

Nayara Gonçalves Emerenciano

**EFEITO *IN SITU* DE DENTIFRÍCIO FLUORETADO E
SUPLEMENTADO COM NANOPARTÍCULAS DE
TRIMETAFOSFATO DE SÓDIO SOBRE A
DESMINERALIZAÇÃO DO ESMALTE E BIOFILME**

Araçatuba – SP
2017

Nayara Gonçalves Emerenciano

**EFEITO *IN SITU* DE DENTIFRÍCIO FLUORETADO E
SUPLEMENTADO COM NANOPARTÍCULAS DE
TRIMETAFOSFATO DE SÓDIO SOBRE A
DESMINERALIZAÇÃO DO ESMALTE E BIOFILME**

Dissertação apresentada à Faculdade de Odontologia da
Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus
de Araçatuba, para obtenção de título de Mestre em Ciência
Odontológica - Área de Concentração: Saúde Bucal da
Criança.

Orientadora: Prof^a. Dr^a. Marcelle Danelon

Coorientador: Prof^o. Titular. Alberto Carlos Botazzo Delbem

Araçatuba – SP
2017

Nayara Gonçalves Emerenciano

Catálogo na publicação (CIP)
Diretoria Técnica de Biblioteca e Documentação – FOA / UNESP

Emerenciano, Nayara Gonçalves.

E53e Efeito *in situ* de dentifrício fluoretado e suplementado com nanopartículas de trimetafosfato de sódio sobre a desmineralização do esmalte e biofilme/Nayara Gonçalves Emerenciano. - Araçatuba, 2017
95 f. ; tab.

Dissertação (Mestrado) – Universidade Estadual Paulista, Faculdade de Odontologia de Araçatuba
Orientadora: Profa. Marcelle Danelon
Coorientador: Prof. Alberto Carlos Botazzo Delbem

1. Cárie dentária 2. Biofilmes 3. Fluoretos 4. Desmineralização 5. Nanopartículas I. T.

Black D5
CDD 617.601

Claudio Hideo Matsumoto
CRB-8/5550

Dados Curriculares

Nayara Gonçalves Emerenciano

Nascimento 16.07.1993

Filiação Joel Emerenciano da Silva
Ivanilda Souto Gonçalves Silva

2011/2015 Curso de Graduação em Odontologia pela Universidade Estadual de Maringá

2014/2015 Desenvolvimento de Projeto de Iniciação Científica, com auxílio do Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq.

2016/2017 Desenvolvimento de Projeto de Mestrado com auxílio da Coordenação de Pessoal de Nível Superior – CAPES

Associações CROSP - Conselho Regional de Odontologia de São Paulo.
SBPqO - Sociedade Brasileira de Pesquisa Odontológica.

COMISSÃO EXAMINADORA

DISSERTAÇÃO PARA OBTENÇÃO DO GRAU DE MESTRE

Profª. Drª. Marcelle Danelon – Orientadora, Professora permanente do Programa de Pós-Graduação em Ciência Odontológica, Departamento de Odontologia Infantil e Social, Disciplina de Odontopediatria da Faculdade de Odontologia - Araçatuba, UNESP - Universidade Estadual Paulista Júlio de Mesquita Filho, Araçatuba.

Profº. Adj. Robson Frederico Cunha – Professor Adjunto do Departamento de Odontologia Infantil e Social, Disciplina de Odontopediatria da Faculdade de Odontologia - Araçatuba, UNESP - Universidade Estadual Paulista Júlio de Mesquita Filho, Araçatuba.

Profº. Drº. Francisco Nunes de Souza Neto - Licenciatura em Química pela Universidade Estadual de Goiás (2008). Mestrado na área de Química de Novos Materiais junto ao Programa de Pós-Graduação em Ciências Moleculares da Universidade Estadual de Goiás (UEG). Doutorado na área de Química de Novos Materiais junto ao Programa de Pós-Graduação em Química da Universidade Federal de São Carlos (UFSCar).

Dedicatória

Nayara Gonçalves Emerenciano

Dedico este trabalho,

Aos meus pais Vâny e Joel

Por acreditarem em mim, por sonharem os meus sonhos junto comigo e principalmente por me ensinar a seguir o caminho de Deus. Cada um do seu jeitinho sempre me apoiando e incentivando, minha mãe, passando horas no telefone e vivendo comigo cada momento, como se estivéssemos perto uma da outra, meu pai, resolvendo todos os meus problemas, como sempre, mas agora a quatrocentos quilômetros de distância. Todos os meus princípios como pessoa e como profissional eu devo a vocês e agradeço muito a Deus por ter me presenteado vocês como pais, vocês são os melhores.

Percebe e entende que os melhores amigos
São aqueles que estão em casa, esperando por ti
Acredita nos momentos mais difíceis da vida
Eles sempre estarão por perto pois só sabem te amar
E se por acaso a dor chegar, ao teu lado vão estar
Pra te acolher e te amparar.
Pois, não há nada como um lar
(Anjos do Resgate)

Agradecimentos Especiais

Nayara Gonçalves Emerenciano

A Deus

Primeiramente agradeço a Deus, por ter me dado o dom da vida e ter colocado no meu caminho as pessoas que citarei ao longo desses agradecimentos. A Ele entrego minha vida todos os dias e graças a Ele recebo diariamente infinitas bênçãos. Também agradeço a Nossa Senhora de Fátima por interceder por mim junto ao seu filho, Jesus, em todos os momentos da minha vida, me ajudando a superar todos os obstáculos.

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

Aos meus irmãos Leonardo e Giovanna,

Aos meus irmãos, Leonardo e Giovanna, por sempre forcerem por mim, por estarem ao meu lado e serem os melhores amigos que existe. Vocês me motivam a ser cada dia uma pessoa melhor. Nunca se esqueçam que são parte de mim e que eu estarei aqui para o que precisarem.

