMP) interferes with the enamel de-remineralization process due to its ability to modify the solubility of dental enamel [da Camara et al., 2015; da Camara et al., 2016]. da Camara et al. [2015, 2016] evaluated the effect of a CT-containing HMP on enamel demineralization and biofilm. In these studies, the authors observed that the association between 1.0% HMP and 1,100 ppm F promoted the lowest surface hardness loss (%SH) and integrated loss of subsurface hardness (ΔKHN). Additionally, it has antimicrobial activity due to its ability to increase the permeability of the bacterial outer membrane [Vaara & Jaakkola, 1989], and inhibitory activity on biofilm formation [Shibata & Morioka, 1982]. Nanotechnology was defined as the creation of functional materials at the nanoscale (1-100 nm) [Samiei et al., 2016]. Nanostructured nanomaterials, nanoparticles in particular, have unique physicochemical properties, such as ultra-small and controllable size, large surface area in relation to mass, high reactivity, and a functionalizable structure [Zhang et al., 2010; He et al., 2015]. Nano-sized phosphates have emerged as an innovative method, aiming to optimize the effect of F toothpaste on the demineralization and remineralization process [Danelon et al., 2015; Dalpasquale et al., 2017]. Dalpasquale et al. [2017] evaluated *in vitro* the effect of CT plus nano-sized HMP (HMPnano) at concentrations of 0.25%, 0.5%, and 1.0% in reducing enamel demineralization. The addition of 0.5% HMPnano to a CT significantly enhances its effects against enamel demineralization.

So far, no study has evaluated whether the addition of HMPnano could affect the biofilm composition and enamel demineralization under cariogenic challenge, simulating a condition of high caries risk. Thus, this study evaluated the effect of a F toothpaste containing HMPnano on enamel demineralization *in situ* and on the composition of biofilm. The null hypothesis was that F toothpaste associated with HMPnano would provide similar anticaries effect when compared to F toothpaste.

3.3 Material and Methods

Experimental Design

This study was approved by the Human Ethical Committee of São Paulo State University (UNESP), School of Dentistry, Araçatuba, Brazil (Protocol: 58549716.8.0000.5420), and all participants read and signed informed consent statements prior to study onset. This crossover double-blind study was conducted in four phases of 7 days each [da Camara et al., 2015]. The sample size of 12 volunteers was based on a previous study [do Amaral et al., 2013], considering as primary outcome the surface and cross-sectional hardness analysis, mean difference between groups (30 and 1300, respectively), standard deviation (20 and 900, respectively), an α-error of 5%, and a β-error of 20%. Volunteers (n=12) aged 20-30 years, who were in good general and oral health [Delbem et al., 2005] were included in the study. The subjects wore an acrylic palatal appliance with sound bovine enamel blocks (4 mm × 4 mm, n = 192), previously polished and selected according to the initial surface hardness (SHi) (baseline). The specimens were allocated to treatments: no F/HMP/HMPnano (Placebo), 1,100 ppm F (1100F), 1100F plus 0.5% micrometric or nano-sized HMP (1100F/HMP; 1100F/HMPnano). After each phase the biofilm was collected for analysis of F, Ca, P, and insoluble extracellular polysaccharides (EPS). In the enamel blocks, the percentage of surface hardness loss (%SH) and integrated loss of subsurface area (ΔKHN) were assessed again. F, Ca, and P content in enamel were determined.

Processing and characterization of nano-sized HMP

The processing and characterization of nano-sized HMP was based on the study by Dalpasquale et al. [2017]. Initially, 70 g of pure HMP ($Na_6P_6O_{18}H_6$, CAS 68915-31-1, average size of 31 ± 33 μm, purity ≥ 95%, Aldrich Chemistry, CAS 68915-31-1, 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. X-ray diffraction (XRD) was used to identify the crystalline structure and estimate the crystallographic coherency domain of HMP, thereafter milled for 48 h (HMPnano). The X-ray diffractograms were obtained from samples in powder form, using Shimadzu XRD 6000 equipment with a CuK radiation source (λ = 1.54056 Å), voltage of 30 kV, and current of 30 mA. Measurements were made continuously in the range of 10 ° ≤ 2θ ≤ 80 ° with a 2 ° sweep speed/min. The

structural identification of the samples was carried out by comparing the diffraction patterns obtained with tabulated patterns available in the databases, BJoint Committee on Powder Diffraction Standards - Powder Difraction File (JCPDS - PDF). The particle morphology of HMP and HMP milled for 48 h (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 with the following components: titanium dioxide, carboxymethyl cellulose, methyl p-hydroxybenzoate sodium, saccharin, mint oil, glycerin, abrasive silica, sodium lauryl sulfate, and deionized water. Toothpastes containing micrometric or nano-sized HMP were prepared (Aldrich Chemistry, CAS 68915-31-1, United Kingdom) at a concentration of 0.5%. In addition, toothpastes without F/HMP/HMPnano (Placebo), as well as with 1,100 ppm F (without HMP/HMPnano, NaF: Vetec, Duque de Caxias, Rio de Janeiro, Brazil) were prepared.

The amounts of total fluoride (TF) and ionic fluoride (IF) were determined [Delbem et al., 2009] using a F^- specific electrode (Orion 9609-BN; Orion Research Inc., Beverly, Mass., USA) connected to an ion analyzer (Orion 720 A+; Orion Research Inc.). The pH levels of toothpaste slurries were determined using a pH electrode (2A09E, Analyser, São Paulo, Brazil) calibrated with standard pH levels of 7.0 and 4.0.

Palatal Appliance Preparation and Treatments

The palatal appliance was prepared in acrylic resin (Jet, Articles Classic Odontológico, São Paulo, Brazil) and four enamel blocks were fixed, using a different device in each phase of the experiment. To allow biofilm accumulation on the enamel blocks, a piece of plastic mesh was fixed to the acrylic appliance, leaving a 1-mm space from the block surface [da Camara et al., 2015]. To provide a cariogenic challenge, the volunteers were instructed to remove the device and drip 30% sucrose solution (Sucrose, Synth, Diadema, Brazil) onto each enamel block 6x/day at predetermined times (8:00 am, 11:00 am, 2:00 pm, 5:00 pm, 7:00 pm, and 9:00 pm) [da Camara et al., 2015]. The appliances were used 24 h/day, and the volunteers brushed their natural teeth 3x/day (08:00 am, 13:00 pm, 21:30 pm) for 2 min, with the palatal appliance in the oral cavity, allowing the natural saliva/toothpaste slurry to come into contact with the enamel blocks

by gently squishing the slurry in the mouth. During a 7-day pre-experimental period and washout periods, the volunteers brushed their teeth with the placebo toothpaste.

Hardness Analysis

The SHi was determined before and after each experimental phase (SHf), using a Shimadzu HMV-2000 microhardness tester (Shimadzu Corp., Kyoto, Japan) under a 25 g load for 10 s [Danelon et al., 2015], followed by calculation of the percentage of surface hardness loss: (%SH =[(SHf-SHi)/SHi]*100). For the cross-sectional hardness measurements, the enamel blocks were longitudinally sectioned through their center and embedded in acrylic resin with the cut face exposed and gradually polished. A sequence of 14 indents was created 100 µm apart at different distances (5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220, and 330 µm) from the outer enamel surface using a Micromet 5114 hardness tester (Buehler, Lake Bluff, USA) and the software Buehler OmniMet (Buehler, Lake Bluff, USA) with a Knoop diamond indenter under a 5 g load for 10 s [Danelon et al., 2013; Dalpasquale et al., 2017]. Integrated hardness (KHN x 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 subsurface regions in enamel, which was named integrated loss of subsurface hardness (ΔKHN; KHN x µm) [Spiguel et al., 2009].