Sou eu quem vai ouvir você
Quando o mundo não puder te entender
Foi Deus que te escolheu pra ser
O melhor amigo que eu pudesse ter
(Anjos do Resgate)

Ao meu namorado Afonso,

Ao meu namorado Afonso, por compartilhar comigo todos os momentos da minha vida. Já são cinco anos juntos, mas nesses últimos dois anos de mestrado enfrentamos a maior dificuldade, a distância. Não vem sendo fácil, mas conseguimos superar graças ao alicerce de compreensão, confiança e respeito que construímos juntos. Você é um dos melhores presentes que Deus me deu! Obrigada pelo amigo, parceiro e namorado que você é. Agradeço por toda compreensão, por me apoiar e incentivar a seguir meus sonhos, todas as minhas conquistas também são suas.

"O amor é paciente, o amor é bondoso. Não inveja, não se vangloria, não se orgulha. Não maltrata, não procura seus interesses, não se ira facilmente, não guarda rancor. O amor não se alegra com a injustiça, mas se alegra com a verdade. Tudo sofre, tudo crê, tudo espera, tudo suporta." 1 Coríntios 13:4-7

A minha vó,

A minha vó, Huelta, por todas as orações, por se preocupar com meu bem-estar e cuidar de mim. Obrigada por estar sempre me esperando quando vou para casa, pelas horas de conversas e conselhos. Te amo!

Aos meus padrinhos José (in memoriam) e Ana,

Uma amizade da juventude da minha mãe me agradeceu vocês como padrinhos, me sinto extremamente abençoada por isso, vocês vieram para exercer da melhor forma possível essa missão, desde pequena me trataram como se eu fosse filha de vocês, e eu sou. Esse ano passamos pelo maior sofrimento que pode existir, a perda do padrinho, foi e está sendo muito difícil, mas ao mesmo tempo aprendemos tantas coisas como família. Hoje ele não está mais fisicamente entre nós, mas sinto muito a presença dele cuidado de todos nós e serei eternamente grata a Deus por ter convivido 24 anos com uma pessoa de caráter e coração inigualável como o dele.

Aos meus amigos Juliana e Vinícius,

A Ju é o presente que a faculdade me deu, minha dupla nos atendimentos e na vida, quantas histórias juntas. Sinto que Deus cruzou os nossos caminhos para nunca mais nos separarmos e isso é tão verdade que meses antes de eu prestar a prova do mestrado ela encontrou o Vini que é de Araçatuba. Vini uma pessoa querida que abre as portas de sua casa para o Afonso e para mim e faz nos sentirmos em família. Obrigada pela amizade de vocês!

A minha querida Orientadora,

Prof^a. Dr^a. Marcelle Danelon,

Ma, difícil traduzir em palavras o carinho que tenho por você. Você é uma pessoa iluminada e transmitiu toda essa luz durante todo o meu mestrado, isso fez com que esses dois anos fossem mais leves e eu tivesse a certeza de que esses são os caminhos de Deus para mim. Muito obrigada por todos os ensinamentos e pela paciência, sem você, com certeza, nada seria possível. Desejo que Deus proteja os seus caminhos e te conceda infinitas bênçãos sempre.

Ao meu Coorientador,

Prof. Alberto Carlos Botazzo Delbem

Agradeço por toda a colaboração durante a execução do trabalho, por estar sempre disposto a ajudar e ensinar.

Ao Prof. Robson,

Obrigada por todos os ensinamentos transmitidos a mim, não só os profissionais, que me fizeram crescer muito, mas também os pessoais, que me tornaram uma pessoa melhor. Obrigada por me ensinar uma odontopediatria de técnicas e de valores, agradeço também por ter aberto as portas da bebê clínica para mim, ela se tornou o meu lugar preferido, onde vivi momentos muito especiais durante o mestrado.

A Equipe Bebê Clínica

Agradeço a todos que participam da bebê clínica, aos funcionários, professores, amigos e pacientes. Em especial agradeço pela amizade da Bárbara, da Carol e do Mário, amigos queridos que fiz na bebê e vou levar para sempre em meu coração.

A minha amiga Mayra,

Obrigada por ter dividido não só o apartamento comigo, mas também os momentos de tristeza e alegria. Obrigada por me ouvir e me aconselhar sempre. Sua amizade é um presente para mim.

As Minhas Queridas amigas,

Isabel, Laís e Luhana

Obrigada por todos os momentos que vivemos juntas, as risadas no laboratório, os almoços de domingo, os atendimentos juntas e todas as conversas. Obrigada pela amizade, levarei vocês em meu coração por toda a vida.

As amigas de turma Ana Paula, Giovanna, Lenara e

Thamires

Meninas, obrigada por terem feito as disciplinas do mestrado serem mais divertidas. Foi muito bom ter entrado no mestrado juntos com vocês e tê-las como colegas de turma. Talvez não estaremos mais todas juntas, mas desejo muito sucesso onde estiverem.

*Aos alunos de Iniciação Científica Fran, Gabriel, Léo,
Ronaldo e Sara*

Obrigada por me receberem na equipe de trabalho com vocês, difícil encontrar alunos tão comprometidos. Tenho certeza que independente do caminho que seguirem terão muito sucesso.

Aos voluntários da pesquisa,

Ser voluntário não é uma missão fácil, necessita de tempo e paciência. Agradeço muito a vocês por terem executado tudo da melhor maneira possível, sem vocês a realização deste trabalho não seria possível.

Aos colegas da pós-graduação,

Agradeço a todos que fazem parte do laboratório de Odontopediatria por estarem sempre dispostos a ajudar e por terem me recebido tão bem.