Fluoride, calcium, and phosphorus in enamel

F present in the enamel was determined as described by Weatherell et al. [1985] and modified by Alves et al. [2007]. Self-adhesive polishing discs (13-mm diameter) and 400-grit silicon carbide (Buehler) were fixed to the bottom of polystyrene crystal tubes (J-10; Injeplast, Sao Paulo, Brazil) and attached to a handpiece (N 270; Dabi Atlante, Ribeirão Preto, Sao Paulo, Brazil) fixed to the top of a modified microscope with a micrometer (Pantec, Sao Paulo, Brazil). One layer of enamel (50.9 ± 0.2 µm) was removed from each block, after addition of 0.5 ml HCl 1.0 mol L^{-1}, and these were kept under constant stirring for 1 hour [Weatherell et al., 1985; Alves et al., 2007]. For F analysis, specific electrode 9409BN (Thermo Scientific, Beverly, Mass., USA) and microelectrode reference (Analyser, Sao Paulo, Brazil) coupled to an ion analyzer (Orion 720A+, Thermo Scientific, Beverly, Mass., USA) were used. The results were expressed in $\mu g/mm^3$. Ca analysis was performed using the Arsenazo III colorimetric method [Fiske

& Subbarow, 1925]. The absorbance readings were recorded at 650 nm, using a plate reader (PowerWave 340, Biotek, Winooski, VT, USA). P was measured according to Fiske and Subbarow [1925], and the absorbance readings were recorded at 660 nm. The results were expressed as $\mu g/mm^3$.

Analysis of dental biofilm composition

The biofilm formed on enamel was collected and stored in microcentrifuge tubes. The biofilm samples were dried in vacuum over P pentoxide for 12 h at room temperature. F was analyzed using an ion specific electrode (Orion 9409 BN) and a potentiometer (Orion 720 A^{plus}). The Ca concentration was analyzed by a colorimetric test [Vogel et al., 1983]. The P concentration was measured using a colorimetric method [Fiske & Subbarow, 1925]. EPS was extracted by adding 1.0 mol L^{-1} NaOH (10 µL/mg dry weight) to the biofilm [Nobre dos Santos et al., 2002; Ccahuana-Vasquez et al., 2007]. The amount of EPS was determined using the phenol-sulfuric acid method [Dubois et al., 1956]. The results were expressed as moles/kilograms (F, Ca, and P) and milligrams/grams (EPS) dry weight.

The ionic activities (IA) of the various species (Ca^{2+}, $CaPO_4^-$, $CaHPO_4^0$, $CaH_2PO_4^+$, PO_4^{3-}, HPO_4^{-2}, $H_2PO_4^-$, CaF^+, HF^0, and F^-) and the degree of saturation (DS) of the solid phases of hydroxyapatite (HA) and calcium fluoride (CaF_2) were calculated from the concentrations (mol/kg) of F, Ca, and P on the biofilm of each toothpaste. All calculations were performed at 37ºC, 7.0 pH, and a density of 1.0 g/cm^3 using the PHREEQC Interactive (version 2.18.3) speciation program [do Amaral et al., 2013; da Camara et al., 2015].

Statistical analysis

SigmaPlot 12.0 software (version 12.0, Systat Software Inc., San Jose, Calif., USA) was used for statistical analysis, and the significance level was set at 5%. The statistical power calculated was 85%, considering all differences between groups for each outcome. Data from the dental biofilm analysis (Ca, P, F and EPS content, and ionic activities) and enamel analysis (%SH, ΔKHN and F, Ca, and P content) exhibited normal (Shapiro–Wilk) and homogeneous (Bartlet) distribution, and were therefore subjected to one-way ANOVA, repeated measures followed by the Student–Newman–Keuls' testing.

3.4 Results

The X-ray diffraction (XRD) pattern of 48 h HMPnano after milling shows broader peaks owing to the smaller crystallites (Figure 1). Figure 2a depicts the SEM images of HMP with large aggregates and particles of smaller sizes (average size of 31 ± 33 μm). Figure 2b portrays the SEM images of HMPnano particles with low size distribution and an average size of 91 ± 34 nm.

Mean (SD) concentration of total F (TF) and ionic fluoride (IF) (n = 3) were as follows: Placebo – 10.5 (0.1) and 10.0 (1.2); 1100F – 1186.0 (33.2) and 1102.4 (28.5); 1100F/HMP – 1168.3 (5.9) and 1136.5 (42.6); and 1100F/HMPnano – 1156.6 (19.7) and 1100.9 (27.1). The mean pH value of the groups was 7.2 (0.3) ranging from 6.8 to 7.7.

The use of 1100F/HMPnano resulted in a 49% decrease in %SH in comparison with 1100F (Table 1). The addition of micrometric HMP to F toothpaste decreased the %SH in 36% when compared with the Placebo group ($p < 0.001$), and was similar to 1100F ($p=0.695$). In addition, the capacity to reduce the lesion body (ΔKHN) was ~ 10% and ~ 55% higher with 1100F/HMP and 1100F/HMPnano, respectively ($p < 0.001$) when compared to 1100F (Table 1).

The addition of HMP and HMPnano to the CT did not influence enamel F concentration, so that its effect was similar to 1100F, except for the Placebo, which showed a lower concentration ($p < 0.001$). With 1100F/HMPnano, the enamel Ca concentration was increased by ~12% and ~78% when compared to 1100F/HMP and 1100F toothpastes ($p < 0.001$). No significant difference was observed between groups regarding enamel P concentrations except for the Placebo, which showed a lower concentration ($p < 0.001$).

As for the biofilm composition, 1100F/HMPnano promoted the highest retention of Ca ($p < 0.001$) and F ($p < 0.001$), when compared with 1100F (Table 1), while the P values were similar for the treatments ($p = 0.084$). 1100F/HMPnano showed lower values for alkali-soluble EPS concentration, when compared with 1100F (~65%) and 1100F/HMP (~60%) ($p < 0.001$). Similar concentrations were observed for the 1100F and 1100F/HMP groups ($p = 0.709$), which were significantly lower than that of the Placebo group ($p < 0.001$).

The ionic activity of CaF^+ and HF^0 for the 1100F/HMPnano group were significantly higher when compared to the other groups ($p < 0.001$), while no significant differences were seen between 1100F/HMPnano and the other groups regarding ionic activity of Ca^{2+}, $CaPO_4^-$, and $CaH_2PO_4^+$ ($p > 0.001$). No significant differences were

observed between the four groups for ionic activity of PO_4^{3-}, HPO_4^{2-}, and $H_2PO_4^-$ ($p > 0.800$). As for phase saturation, the 1100F/HMPnano group showed the highest supersaturation with respect to HA and CaF_2 ($p < 0.001$).

3.5 Discussion

Studies have shown an additional effect of nano-sized phosphates in preventing enamel demineralization and promoting remineralization when added to fluoridated formulations [Danelon et al., 2015; Dalpasquale et al., 2017; Danelon et al., 2017]. The present results showed that the addition of 0.5%HMPnano to 1100F led to superior anticaries effects when compared to the conventional toothpaste. Thus, the null hypothesis was rejected.

Our study showed that the addition of HMPnano to conventional fluoridated toothpastes at a concentration of 0.5% was able to reduce enamel demineralization by 49% when compared to the 1100F group. Considering micrometric HMP, da Camara et al. [2015, 2016] observed that the addition of 1.0%HMP to a CT significantly reduces enamel demineralization when compared to 1100F. Unlike the previous findings, our results show that it is possible to reduce the particle concentration obtaining an additional result when compared to 1100F; however, if this is in its nano-sized form, its effect will be better and more effective.

The supplementation of 1100F with 0.5% HMPnano resulted in a ~50% reduction in mineral loss (%SH and ΔKHN) when compared to 1100F. Our findings were far superior when compared to those obtained by da Camara et al. [2015], in which the reduction was ~25% with micro-sized 1.0% HMP when compared to 1100F. This synergistic effect is in line with previous *in vitro* findings with a CT supplemented with 0.5% HMPnano [Dalpasquale et al., 2017] and can be attributed to a higher ability to prevent enamel mineral loss, justifying the use of nano-sized HMP. It is noteworthy that HMP does not improve F enamel uptake [da Camara et al., 2015; da Camara et al., 2016; Dalpasquale et al., 2017] as observed in the present study (Table 1). However, Ca concentration in enamel is higher using 1.0% HMP [da Camara et al., 2015] or 0.5% HMPnano (Table 1). Notwithstanding, 1.0% HMP [da Camara et al., 2015] and 0.5% HMPnano (Table 2) produces higher supersaturation with respect to CaF_2 and HA compared to 1100F. Thus, enamel Ca concentrations are increased, it is probably due to formation of HMP-Ca^{2+} layer on enamel, which reduces acid diffusion into enamel [van

Dijk, 1980; da Camara et al., 2015] and supersaturation with respect to CaF_2 that can reduce EPS in the biofilm (Table 1).