*Aos docentes da Disciplina de Odontopediatria da
Faculdade de Odontologia de Araçatuba, UNESP,*

Prof. Titular Célio Percinoto, Prof. Adj. Robson Frederico Cunha, Prof. Ass. Dr. Juliano Pelim Pessan, Profa. Dra. Rosângela dos Santos Nery, Profa.

Adj. Sandra Maria Herondina Ávila de Aguiar, Profa. Ass. Dra. Cristiane Duque, pela agradável convivência e conhecimentos transmitidos.

Aos alunos de Pós-Doutorado da Universidade Federal de São Carlos - Francisco Nunes de Souza Neto, Luiz Fernando Gorup,

Obrigada pela síntese e caracterização das nanopartículas de trimetafosfato de sódio (TMPnano). A ajuda de vocês foi fundamental para a realização deste trabalho.

A Faculdade de Odontologia de Araçatuba,

Na pessoa dos professores: Prof. Titular Wilson Roberto Poi, digníssimo Diretor e Prof. Titular João Eduardo Gomes Filho, digníssimo Vice-Diretor.

Ao Curso de Pós-Graduação em Ciência Odontológica da Faculdade de Odontologia de Araçatuba-UNESP,

Na pessoa do Coordenador Prof. Adj. Luciano Tavares Angelo Cintra.

À Valéria, Cristiane e Lílian da Seção de Pós-Graduação da Faculdade de Odontologia de Araçatuba-UNESP,

Pelo profissionalismo e atenção sempre carinhosa.

Ao Frigorífico FRIBOI,

Pela permissão da coleta dos dentes bovinos.

A Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e a Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

Pela concessão de recursos a mim e ao aluno de iniciação Científica Gabriel Pereira Nunes.

Epígrafe

Nayara Gonçalves Emerenciano

*Pedi e recebereis
buscai, e achareis
batei e abrir-se-vos-á
Pois todo o que pede, recebe
e quem busca, acha
e ao que bate, abrir-se-lhe-á.*

Mateus 7:7,8

Resumo

Nayara Gonçalves Emerenciano

EMERENCIANO, N.G. **Efeito *in situ* de dentifrício fluoretado e suplementado com nanopartículas de trimetafosfato de sódio sobre a desmineralização do esmalte e biofilme.** 2017 93f. Dissertação (Mestrado em Ciência Odontológica, área de concentração Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2017.

Objetivo: Avaliar o efeito de um dentifrício fluoretado contendo trimetafosfato de sódio de tamanho nanométrico (TMPnano) na desmineralização do esmalte *in situ* e composição do biofilme. Métodos: Este estudo foi duplo-cego cruzado consistindo em quatro fases experimentais (7 dias cada) com 12 voluntários que utilizavam dispositivos orais contendo quatro blocos de esmalte bovino. O desafio cariogênico foi realizado por solução de sacarose 30% (6x/dia). Os tratamentos com dentífricos (3x/dia) foram os seguintes: sem F/TMP/TMPnano (Placebo), 1100 ppm F (1100F), 1100F mais 3% de TMP micrométrico ou nanométrico (1100F/TMP; 1100F/TMPnano). A porcentagem de perda de dureza da superfície (%SH) e a perda integrada de dureza de subsuperfície (Δ KHN), bem como o cálcio (Ca), o fósforo (P) e o fluoreto (F) foram determinados. Além disso, o biofilme formado nos blocos foi analisado quanto às concentrações de polissacarídeos extracelulares (EPS), F, Ca, P. Os dados foram analisados utilizando ANOVA 1-critério de medidas repetidas seguidas pelo teste Fisher LSD ($p < 0,001$). Resultados: 1100F/TMPnano promoveu menor %SH e Δ KHN entre todos os grupos ($p < 0,001$). A adição de TMPnano a 1100F não aumentou a absorção de F no esmalte, mas aumentou significativamente as concentrações de Ca do esmalte ($p < 0,001$). 1100F/TMPnano apresentou valores mais baixos de concentração de EPS quando comparados com 1100F (~ 80%) ($p < 0,001$).

Quanto os graus de saturação, os grupos 1100F/TMP e 1100F/TMPnano mostraram a maior saturação em relação ao HA e similares entre si para CaF_2 ($p > 0,001$). A atividade iônica de CaF^+ e HF^0 para os grupos 1100F/TMP e 1100F/TMPnano foi semelhante ($p > 0,001$). Conclusão: 1100F/ TMPnano promoveu um efeito protetor maior contra a desmineralização do esmalte e afetou significativamente a composição do biofilme formado *in situ*, quando comparado ao dentífrico com 1100F. Relevância clínica: Essa formulação testada pode ser uma alternativa viável para pacientes com alto risco de cárie.

Palavras-chave: Cárie; Biofilme; Fluoreto; Desmineralização; Nanopartículas.

Abstract

Emerenciano, NG. ***In situ* effect of fluoride toothpaste and supplemented with nano-sized sodium trimetaphosphate on enamel demineralization and biofilm.** 2017 93f. Dissertação (Mestrado em Ciência Odontológica, área de concentração Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2017.