These findings are supported by the significantly higher Ca concentrations in enamel seen for the 1100F/HMPnano group (78%) when compared to 1100F, which seems to be related to a more resistant enamel. Conversely, HMPnano did not seem to have any effect on enamel F and phosphate concentrations, which is also in line with a previous *in vitro* study assessing the effects of HMPnano when added to a 1,100 ppm F toothpaste [Dalpasquale et al., 2017]. Thus, it can be assumed that the mechanism of action of HMPnano containing F toothpastes is somehow different from that described for toothpastes containing F as the only active anticaries ingredient. Furthermore, the procedure used to synthesize HMPnano promoted more reactive particles with increased adsorption on 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, as demonstrated by Dalpasquale et al. [2017].

Based on previous studies and confirmed by the present results, it is known that the association of HMPnano and F reduces mineral loss, and its effect may be explained by the adsorption of HMPnano on enamel, even with the presence of biofilm on the enamel surface. This effect is greater when the salt is in its nano-sized form, as already shown by the study of Dalpasquale et al. [2017]. This interaction may result in a barrier that reduces acid diffusion, reducing enamel demineralization while favoring high incorporation of Ca^{2+} in enamel. HMP is a negatively charged cyclic phosphate as demonstrated by other studies [Choi et al., 1993; da Camara et al., 2015; da Camara et al., 2016]. Furthermore, HMP retains charged ions of CaF^{+} and Ca^{2+} by replacement of Na^{+} in the cyclic structure, leading to a reticular formation via Ca^{2+} binding to one or more HMP molecules [van Wazer & Campanella, 1950], and in its nano-sized form (HMPnano) it retains with greater intensity. However, HMP cannot consider a source of phosphate to enamel or biofilm [da Camara et al., 2015] since it does not undergo spontaneous hydrolysis [Choi et al., 1993].

The use of 1100F/HMPnano promoted significant changes in the biofilm composition, providing more insights into the mechanisms of action of this association. It is noteworthy that the increase in F concentrations in the biofilm is often correlated with Ca concentration [Whitford et al., 2002; Pessan et al., 2006; Pessan et al., 2008], as observed for the 1100F group when compared to the Placebo group (Table 1). The addition of micro-sized 0.5% HMP to 1100F toothpaste presented similar outcomes,

showing no additional effect on the biofilm. Nevertheless, micro-sized 0.5%HMP presented smaller depth of lesion (ΔKHN) and higher Ca concentration in enamel when compared to 1100F (Table 1). The lower cariogenic challenge observed in the present study can also have contributed to lower mineral loss of the 1100F toothpaste when compared to *in vitro* study [da Camara et al., 2016]. This effect is due to the HMP capacity of enamel adsorption and reduction of acid diffusion into enamel [da Camara et al., 2016]. Considering the effect in the biofilm, micro-sized 0.5% HMP did not interfere with Ca retention, unlike the findings for micro-sized 1.0% HMP in a previous study [da Camara et al., 2015]. However, the 0.5% HMP in its nano-sized form led to higher F and Ca retention in the biofilm as a consequence of its more reactive and smaller particles. In the 1100F/HMPnano group, the biofilm served as a reservoir of F and Ca ions during the high cariogenic challenge (30% sucrose solution), which could contribute to minimize the enamel mineral loss (%SH and ΔKHN).

The synergistic effect of HMPnano and F in the toothpaste was also assessed considering the ionic activity of F, Ca and phosphate. The ionic activity of CaF^+ and HF^0 for the 1100F/HMPnano were significantly higher when compared to the other groups. In addition, only the 1100F/HMPnano toothpaste was supersaturated in relation to CaF_2, when compared to 1100F and 1100F/HMP groups. As the biofilm was under acidic conditions, CaF^+ and Ca^{2+} species might have reacted with $H_2PO_4^-$, increasing the ionic activity of neutral ion HF^0. As their diffusion coefficients into enamel lesions are thousand-fold higher than their charged counterparts, they would not be impeded by the charged enamel surface [Cochrane et al., 2008]. This mechanism is strengthened when considering the two different particle sizes used in the present study: due to the higher ratio of surface area per volume of nano-sizeds, as well as to their higher percentage of atoms on the surface compared to larger (micrometric) particles, nanoparticles can be regarded as more reactive than microparticles. Analysis of the biofilm shows that nano-sized HMP, in fact, increases the concentration of those reactive compounds that might act on the inner part of the subsurface lesion [Cochrane et al., 2008]. Nonetheless, the mechanisms proposed above need to be carefully considered, given that data from ionic activity were calculated based on the total ion concentration from the whole biofilm, which comprises not only the free ions in the biofilm fluid, but also ionizable and firmly bound pools. Such calculations, however, are useful for an overall comparison between groups, providing insights on the reasons why HMP nano promoted a significantly higher protective effect on enamel.

Regarding alkali-soluble EPS concentration, our results showed an expressive reduction (64%) for the HMPnano toothpaste when compared with 1100F, which was not observed for micro-sized 1.0% HMP added to 1100F toothpaste [da Camara et al., 2015]. This study hypothesized that 1.0% HMP reduces the calcium concentration in the biofilm. It is noteworthy that more fluoride in the biofilm is related with higher Ca concentration [Whitford et al., 2002; Pessan et al., 2006; Pessan et al., 2008], as observed for 1100F and 1100F plus micrometric 0.5% HMP when compared to the placebo (Table 1). In addition, the reduction of EPS is related to Ca concentration between 1×10^{-3} and 1×10^{-4} mol/L [Boyd, 1978]. A lower percentage of HMP did not interfere with calcium in the biofilm as verified in the previous study [da Camara et al., 2015], and 1100F and 1100F/HMP showed similar ability to reduce the EPS in the biofilm (Table 1). Thus, the higher F and Ca concentrations produced by 1100F/HMPnano in the biofilm may have been responsible for the reduction of bacterial metabolism and EPS production [Van loveren, 2001; Marquis et al., 2003]. Notwithstanding, the HMP presents capacity to alter the permeability and glucose transport, since it forms a strong complex with Mg^{2+} in the outer bacterial membrane, leading to an antimicrobial effect [Vaara & Jaakkola, 1989] and thus the results of our study showed that this effect can be increased when phosphate is used in its nano-sized form. The HMP in its nano-sized form can lead to reduction of EPS since its particles are more reactive. However, these data are not supported by the literature because a report showed antimicrobial action over cariogenic bacterial in concentrations over 6.0% [da Camara et al., 2015]. These data are based on an *in situ* demineralization protocol, and we suggest that other similar studies should be conducted with biofilm accumulation and different cariogenic challenges (frequency x exposure to sucrose), to simulate patients with different caries activities. Studies analyzing the inorganic composition of saliva and plaque fluid, *in situ* remineralization protocol, and *in vivo* studies would be of great importance to confirm our findings.

We conclude that 1100F/HMPnano promoted a greater protective effect against enamel demineralization and significantly affected the composition of biofilm formed *in situ* when compared to 1100F toothpaste. Thus, this toothpaste could be an alternative for patients at high caries risk and activity.

3.6 References

Alves KMRP, Pessan JP, Brighenti FL, Franco KS, Oliveira FAL, Buzalaf MAR, Sassaki KT, Delbem ACB. In vitro evaluation of the effectiveness of acidic fluoride dentifricies Caries Res 2007;41:263-267.

Boyd RF. The effect of some divalent cations on extracellular polysaccharide synthesis in Streptococcus salivarius. J Dent Res 1978;57:380-383.

Browne D, Whelton H, O'Mullane D. Fluoride metabolism and fluorosis. J Dent 2005;33:177-186.

Ccahuana-Vasquez RA, Tabchoury CPM, Tenuta LMA, Del Bel Cury AA, Vale GC, Cury JA. Effect of frequency of sucrose exposure on dental biofilm composition and enamel demineralization in the presence of fluoride. Caries Res 2007;41:9-15.

Choi IK, Wen WW, Smith RW. Technical note the effect of a long chain phosphate on the adsorption of collectors on kaolinite. Miner Eng 1993;6:1191-1197.

Cochrane HJ, Saranathan S, Cai F, Cross K J, Reynolds EC. Enamel subsurface lesion remineralisation with casein phosphopeptide stabilized solutions of calcium phosphate and fluoride. Caries Res 2008;42:88-97.

da Camara DM, Pessan JP, Francati TM, Santos Souza JA, Danelon M, Delbem AC. Synergistic effect of fluoride and sodium hexametaphosphate in toothpaste on enamel demineralization in situ. J Dent 2015;43:1249-1254.

da Camara DM, Pessan JP, Francati TM, Souza JA, Danelon M, Delbem AC. Fluoride toothpaste supplemented with sodium hexametaphosphate reduces enamel demineralization in vitro. Clin Oral Investig 2016;20:1981-1985.

Dalpasquale G, Delbem ACB, Pessan JP, Nunes GP, Gorup LF, Souza-Neto FN, Camargo ER, Danelon M. Effect of the addition of nano-sized sodium hexametaphosphate to fluoride toothpastes on tooth demineralization: an in vitro study. Clin Oral Investig 2017;21:1821-1827.

Danelon M, Takeshita EM, Sassaki KT, Delbem ACB. In situ evaluation of a low fluoride concentration gel with sodium trimetaphosphate in enamel remineralization. Am J Dent 2013;26:15-20.

Danelon M, Pessan JP, Neto FN, de Camargo ER, Delbem AC. Effect of toothpaste with nano-sized trimetaphosphate on dental caries: In situ study. J Dent 2015;43:806-813.

Danelon M, Pessan JP, Souza-Neto Francisco Nunes, de Camargo ER, Delbem ACB. Effect of fluoride toothpaste with nano-sized trimetaphosphate on enamel demineralization: An in vitro study. Arch Oral Biol 2017;78:82-87.

Delbem AC, Carvalho LP, Morihisa RK, Cury JA. Effect of rinsing with water immediately after APF gel application on enamel demineralization in situ. Caries Res 2005;39:258-60.

Delbem AC, Sassaki KT, Vieira AE, Rodrigues E, Bergamaschi M, Stock SR, Cannon ML, Xiao X, De Carlo F, Delbem ACB. Comparison of methods for evaluating mineral loss: hardness versus synchrotron microcomputed tomography. Caries Res 2009;43:359-365.

do Amaral JG, Martinhon CC, Delbem ACB. Effect of low-fluoride toothpastes supplemented with calcium glycerophosphate on enamel demineralization in situ. Am J Dent 2013;26:75-80.

Dubois M, Grilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350-356.

Fiske CH, Subbarow Y. The colorimetric determination of phosphorus. J Biol Chem 1925;66:375-400.

He L, Deng D, Zhou X, Cheng L, ten Cate JM, Li J, Li X, Crielaard W. Novel tea polyphenol-modified calcium phosphate nanoparticle and its remineralization potential. J Biomed Mater Res B Appl Biomater 2015;8:1525-31.

Marquis RE, Clock SA, Mota-Meira M. Fluoride and organic weak acids as modulators of microbial physiology. FEMS Microbiol Rev 2003;26:493-510.

Nobre dos Santos M, Melo dos Santos L, Francisco SB, Cury JA. Relationship among dental plaque composition, daily sugar exposure and caries in the primary dentition. Caries Res 2002;36:347-352.

Pessan JP, Sicca CM, de Souza TS, da Silva SMB, Whitford GM, Buzalaf MAR. Fluoride concentrations in dental plaque and saliva after the use of a fluoride toothpaste preceded by a calcium lactate rinse. Eur J Oral Sci 2006;114:489-493.

Pessan JP, Silva SMB, Lauris JRP, Sampaio FC, Whitford GM, Buzalaf MAR. Fluoride uptake by plaque from water and from toothpaste. J Dent Res 2008;87:461-465.

Samiei M, Farjami A, Dizaj SM, Lotfipour F. Nanoparticles for antimicrobial purposes in Endodontics: a systematic review of in vitro studies. Mater Sci Eng C 2016;58:1269-78.

Shibata H, Morioka T. Antibacterial action of condensed phosphates on the bacterium Streptococcus mutans and experimental caries in the hamster. Arch Oral Biol 1982;27:809-816.

Spiguel MH, Tovo MF, Kramer PF, Franco KS, Alves KM, Delbem AC. Evaluation of laser fluorescence in the monitoring of the initial stage of the de-/remineralization process: an in vitro and in situ study. Caries Res 2009;43:302-307.

Vaara M, Jaakkola J. Sodium hexametaphosphate sensitizes Pseudomonas aeruginosa, several other species of Pseudomonas, and E. coli to hydrophobic drugs. Antimicrob. Agents Chemother 1989;3:1741-1747.

van Dijk JW, Borggreven JM, Driessens FC. The effect of some phosphates and a phosphonate on the electrochemical properties of bovine enamel. Arch Oral Biol 1980;25:591-595.

Van Loveren B. Antimicrobial activity of fluoride and its in vivo importance: identification of research questions. Caries Res 2001;35:65-70.

van Wazer JR, Campanella DA. Structure and properties of the condensed phosphates. IV. Complex ion formation in polyphosphate solutions. J Am Chem Soc 1950;72:655-63.

Vogel GL, Chow LC, Brow WL. A microanalytical procedure for the determination of calcium, phosphate and fluoride in enamel biopsy samples. Caries Res 1983;17:23-31.

Weatherell JA, Robinson C, Strong M, Nakagaki H. Micro-sampling by abrasion. Caries Res 1985;19:97-102.

Whitford GM, Wasdin JL, Schafer TE, Adair SM. Plaque fluoride concentrations are dependent on plaque calcium concentrations. Caries Res 2002;36:256-265.

Zhang L, Pornpattananangku D, Hu CM, Huang CM. Development of nanoparticles for antimicrobial drug delivery. Curr Med Chem 2010;17:585-594.

Contributions made by each author to the paper

Study's idea and design: MD, ACBD and JPP.

Synthesis and characterization of nano-sized HMP: ERC

Accomplishment of experiments: LSGG, MD, MPS, JPP and ACBD.

Data analysis: ACBD, MD, LSGG, ERC and JPP.

Manuscript preparation: LSGG, ACBD, JPP, MPS, ERC and MD.

Table legend

Table 1: Mean (SD) of variables analyzed according to the toothpaste treatments

Table 2. Ionic activity of ions species and phase saturation from dental biofilms treated with different toothpastes

Figure legends

Figure 1. X-ray diffraction patterns of HMP before (HMP) and after (HMPnano) grinding of powder for 48 h in ball mill. The possible phases $(NaPO_3)_6$ PDF# 3643 Sodium hexametaphosphate, $NaPO_3$ PDF# 76788 Sodium metaphosphate, $Na_2H_2P_2O_7$ PDF# 10187 Disodium dihydrogen diphosphate, NaH_2PO_4 (H_2O) DF#11651 Sodium dihydrogen phosphate monohydrate, NaH_2PO_4 PDF#11657 Sodium dihydrogen phosphate and $Na_5P_3O_{10}$ PDF# 11652 Pentasodium triphosphate.

Figure 2. SEM images of sodium hexametaphosphate particles. a HMP and b HMPnano after grinding of powder for 48 h in ball mil.

Table 1: Mean (SD) of variables analyzed according to the toothpaste treatments

Analysis	Toothpastes			
	Placebo	**1100F**	**1100F/HMP**	**1100F/HMPnano**
%SH, KHN	-53.8[a] (8.7)	-35.3[b] (9.4)	-34.3[b] (4.5)	-18.7[c] (5.5)
ΔKHN, KHN x µm	6,244.6[a] (909.0)	3,663.4[b] (305.7)	3,280.6[c] (368.6)	1,655.4[d] (391.8)
Enamel, µg/mm^3				
Fluoride	0.18[a] (0.03)	0.29[b] (0.03)	0.29[b] (0.05)	0.25[b] (0.05)
Calcium	228.9[a] (57.0)	381.5[b] (68.1)	608.3[c] (76.1)	678.6[d] (67.3)
Phosphorus	287.5[a] (49.1)	342.7[b] (81.1)	331.7[b] (81.5)	390.4[b] (68.6)
Biofilm, mol/kg				
Fluoride	2.74E-04[a] (9.29E-05)	6.81E-04[b] (5.26E-04)	5.27E-04[b] (1.97E-04)	1.28E-03[c] (1.01E-03)
Calcium	6.63E-02[a] (2.94E-02)	1.13E-01[b] (2.64E-02)	1.10E-01[b] (3.94E-02)	1.24E-01[c] (2.29E-02)
Phosphorus	7.91E-02[a] (2.48E-02)	9.60E-02[a] (3.94E-02)	1.12E-01[a] (5.90E-02)	1.13E-01[a] (3.68E-02)
Biofilm: EPS, mg/g	434.2[a] (156.3)	239.4[b] (97.3)	225.4[b] (71.7)	87.4[c] (43.4)

Different superscript letters indicate significant differences among the treatments for each variable separately. One-way ANOVA, repeated measures followed Student-Newman-Keuls' test (n=12, $p < 0.001$).