Objective: To evaluate the effect of a fluoride toothpaste containing nano-sized sodium trimetaphosphate (TMPnano) on enamel demineralization *in situ* and composition of the biofilm. Methods: This crossover double-blind study consisted of four phases (7 days each) and 12 volunteers who wore oral appliances containing four enamel bovine blocks. The cariogenic challenge was performed by 30% sucrose solution (6x/day). The toothpaste treatments (3x/day) were as follows: no F/TMP/TMPnano (Placebo), 1,100 ppm F (1100F), 1100F plus 3% micrometric or nano-sized TMP (1100F/TMP; 1100F/TMPnano). Percentage of surface hardness loss (%SH), and integrated loss of subsurface hardness (Δ KHN), as well as enamel calcium (Ca), phosphorus (P), and fluoride (F) were determined. Moreover, biofilm formed on the blocks were analyzed for F, Ca, P, and insoluble extracellular polysaccharide (EPS) concentrations. Data were analyzed using one-way ANOVA, repeated measures followed by Fisher LSD test ($p < 0.001$). Results: 1100F/TMPnano promoted the lowest %SH and Δ KHN among all groups ($p < 0.001$). The addition of TMPnano to 1100F did not enhance enamel F uptake, but significantly increased enamel Ca concentrations ($p < 0.001$). 1100F/TMPnano showed lower values of EPS concentration when compared with 1100F (~80%) ($p < 0.001$). As for phase saturation, the 1100F/TMP and 1100F/TMPnano groups showed the highest supersaturation with respect to HA and similar to each other

for CaF_2 ($p > 0.001$). The ionic activity of CaF^+ and HF^0 for the 1100F/TMP and 1100F/TMPnano groups were similar ($p > 0.001$). Conclusion: 1100F/TMPnano promoted a greater protective effect against enamel demineralization and significantly affected the composition of biofilm formed *in situ* when compared to 1100F toothpaste. Clinical Significance: This toothpaste could be a viable alternative to patients at high risk of caries.

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

Lista de Figuras

Nayara Gonçalves Emerenciano

LISTA DE FIGURAS

Figure 1: **A** Enamel block preparation. **B** Hardness analysis. **C** Treatments. **D** Analysis of dental biofilm composition. **E** Hardness Analysis. **F** Cross-sectional. **G** Fluoride, calcium, and phosphorus in enamel. **H** Integrated loss of subsurface hardness.

Figure 2: X-ray patterns of the micrometric TMP and of the nano-sized TMP after milling for 48 h.

Figure 3: SEM images of the TMP powders (a) before milling and (b) after milling for 48 h.

Lista de Tabelas

LISTA DE TABELAS

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

Lista de Abreviaturas

LISTA DE ABREVIATURAS

Am Ante Meridiem

ANOVA Analysis of Variance

°C Degrees Celsius

Ca Calcium

Ca⁺² Calcium ion

CaF⁺ Calcium fluoride ion

CaF₂ Loosely bound fluoride

CaPO₄⁻ Calcium phosphate ion

CaPOH⁻ Phosphate hydrogenated calcium ion

CaHPO₄⁰ Neutral Calcium Phosphate

CaH₂PO₄⁺⁴ Dihydrogenated calcium phosphate

CT Conventional toothpaste

DS Degree of saturation

EPS Insoluble extracellular polysaccharides

F Fluoride

FI Ionic fluoride

FT Total fluoride

g Gram

h Hour

HCl Hydrochloric acid

HF⁰ Neutral hydrogen fluoride

HPO₄⁻² Hydrogenated phosphate ion

H₂PO₄⁻ Dihydrogen phosphate ion

KHN Knoop hardness unit
L Liter
IF ionic fluoride
M Molar
Min Minutes
mg Milligram
mg/g Milligram per gram
ml Milliliter
mm Millimeter
Mol/L Mol/liter
mol L⁻¹ Mol per liter
mol/kg Mol/kilograms
mV Millivolts
n Volunteers number
Na⁺ Sodium Ion
NaF Sodium Fluoride
NaOH Sodium hydroxide
nm Nanometers
P Phosphor
P₂O₅ Diphosphorus pentoxide
PO₄³⁻ Orthophosphate
pH Hydrogen potential
ppmF Parts per million of fluoride
pm Post Meridiem
s Seconds

SD Standard deviation

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 sodium trimetaphosphate

μg Microgram

$\mu\text{g}/\text{mm}^3$ Microgram per cubic millimeter

$\mu\text{L}/\text{mg}$ Microliters/milligram

μm Micrometer

ΔKHN Integrated loss of subsurface hardness

XRD X-ray diffraction

Sumário

SUMÁRIO

ABSTRACT	37
INTRODUCTION	39
MATERIALS AND METHODS	41
RESULTS	47
DISCUSSION	49
ACKNOWLEDGMENTS	55
REFERENCES	56
ANEXOS	71

Capítulo 1

Nayara Gonçalves Emerenciano

***In situ* effect of fluoride toothpaste and supplemented with nano-sized sodium trimetaphosphate on enamel demineralization and biofilm**

N.G. Emerenciano^a, A.C.B. Delbem^a, J.P. Pessan^a, G.P. Nunes^a, E.R. Camargo^b,
M. Danelon^a.

^aSão Paulo State University (UNESP), School of Dentistry, Araçatuba

^aDepartment of Pediatric Dentistry and Public Health

Rua José Bonifácio 1193 Araçatuba, SP - Cep 16015-050 - Brazil

^bLIEC, Federal University of São Carlos (UFSCar),

^bDepartment of Chemistry

Rodovia Washington Luís, São Carlos, SP - Cep13565-905 - Brazil

Running Head: Fluoride toothpaste with nano-sized trimetaphosphate

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

Corresponding author:

Marcelle Danelon

^aSão Paulo State University (UNESP), School of Dentistry, Araçatuba

Department of Pediatric Dentistry and Public Health

Rua José Bonifácio 1193

16015-050 Araçatuba – SP - Brazil

Tel. +55 18 3636 3235

Fax +55 18 3636 3332

Email:marcelledanelon@hotmail.com

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

(Anexo A)

Declaration of Interests

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.