Table 2. Ionic activity of ions species and phase saturation from dental biofilms treated with different toothpastes

Ion activity, mol/kg	**Toothpastes**			
	Placebo	**1100F**	**1100F/HMP**	**1100F/HMPnano**
Ca^{2+}	1.39E-02[a] (1.72E-02)	1.68E-02[a] (7.20E-03)	1.52E-02[a] (9.61E-03)	1.65E-02[a] (4.54E-03)
$CaPO_4^-$	1.13E-03[a] (5.06E-04)	1.68E-03[a] (5.24E-04)	2.63E-03[a] (3.14E-03)	2.01E-03[a] (5.04E-04)
$CaHPO_4^0$	3.86E-02[a] (1.73E-02)	5.80E-02[b] (1.75E-02)	5.80E-02[b] (2.15E-02)	6.72E-02[b] (1.83E-02)
$CaH_2PO_4^+$	1.59E-02[a] (4.51E-02)	4.05E-03[a] (1.27E-03)	4.11E-03[a] (1.53E-03)	4.83E-03[a] (1.26E-04)
PO_4^{3-}	4.23E-08[a] (1.75E-08)	3.40E-04[a] (1.18E-08)	4.76E-08[a] (3.83E-08)	3.88E-08[a] (2.09E-08)
HPO_4^{2-}	6.78E-03[a] (3.73E-03)	6.10E-03[a] (3.75E-03)	8.38E-03[a] (6.74E-03)	1.14E-02[a] (1.64E-02)
$H_2PO_4^-$	1.15E-02[a] (4.81E-03)	8.88E-03[a] (5.92E-03)	1,15E-02[a] (1.08E-02)	9.53E-03[a] (3.98E-03)
F^-	1.88E-04[a] (6.06E-05)	4.01E-04[a] (2.77E-04)	3.33E-04[a] (1.44E-04)	7.85E-04[a] (6.32E-04)
CaF^+	2.06E-05[a] (1.42E-05)	8.99E-05[b] (9.91E-05)	5.14E-05[b] (2.62E-05)	1.35E-04[c] (9.46E-05)
HF^0	3.51E-08[a] (1.10E-08)	7.49E-08[b] (5.17E-08)	6.23E-08[b] (2.69E-08)	1.46E-07[c] (1.18E-07)
Degree of Saturation				
HA	15.22[a] (1.58)	16.54[b] (0.46)	16.36[b] (0.69)	16.80[c] (0.41)
CaF_2	0.84[a] (0.56)	1.69[b] (0.69)	1.56[b] (0.34)	2.28[c] (0.49)

Distinct superscript letters indicate statistical significance among the toothpastes for each ions species or solid phase (Student-Newman-Keuls's test; $p < 0.05$). Values between parentheses indicate the standard deviation of the mean.

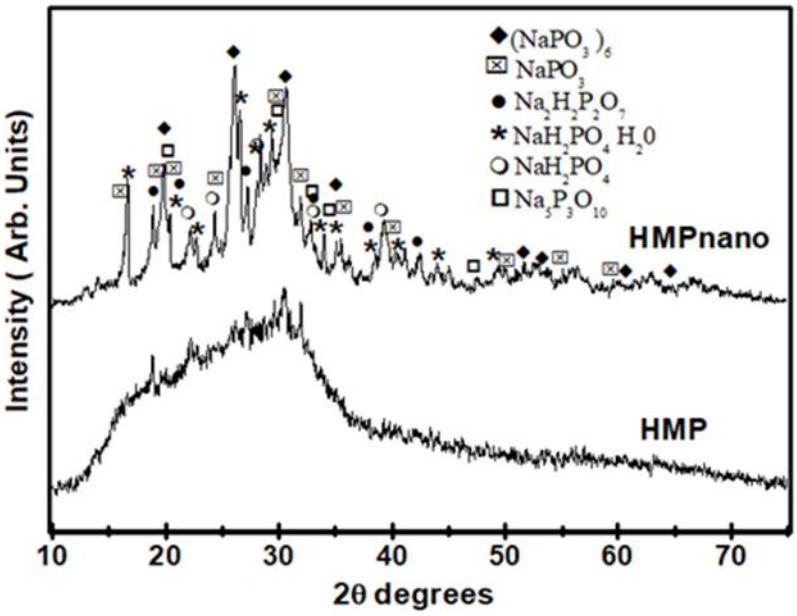

Figure 1. X-ray diffraction patterns of HMP before (HMP) and after (HMPnano) grinding of powder for 48 h in ball mill. The possible phases $(NaPO_3)_6$ PDF# 3643 Sodium hexametaphosphate, $NaPO_3$ PDF# 76788 Sodium metaphosphate, $Na_2H_2P_2O_7$ PDF# 10187 Disodium dihydrogen diphosphate, NaH_2PO_4 (H_2O) DF#11651 Sodium dihydrogen phosphate monohydrate, NaH_2PO_4 PDF#11657 Sodium dihydrogen phosphate and $Na_5P_3O_{10}$ PDF# 11652 Pentasodium triphosphate.

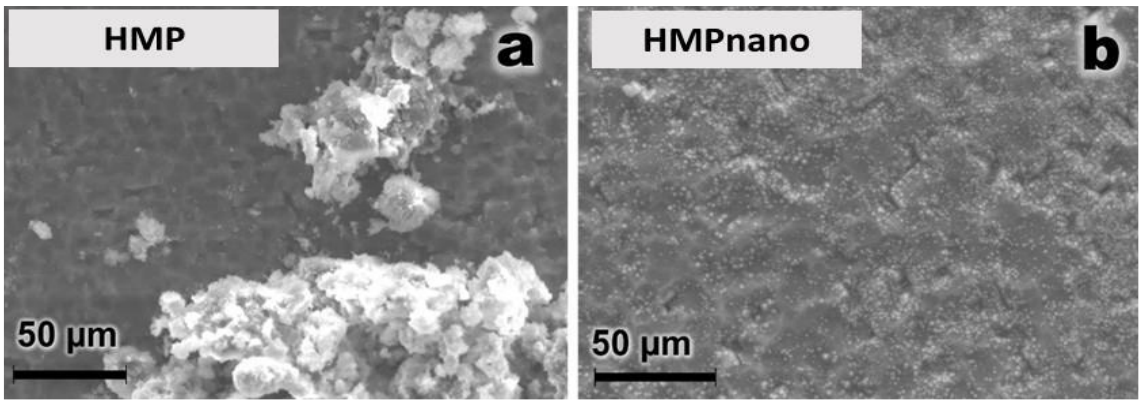

Figure 2. SEM images of Sodium hexametaphosphate particles, a) HMP and b) HMPnano after grinding of powder by 48h in ball mill.

Conclusão Geral

4. CONCLUSÃO GERAL

Conclui-se que a adição de HMPnano a um dentifrício convencional promoveu um efeito remineralizador significantemente maior em lesões artificiais de cárie e demonstrou um maior efeito protetor contra a desmineralização e biofilme *in situ*.