Abstract

Objective: To evaluate the effect of a fluoride toothpaste containing nano-sized sodium trimetaphosphate (TMPnano) on enamel demineralization *in situ* and composition of the biofilm. Methods: This crossover double-blind study consisted of four phases (7 days each) and 12 volunteers who wore oral appliances containing four enamel bovine blocks. The cariogenic challenge was performed by 30% sucrose solution (6x/day). The toothpaste treatments (3x/day) were as follows: no F/TMP/TMPnano (Placebo), 1,100 ppm F (1100F), 1100F plus 3% micrometric or nano-sized TMP (1100F/TMP; 1100F/TMPnano). Percentage of surface hardness loss (%SH), and integrated loss of subsurface hardness (Δ KHN), as well as enamel calcium (Ca), phosphorus (P), and fluoride (F) were determined. Moreover, biofilm formed on the blocks were analyzed for F, Ca, P, and insoluble extracellular polysaccharide (EPS) concentrations. Data were analyzed using one-way ANOVA, repeated measures followed by Fisher LSD test ($p < 0.001$). Results: 1100F/TMPnano promoted the lowest %SH and Δ KHN among all groups ($p < 0.001$). The addition of TMPnano to 1100F did not enhance enamel F uptake, but significantly increased enamel Ca concentrations ($p < 0.001$). 1100F/TMPnano showed lower values of EPS concentration when compared with 1100F (~80%) ($p < 0.001$). As for phase saturation, the 1100F/TMP and 1100F/TMPnano groups showed the highest supersaturation with respect to HA and similar to each other for CaF_2 ($p > 0.001$). The ionic activity of CaF^+ and HF^0 for the 1100F/TMP and 1100F/TMPnano groups were similar ($p > 0.001$). Conclusion: 1100F/TMPnano promoted a greater protective effect against enamel demineralization and significantly affected the composition of

biofilm formed *in situ* when compared to 1100F toothpaste. Clinical Significance:
This toothpaste could be a viable alternative to patients at high risk of caries.

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

Introduction

Dental caries is a disease characterized as biofilm-sugar-dependent [Paes Leme et al., 2006; Sheiham and James, 2015]. The presence of biofilm in the oral cavity associated with the addition of sugars from the diet stimulates the proliferation and adhesive qualities of microorganisms [Sheiham and James, 2015] and allows them to produce acids promoting a decrease in oral pH, resulting in demineralization of dental surface [Thakahashi and Nyvad, 2011]. Fluoride (F) is the main auxiliary in maintaining the mineral balance of the tooth and when incorporated into the enamel hydroxyapatite, forms fluorapatite as a mineral, improving resistance to acid challenge [ten Cate, 1990; Chow, 1990; Moreno, 1993], once its critical pH is 4.5, while for hydroxyapatite is about 5.5 [Buzafaf et al., 2011].

Fluoride toothpastes have been highlighted as the topical administration form most used by the population due to its wide availability, contributing to the reduction of caries [Marinho et al., 2003]. A systematic review [Walsh et al., 2010] evaluating the effectiveness of fluoridated toothpastes of different concentrations of F showed that only toothpastes with concentrations higher than 1000 ppm F have efficacy in the prevention of dental caries in children and adolescents. Despite the numerous sources of F, there is still a significant proportion of infants and preschoolers affected by caries disease with a strong polarization. Mattos-Graner et al. [1996] and Vanobbergen et al. [2001] observed that 15-17% of children have approximately 50% of the lesions, showing that there are still individuals who do not have access to preventive measures and they are high-risk of developing the disease. Therefore, it is necessary to identify these high-

risk groups at an early stage and to use approaches more effective in these communities to reduce their incidence [Ferreira et al., 2007].

Given these findings, it would be interesting to increase the effectiveness of toothpastes, providing an increase in decreasing rates of disease. Recently, studies have demonstrated that it is possible to increase the effectiveness of a toothpaste without increasing the concentration of F. The addition of inorganic and organic phosphates to conventional toothpastes (1100 ppm F-CT) may be a strategy to increase their anticaries effect [de Castro et al., 2015; Danelon et al., 2017]. Among the phosphates studied, micrometric sodium trimetaphosphate (TMP) has been extensively tested at different vehicle and concentrations of F, showing to reduce mineral loss and increase the process of remineralization, because it has the ability to modify the surface of the hydroxyapatite, through its adsorption [Danelon et al., 2014; de Castro et al., 2015]. The *in vitro* study, by de Castro et al. [2015] showed that the association of TMP at 3% concentration in CT reduced enamel demineralization by 61% (Δ KHN) when compared to 1100 ppm F.

Another strategy to increase the effect of topical formulations containing F would be the use of phosphate nano-sized [Danelon et al., 2015]. Studies using an *in vitro* and *in situ* enamel caries model [Danelon et al., 2015; Danelon et al., 2017], found that a toothpaste formulation containing 1100 ppm F associated with nano-sized 3%TMP (TMPnano) showed better results in inhibiting enamel demineralization and promoting the remineralization of early caries lesions when compared to formulations containing 1100 ppm F and 1100 ppm F associated with 3% TMP. However, no study so far has evaluated the effect of the association of TMPnano to F on demineralization and biofilm *in situ*, under

conditions of high sucrose exposure, simulating a high risk of caries. Thus, the study of a new formulation with these characteristics would have an important impact on dentistry besides introduce a knowledge about the synergism between F and TMPnano in different formulations.

So far, no study has evaluated whether the addition of TMPnano could affect the biofilm composition and enamel demineralization under cariogenic challenge, simulating a condition of high caries risk. Thus, this study evaluate the effect of a fluoride toothpaste containing nano-sized sodium trimetaphosphate (TMPnano) on enamel demineralization *in situ* and composition of the biofilm. The null hypothesis was that F toothpaste associated with TMPnano would provide similar anticaries effect when compared to F toothpaste without TMPnano or micrometric TMP.