Anexos

5.1 ANEXO A

COMITÊ DE ÉTICA (Capítulo 1)

FACULDADE DE ODONTOLOGIA - CÂMPUS DE ARAÇATUBA - JÚLIO DE

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: EFEITO DA ADIÇÃO DE NANOPARTÍCULAS DE HEXAMETAFOSFATO DE SÓDIO EM DENTIFRÍCIOS FLUORETADOS SOBRE A REMINERALIZAÇÃO DENTÁTIA: ESTUDO IN SITU

Pesquisador: Alberto Carlos Botazzo Delbem

Área Temática:

Versão: 3

CAAE: 45716715.0.0000.5420

Instituição Proponente: Faculdade de Odontologia do Campus de Araçatuba - UNESP

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.235.101

Apresentação do Projeto:

O estudo será duplo-cego e cruzado consistindo em três fases com duração de 3 dias cada e washout de 7 dias entre uma etapa e outra, para eliminar possíveis efeitos residuais dos tratamentos [Delbem et al., 2010; Afonso et al., 2013; Danelon et al.,
2014]. O projeto será submetido pelo Comitê de Ética de Pesquisa em Humanos pela Plataforma Brasil. Doze voluntários com idade entre 18 e 33
anos e boa saúde geral e bucal e com fluxo salivar normal serão selecionados. Blocos de esmalte (4 mm × 4 mm, n = 144) serão obtidos de dentes incisivos bovinos e mantidos em solução de formol a 2%, pH 7,0 durante 30 dias antes de qualquer procedimento experimental [Delbem e Cury, 2002]. Esses blocos terão sua
superfície de esmalte polida, permitindo sua seleção através da determinação da dureza de superfície inicial (SH). Os blocos serão desmineralizados
e submetidos ao teste de dureza de superfície pós-desmineralização (SH1). Serão confeccionados dispositivos para a arcada superior com quatro espaços (4 mm × 4 mm), nos quais serão fixados quatro blocos de esmalte bovino desmineralizados. Os
grupos consistirão em três tratamentos: 1)sem F/HMPnano (Placebo), 2) 1100 ppm F (1100 ppm F), 3) 1100 ppm F associado à 0,5% HMP

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Continuação do Parecer: 1.235.101

nanoparticulado. Após três dias do período de remineralização os quatro blocos serão removidos do dispositivo para análise da dureza de superfície final (SH2) e porcentagem de recuperação de dureza de superfície (%SHR), dureza em secção longitudinal para o cálculo da perda integrada de dureza de subsuperfície (KHN), microtomografia computadorizada (gHApcm-3), e da concentração de F presente no esmalte após o período de remineralização. Para análise estatística, serão considerados como variáveis os valores de %SHR, KHN, gHApcm-3 e o conteúdo de F no esmalte e, como fator de variação, os dentifrícios experimentais.

Objetivo da Pesquisa:

O objetivo deste subprojeto será avaliar in situ o efeito da adição de hexametafosfato de sódio (HMP) nanoparticulado em dentifrícios com 1100 ppm F no processo de remineralização do esmalte dentário.

Avaliação dos Riscos e Benefícios:

Mínimos. O voluntário poderá sofrer algum desconforto com o uso do dispositivo palatino.

Benefícios: Desenvolvimento de um produto, o qual será mais eficiente no controle da cárie dentária.

Comentários e Considerações sobre a Pesquisa:

O protocolo de pesquisa está bem elaborado e a metodologia é adequada. Os resultados trarão contribuição área de pesquisa.

Considerações sobre os Termos de apresentação obrigatória:

Os termos obrigatórios foram devidamente apresentados.

Recomendações:

Não há.

Conclusões ou Pendências e Lista de Inadequações:

Não há.

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Plataforma Brasil

Continuação do Parecer: 1.235.101

Considerações Finais a critério do CEP:

O CEP o protocolo e informa que, de acordo com a Resolução 466 CNS, de 12/12/2012 (título X, seção X.1., art. 3, item b, e, título XI, seção XI.2., item d), há necessidade de apresentação de relatórios semestrais, devendo o primeiro relatório ser enviado até 18/03/2016.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Projeto Detalhado / Brochura Investigador	Projeto CEP.docx	17/05/2015 18:24:36		Aceito
Folha de Rosto	Folha de rosto CEP.pdf	26/05/2015 09:22:29		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE 1.pdf	03/08/2015 09:10:29		Aceito
Outros	CV.pdf	24/08/2015 20:40:36	Alberto Carlos Botazzo Delbem	Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_518446.pdf	26/08/2015 09:38:47		Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

ARACATUBA, 16 de Setembro de 2015

Assinado por:
André Pinheiro de Magalhães Bertoz
(Coordenador)

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5.2 ANEXO B

COMITÊ DE ÉTICA (Capítulo 2)

UNESP - FACULDADE DE ODONTOLOGIA-CAMPUS DE ARAÇATUBA/ UNIVERSIDADE

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Efeito da adição de nanopartículas de hexametafosfato de sódio em dentifrícios fluoretados sobre o processo de desmineralização dentária: estudo in situ.

Pesquisador: Marcelle Danelon

Área Temática:

Versão: 1

CAAE: 58549716.8.0000.5420

Instituição Proponente: Faculdade de Odontologia do Campus de Araçatuba - UNESP

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.768.104

Apresentação do Projeto:

O objetivo do presente estudo será avaliar, in situ, a capacidade de um dentifrício contendo hexametafosfato de sódio nanoparticulado (HMPnano) associado ao fluoreto (F), em reduzir a desmineralização do esmalte dentário bovino. Serão realizados 4 períodos experimentais com duração de 7 dias cada, e washout de 7 dias entre eles, sendo um estudo cego e cruzado. Blocos de esmalte bovinos (n=192) serão selecionados através da dureza de superfície inicial (SHi) e a seguir doze voluntários (n=12) utilizarão dispositivos palatinos contendo 4 blocos de esmalte, durante 7 dias em 4 fases experimentais: 1) dentifrício sem F/HMPnano (Placebo); 2) dentifrício 1100 ppm F (1100 ppm F); 3) dentifrício 1100 ppm associado a 0,5%HMP microparticulado (1100 0,5%HMPmicro) e 4) dentifrício 1100 ppm associado a 0,5%HMP nanoparticulado (1100 0,5%HMPnano). Os desafios cariogênicos serão produzidos pelo uso uma solução de sacarose a 30%. Nos blocos de esmalte, serão determinadas a dureza de superfície final (SHf) para o cálculo da porcentagem de perda de dureza de superfície (%SH). Será utilizado o teste estatístico mais ade[illegible]ado à distribuição dos dados, através do programa estatístico software Sigmaplot© para Windows versão 12.0, com significância ao nível de 5%.

Objetivo da Pesquisa:

O objetivo do presente estudo será avaliar, in situ, a capacidade de um dentifrício contendo

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Bairro: VILA MENDONCA **CEP:** 16.015-050
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Telefone: (18)3636-3200 **Fax:** [illegible]336-3332 **E-mail:** andrebertoz@foa.unesp.br

UNESP - FACULDADE DE ODONTOLOGIA-CAMPUS DE ARAÇATUBA/ UNIVERSIDADE

Plataforma Brasil

Continuação do Parecer: 1.768.104

hexametafosfato de sódio nanoparticulado (HMPnano) associado ao fluoreto (F), em reduzir a desmineralização do esmalte dentário bovino.

Avaliação dos Riscos e Benefícios:

Riscos:

O Risco será Mínimo, uma vez que os voluntários poderão apresentar um leve desconforto durante a utilização do dispositivo palatino.

Benefícios:

Espera-se, com este projeto desenvolver uma formulação dentifrícia com 1100 ppm F e eficácia superior à de um dentifrício padrão ou comercial (1100 ppm F) mantendo a estabilidade do fluoreto no dentifrício. Entender o mecanismo de ação da associação F/HMPnano no processo da cárie dentária.

Com os resultados parciais ou totais obtidos, a divulgação será realizada em congressos nacionais e internacionais e em periódicos de impacto (dois artigos).

Pretende-se, também, o depósito de patente junto ao Instituto Nacional da Propriedade Industrial – I.N.P.I./S.P. de formulação dentifrícia com 1100 ppm F suplementado com hexametafosfato de sódio nanoparticulado

Comentários e Considerações sobre a Pesquisa:

Objetivos são claros e bem definidos.

A metodologia proposta é capaz de atender os objetivos do estudo

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos de apresentação obrigatória foram apresentados.

Recomendações:

Não há.

Conclusões ou Pendências e Lista de Inadequações:

O CEP aprova o projeto.

Considerações Finais a critério do CEP:

Não havendo pendências, o CEP propõe a aprovação do projeto de pesquisa salientando que, de acordo com a Resolução 466 CNS de 12/12/2012 (título X, seção X.1., art. 3, item b, e, título XI, seção XI.2., item d), há necessidade de apresentação de relatórios semestrais, devendo o primeiro relatório ser enviado até 01/04/2017 O CEP reitera a necessidade de entrega de uma via (não cópia) do TCLE ao sujeito participante da pesquisa e solicita ao pesquisador responsável leitura da carta circular 003/2011 CONEP/CNS antes do início do projeto.