Material and Methods

Experimental Design

This study was approved by the Human Ethical Committee of Araçatuba Dental School, São Paulo State University, Brazil (Protocol: 61591416.1.0000.5420) (Anexo B), and all participants read and signed informed consent statements prior to the study initiation. The experimental design is presented in Figure 1. This crossover double-blinded study was performed in 4 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 the primary outcome as from surface and cross-sectional hardness analysis, the mean difference among the groups (30 and 1300, respectively), standard deviation (20 and 9000, respectively), an α -error of 5%, and a β -error of 20%. Volunteers

(n=12) aged 22-33 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 (Fig. 1A) (Anexo C) and selected using an initial surface hardness (SHi) (baseline) (Fig. 1B). Then, the specimens were allocated to 4 treatments: no F/TMP/TMPnano (Placebo), 1,100 ppm F (1100F), 1100F plus 3% micrometric or nano-sized TMP (1100F/TMP; 1100F/TMPnano). After each phase of 7 days of cariogenic challenge, the biofilm was collected for the analysis of F, Ca, P, and insoluble extracellular polysaccharides (EPS). In the enamel blocks, Percentage of surface hardness loss (%SH), and integrated loss of subsurface area (Δ KHN) was assessed again. F, Ca, and P content in the enamel were determined.

Synthesis and characterization of nano-sized sodium trimetaphosphate

The synthesis and characterization of nano-sized TMP was based on the study by Danelon et al. [2015, 2017]. To prepare nano-sized TMP, 70 g of pure (micrometric) sodium trimetaphosphate ($\text{Na}_3\text{O}_9\text{P}_3$, Aldrich, purity \geq 95% CAS 7785-84-4) was ball milled using 500 g of zirconia spheres in 1 L of isopropanol. After 48 h, the material was filtered and sealed with aluminum foil, and the vials were dried at 75°C to evaporate the hexane. The particle morphology of TMP and TMP milled for 48 h (TMPnano) was analyzed by scanning electron microscopy (SEM). The SEM images were collected using a Philips XL-30 FEG (Anexo D).

Toothpaste formulation and fluoride and pH assessment

The experimental toothpastes were prepared in a laboratory and had the following ingredients: 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 TMP were prepared ($\text{Na}_3\text{O}_9\text{P}_3$, Aldrich, purity $\geq 95\%$ CAS 7785-84-4) at concentration of 3% micrometric TMP (TMP) or nano-sized TMP (TMPnano). To these toothpastes, NaF (Merck, CAS 7681-49-4, Germany) was added to reach a concentration of 1100 ppm F. In addition, toothpaste without TMP/TMPnano and F (Placebo), as well as with 1100 ppm F (without TMP/TMPnano) were prepared. The toothpastes used in this study were stored at room temperature and kept properly closed to prevent any change of the samples (Anexo E).

The total fluoride (TF) and ionic fluoride (IF) amounts 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 the 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 (Fig. 1C) (Jet, Articles Classic Odontológico, São Paulo, Brazil), and four enamel blocks were fixed, with a different device used in each phase of the experiment. In order to allow biofilm

accumulation on the enamel blocks, a piece of plastic mesh was fixed to the acrylic appliance, leaving a space of 1 mm 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) and five minutes later, the device was reinserted into the mouth [da Camara et al., 2015] (Fig. 1D). The volunteers were instructed to use the appliances during the entire day (including night time), except when drinking or eating anything, and brushed their natural teeth 3x/day (08:00 am, 13:00 pm, 21:30 pm) for 2 min, with 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. Following, the devices were removed from the oral cavity and gently rinsed with tap water; volunteers then brushed their natural teeth and rinsed the mouth as usual, returning the devices to the oral cavity immediately afterwards. During a 7-day pre-experimental period and washout periods, the volunteers brushed their teeth with the placebo toothpaste (Anexo F).

Hardness Analysis

The enamel surface hardness was determined before (SH_i) (Fig. 1B) and after (Fig. 1E) each phase in each specimen, using a Shimadzu HMT-2000 microhardness tester (Shimadzu Corp., Kyoto, Japan) under a 25 g load for 10 s [Danelon et al., 2015] and next, calculate the percentage of surface hardness loss ($\%SH = [(SH_f - SH_i) / SH_i] * 100$). For the cross-sectional hardness

measurements, the enamel blocks were longitudinally sectioned (Fig. 1F) through their center and embedded in acrylic resin with the cut face exposed and gradually polished. A 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, Lake Bluff, USA) with a Knoop diamond indenter under a 5 g load for 10 s. Integrated hardness (KHN $\times \mu\text{m}$) for the lesion into sound enamel was calculated by the trapezoidal rule (GraphPad Prism, version 3.02) and subtracted from the integrated hardness for sound enamel to obtain the integrated area of the subsurface regions in the enamel, which was named integrated loss of subsurface hardness (ΔKHN ; KHN $\times \mu\text{m}$) (Fig. 1F) [Danelon et al., 2015] (Anexo G).

Fluoride, calcium and phosphorus in enamel

F present in the enamel was determined as described by Weatherell et al. [1985], as modified by Alves et al. [2007]. 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, SP, Brazil). One layer of enamel ($50.9 \pm 0.2 \mu\text{m}$) was removed from each block, after the 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, modified by Alves et al., 2007]. The analysis of F was performed in 0.30 mL of that solution after addition of the same volume of TISAB II, modified with NaOH, using an specific electrode 9409BN (Thermo Scientific,

Beverly, MA, USA) and microelectrode reference (Analyser, São Paulo, Brazil) coupled to an ion analyzer (Orion 720A+, Thermo Scientific, Beverly, MA, USA) was used. The results were expressed in $\mu\text{g}/\text{mm}^3$. Ca analysis was performed using the Arsenazo III colorimetric method [Fiske and 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 (Fig. 1G). The results were expressed as $\mu\text{g}/\text{mm}^3$ (Anexo H).

Analysis of dental biofilm composition

The biofilm formed on the enamel was collected and stored in microcentrifuge tubes. The biofilm samples were dried in vacuum over P_2O_5 (Vetec Quimica Fina Ltda., Duque de Caxias, Rio de Janeiro, Brazil) for 12 h at room temperature. After extraction for 3 h at room temperature with 0.5 mol/L hydrochloric acid (250 $\mu\text{L}/\text{mg}$, biofilm wet weight) under constant agitation, the same volume of NaOH (0.5 mol L^{-1}) was added [Nobre dos Santos et al., 2002; da Camara et al., 2015]. The samples were then centrifuged (11,000 $\times g$) for 1 min and the supernatant retained for determination of F, Ca and P. 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] (Fig. 1D). The P concentration was measured using a colorimetric method [Fiske and Subbarow, 1925] (Fig. 1D). Insoluble extracellular polysaccharide (EPS) was extracted by adding 1.0 mol L^{-1} NaOH (10 $\mu\text{L}/\text{mg}$ dry weight) to the biofilm [Nobre dos Santos et al., 2002; Ccahuana-Vásquez et al.,

2007]. The amount of EPS was determined using the phenol-sulfuric acid method [Dubois et al., 1956] (Fig. 1D). The results were expressed as mol/kg of F, Ca, and P; and mg/g of EPS (dry weight) (Fig. 1D).

The ionic activities (IA) of the various species (Ca^{2+} , CaPO_4^- , CaHPO_4^0 , $\text{CaH}_2\text{PO}_4^+$, 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 37 °C, 7.0 pH, and a density of 1.0 g/cm³ using the PHREEQC Interactive (version 2.18.3) speciation program [do Amaral et al., 2013; da Camara et al., 2015] (Anexo I).

Statistical analysis

SigmaPlot 12.0 software was used for statistical analysis, and the significance level was set at 5%. 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 a normal (Shapiro–Wilk) and homogeneous (Bartlett) distribution, and were therefore subjected to one-way repeated measures ANOVA, followed by the Fisher LSD test.

Results

The milling processing reduced the particle size of the TMP powders without affecting its crystalline structure. The X-ray diffraction (XRD) pattern of the nano-sized TMP after 48 h of milling (Fig. 2) shows broader peaks due the

smaller crystallites, which could be used to estimate an average particle size of 22.7 nm. The Fig. 3 shows the SEM images of the TMP powder (a) before milling and (b) after 48 h of milling, where it is possible to clearly see the particles agglomerated before the milling.

Mean (SD) concentration of total F (TF) and ionic F (IF) (n = 3) were: Placebo – 10.7 (1.1) and 10.9 (0.4), 1100F - 1162.0 (44.1) and 1157.2 (16.8), 1100F/TMP - 1162.0 (44.1) and 1157.2 (16.8), and 1100F/TMPnano - 1162.0 (44.1) and 1157.2 (16.8). The pH value from the groups was 7.5 (0.2) ranging from 7.1 to 7.7.

Mean (SD) initial surface hardness (SH) for all blocks was 366.2 (1.0), and the means varied between 364.5 (1.0) and 367.4 (1.1). The use of 1100F/TMPnano resulted in a 49% and 35% decrease in %SH in comparison with 1100F and 1100F/TMP (p < 0.001) (Table 1). In addition, the capacity to reduce the lesion body (Δ KHN) was ~16% and ~60% higher with 1100F/TMP and 1100F/TMPnano when compared to 1100F (p < 0.001) (Table 1).

The addition of TMP and TMPnano 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/TMPnano, enamel Ca concentration was increased by ~ 62% and ~135% when compared to 1100F/TMP and 1100F toothpastes (p < 0.001). P concentrations in enamel were ~ 76% and ~ 58% higher than the 1100F/TMPnano when compared with the 1100F and 1100F/TMP groups (p < 0.001). No significant difference was observed among the 1100F and 1100F/TMP groups regarding enamel P concentrations except for the Placebo, which showed a lower concentration (p < 0.001)

As for the biofilm composition, 1100F/TMP and 1100F/TMPnano promoted the highest and similar retention of Ca ($p < 0.001$) and similar values of F when compared with 1100F (Table 1), while the P values were similar for the 1100F/TMP and 1100/TMPnano treatments ($p = 0.084$). 1100F/TMPnano showed lower values for alkali-soluble EPS concentration, when compared with 1100F (~ 80%) and 1100F/TMP (~ 60%) ($p < 0.001$).

The ionic activity of CaF^+ and HF^0 for the 1100F/TMP and 1100F/TMPnano groups were similar ($p > 0.001$); no significant differences were seen between 1100F/TMPnano and the other fluoride groups (i.e. 1100F and 1100F/TMP) regarding ionic activity of Ca^{2+} , CaPO_4^- , and $\text{CaH}_2\text{PO}_4^+$ ($p > 0.001$). No significant differences were observed among 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/TMP and 1100F/TMPnano groups showed the highest supersaturation with respect to HA and similar to 1100F for CaF_2 ($p > 0.001$).

Discussion

Toothpastes can be used as a vehicle for substances that contribute to improve the oral health of individuals [Cury and Tenuta, 2013]. The present study evaluated the effect of a fluoride toothpaste containing nano-sized sodium trimetaphosphate (TMPnano) on enamel demineralization and on the composition of the biofilm formed *in situ*. The results of this study showed that the addition of TMPnano 1100F showed higher anticaries effect compared to CT and its counterpart (1100F/TMP). Thus, the null hypothesis was rejected.