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UNESP - FACULDADE DE ODONTOLOGIA-CAMPUS DE ARAÇATUBA/ UNIVERSIDADE

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Continuação do Parecer: 1.768.104

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_770109.pdf	09/08/2016 12:04:15		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.pdf	09/08/2016 12:03:42	Marcelle Danelon	Aceito
Projeto Detalhado / Brochura Investigador	Projeto.docx	09/08/2016 11:41:01	Marcelle Danelon	Aceito
Folha de Rosto	FR.pdf	09/08/2016 11:34:32	Marcelle Danelon	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

ARACATUBA, 10 de Outubro de 2016

Assinado por:
André Pinheiro de Magalhães Bertoz
(Coordenador)

Endereço: JOSE BONIFACIO 1193
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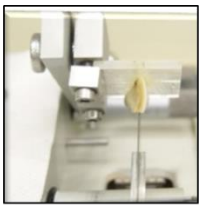
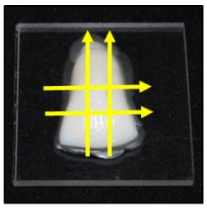
5.3 ANEXO C

PREPARO DOS BLOCOS DE ESMALTE

Colagem em placa de acrílico

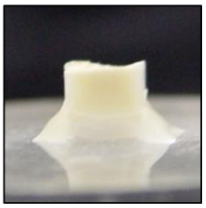


Separação da superfície vestibular/lingual



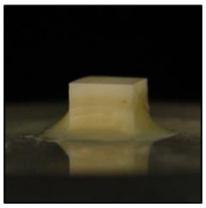
Superfície vestibular

Blocos de 4mm x 4mm



Planificação da dentina

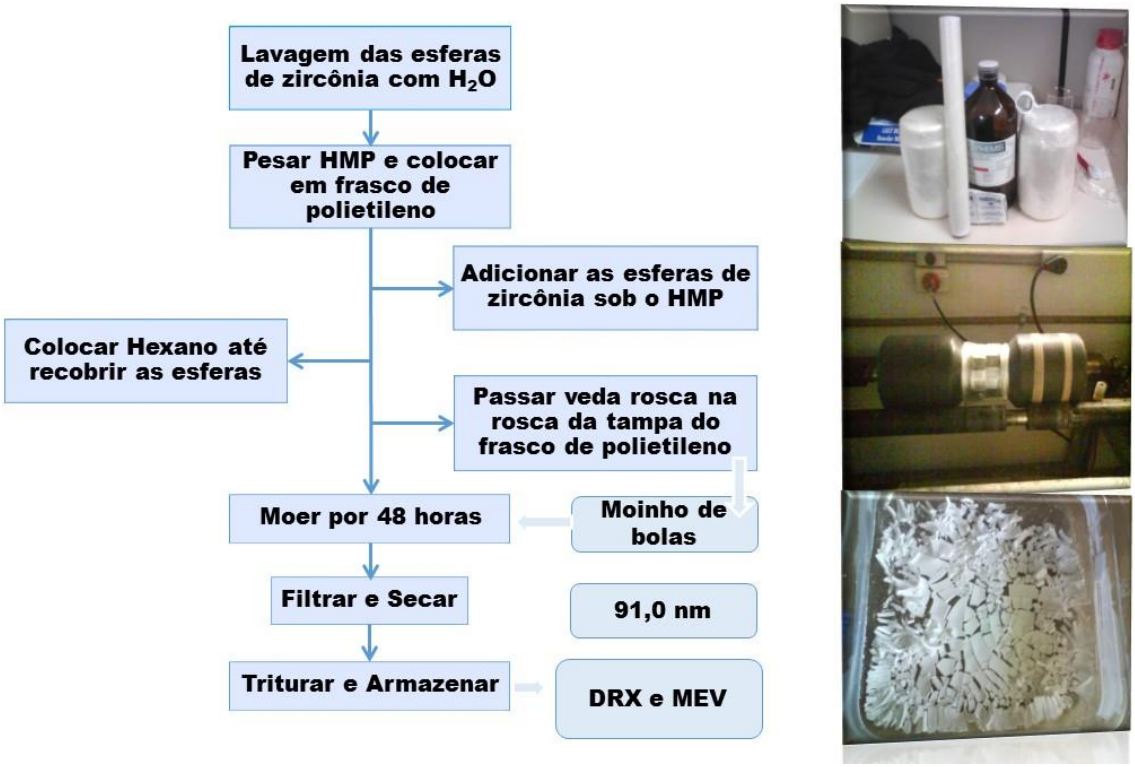
Politriz BUEHLER



Polimento do esmalte

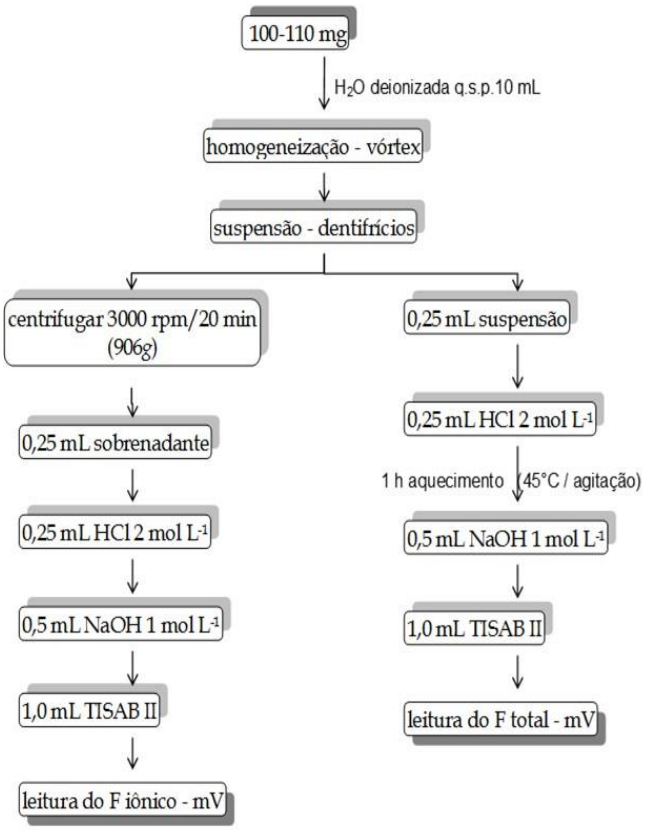
5.4 ANEXO D

PROCESSAMENTO E CARACTERIZAÇÃO DO HEXAMETAFOSFATO DE SÓDIO NANOPARTICULADO



5.5 ANEXO E

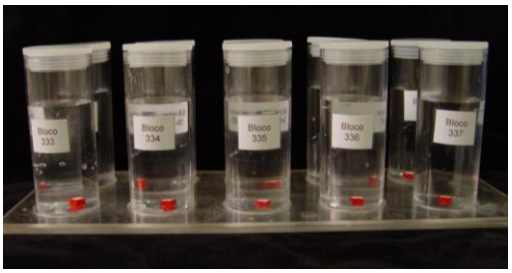
DOSAGEM DE FLUORETO NOS DENTIFRÍCIOS EXPERIMENTAIS

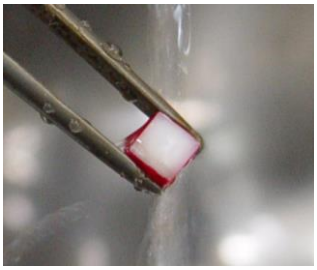


- Eletrodo específico para F; Orion 9409-BN
- Microeletrodo de referência
- Analisador de íons

5.6 ANEXO F

INDUÇÃO DE LESÃO DE CÁRIE ARTIFICIAL





16 horas

5.7 ANEXO G

DISPOSITIVO PALATINO (Capítulo 1)

Kit fornecido ao voluntário a cada período experimental

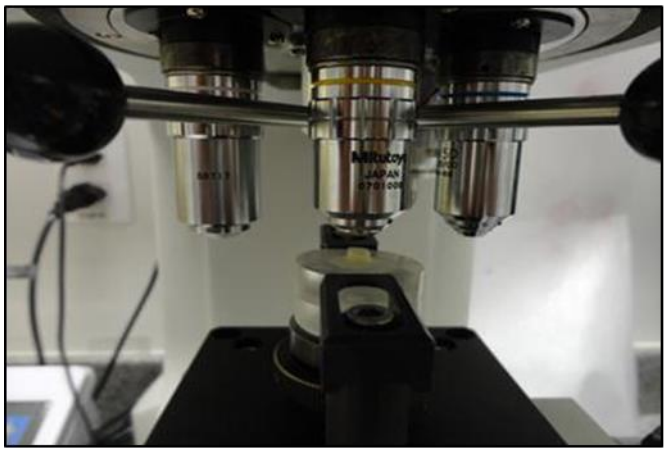
5.8 ANEXO H

DISPOSITIVO PALATINO (Capítulo 2)

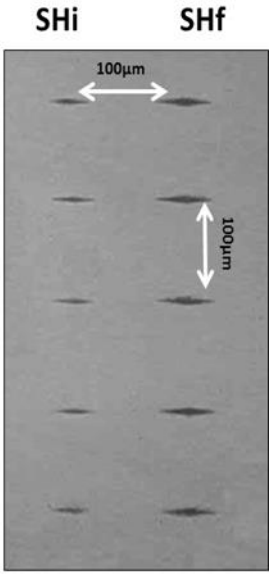
Kit fornecido ao voluntário a cada período experimental.

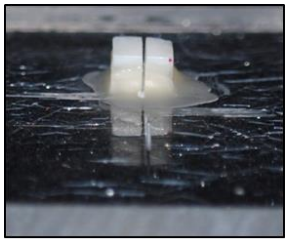
5.9 ANEXO I

ANÁLISE DA DUREZA SUPERFICIAL E LONGITUDINAL DO ESMALTE



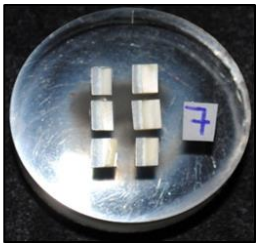
Microdurômetro Buehler
Carga 25 gramas
Tempo 10 segundos





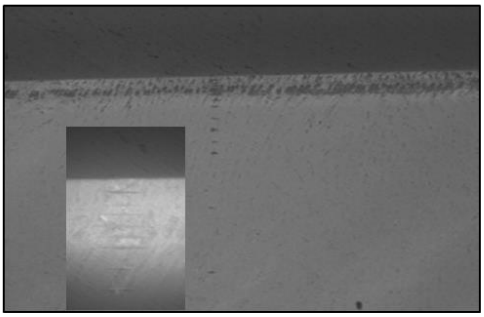
Secção dos blocos de esmalte no sentido longitudinal

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

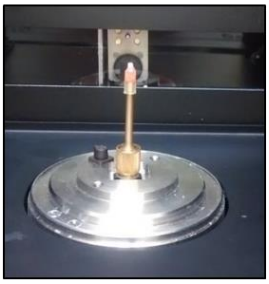
5.10 ANEXO J

ANÁLISE DA CONCENTRAÇÃO MINERAL DO ESMALTE PELA MICROTOMOGRAFIA COMPUTADORIZADA

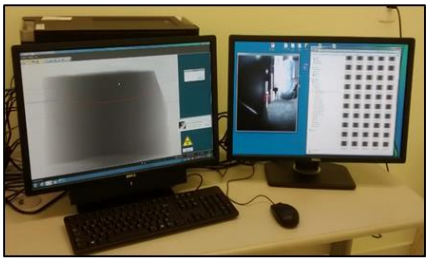
Blocos com espessura de 1 mm

Fixação dos blocos

Bruker Micro-CT



Blocos no interior do Micro-CT



NRecon sofware

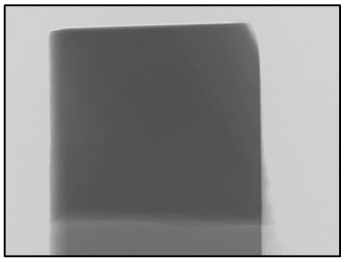
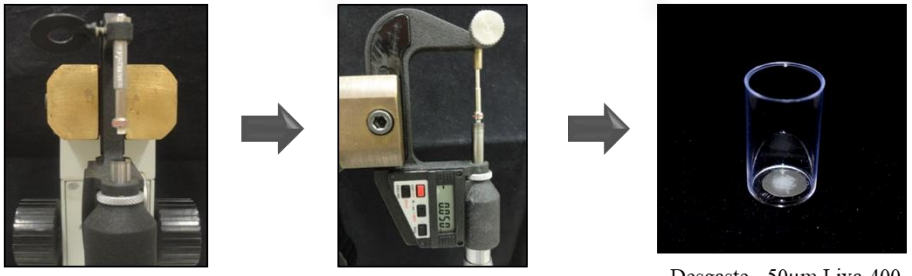


Imagem gerada após leitura pelo Micro-CT

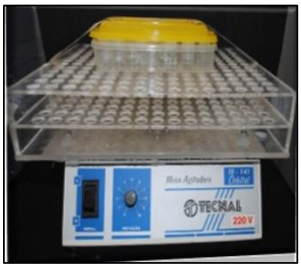
5.11 ANEXO K

ANÁLISE DE FLUORETO, CÁLCIO E FÓSFORO NO ESMALTE



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

Desgaste ~50µm Lixa 400 (CARBIMET - BUEHLER)



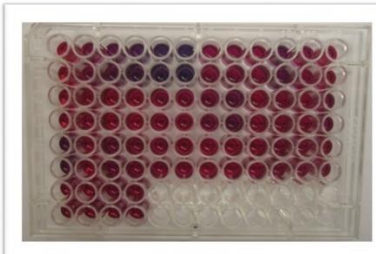
0,5 ml de HCl 1 mol/L

Agitação por 1 hora

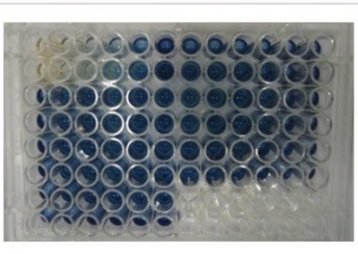
0,25 mL da amostra + 0,25 mL TISAB ll modificado com NaOH.

Akabane et al., 2018.

Espectrofotômetro de microplaca EONC, Biotek, USA



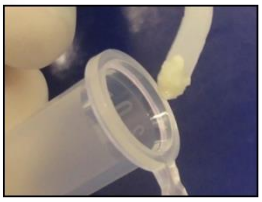
Cálcio - Método colorimétrico Arsenazo III, Fiske e Subbarow, 1925.



Fósforo - Método colorimétrico Fiske e Subbarow, 1925.

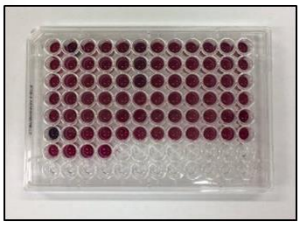
5.12 ANEXO L

ANÁLISE DA COMPOSIÇÃO DO BIOFILME DENTÁRIO

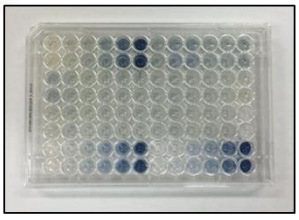


Biofilme Coletado

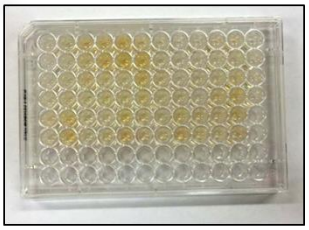
Fluoreto - Eletrodo específico (Orion 9409) - Eletrodo de referência (Orion 900200) - Analisador de íons (Orion 720A+).



Cálcio - Método colorimétrico Arsenazo III, Vogel et al., 1983.



Fósforo - Método colorimétrico, Fiske e Subbarow, 1925.



EPS - Método Fenol-Sulfúrico Dubois, 1956.

5.13 ANEXO M

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
cre@karger.com

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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).

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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

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For legal reasons, we must receive your **'Submission Statement'** with your original (hand-written) signature. Please download, print, sign and either fax or scan it to make it legally binding.

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. Reviews are not subject to page charges.

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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 patent(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 the 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 purpose.

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).

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(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.

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