The concentration of TMPnano tested in this study was based on the F/TMP and F/TMPnano ratio of the studies of Danelon et al. [2015 and 2017],

who demonstrated in *in situ* and *in vitro* studies that the addition of 3% TMPnano to a CT significantly increased the anticaries effect. Our study confirms previous findings [Danelon et al., 2015; Danelon et al., 2017] that when TMPnano was added to the CT at a concentration of 3%, it reduced enamel demineralization by 49% when compared to the 1100F group (Table 1), even in the presence of biofilm. Biofilms formed in the presence of sucrose have higher cariogenicity when compared to those formed in the absence of sugar [Paes Leme et al., 2006], and their cariogenic potential is directly linked to the exposure frequency [Paes Leme et al., 2004], since the frequent exposure to sucrose changes the microbiological and biochemical composition of the biofilm. Van Houte [1980] and Marsh [1994] reported that frequent exposure to fermentable sugars causes frequent reductions in pH, selecting acidogenic and aciduric microorganisms, making the biofilm increasingly cariogenic. In the deeper demineralization regions (Δ KHN), the use of 1100F/TMPnano reduced the lesion body by 51% and 60% when compared to 1100F/TMP and 1100F treatments (Table 1). These findings corroborate those of Danelon et al. [2015], who observed that CT supplemented with TMPnano increased the mineral gain by 44% when compared to its counterparts with micrometric TMP.

The effect of TMPnano on %SH and Δ KHN may be directly related to the amount of Ca and P retained in enamel when this formulation was used, as there was an increase (62% and 58%) (Table 1) when compared to 1100F/TMP. These findings reinforce the hypothesis that the nano-sized phosphate salt has a higher adsorption capacity on the enamel surface, since its greater proportion of surface area by volume, as well as higher percentage of atoms in the surface compared to micrometric particles, favors the greater reactivity, as already demonstrated in

previous studies [Danelon et al., 2015; Danelon et al., 2017]. Thus, Ca^{++} and CaF^+ ions present in the saliva replace the Na^+ of the TMP/TMPnano molecule, and during pH reduction (demineralization) there is formation of neutral species (CaHPO_4^0 e HF^0) [Danelon et al., 2015; Souza et al., 2016], which have greater diffusion capacity in enamel in relation to the ions, leading to the reduction of enamel demineralization. Still, the treatment with TMPnano did not promote an increase in the retention of F in enamel, leading to the assumption that its effect is related to modification of the enamel surface, promoting the formation of a protective layer, as previously mentioned and demonstrated by other studies [Takeshita et al., 2015; Takeshita et al., 2016; Danelon et al., 2015].

Studies show that a highly cariogenic biofilm has a low concentration of Ca, P and F in its matrix [Cury et al., 1997; Paes Leme et al., 2004; Paes Leme et al., 2006]. However, treatments with 1100F/TMP and 1100F/TMPnano led to a higher retention of Ca and F in the biofilm (Table 1), demonstrating that these associations promoted significant changes in biofilm composition. These results corroborate those reported by Takeshita et al. [2015], which showed an increase in Ca and F retention in biofilm when phosphate was used in a low fluoride toothpaste supplemented with TMP. The deposition of Ca is directly related to an increase in the retention of F in the biofilm, being responsible for the decrease of enamel demineralization [Souza et al., 2016], as observed for the 1100F/TMPnano group that presented the highest values of Ca and F in the biofilm (Table 1) and consequently lower mineral loss in relation to 1100F, suggesting that in these groups the biofilm served as a reservoir of F and Ca ions during the high cariogenic challenge (i.e. 30% sucrose, 6x/day), which could contribute to minimize the enamel mineral loss. Thus, it is possible that the

TMPnano can be adsorbed to the biofilm in greater proportion by calcium bridging [Rose et al., 1996] and that, as well as for TMP, it acts as sites for further Ca^{2+} and CaF^+ binding. However, the above mentioned considerations should be interpreted within the limitations of this study, since the correlation between F levels throughout the biofilm is known to be weak [Vogel, 2011]. Despite this limitation, whole biofilm fluoride is a useful approach when studying the effect of different fluoridated formulations on enamel demineralization, especially when little is known about a formulation to be tested, which is the case of toothpastes supplemented with TMPnano.

Aiming to evaluate the synergistic effect of TMPnano and F on toothpastes, the activity of ionic species in the biofilm was also evaluated, considering the fluoride, calcium and phosphate data (Table 2). Treatment with the 1100F/TMPnano toothpaste presented higher activity for CaF^+ and HF^0 (Table 2) in relation to the other fluoridated toothpastes; namely, in the absence of F and TMP in the formulation (Placebo toothpaste), the activity of these ions is strongly reduced and consequently there is a larger lesion body (Table 1). Thus, we can assume that the effect of TMP/TMPnano in the demineralization process may be directly related to the increased activity of these species. Furthermore, 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 TMPnano promoted a significantly higher protective effect on enamel.

Regarding the effects of treatments on the concentration of alkali-soluble EPS in the biofilm, the high concentration observed for Placebo (Table 1) shows that the absence of F under cariogenic challenges leads to the formation of a highly cariogenic biofilm [Vale et al., 2007; Takeshita et al., 2015]. Our results show a significant reduction (60 and 80%) for the treatments with 1100F/TMP and 1100F/TMPnano when compared to 1100F. These findings agree with the studies of Takeshita et al. [2015] and Souza et al. [2016], confirming that both TMP and TMPnano present indirect action in the formation of EPS. It is worth mentioning that Ca concentrations varying between 1×10^{-3} and 1×10^{-4} mol/l (0.04 and 0.004 $\mu\text{g}/\text{mg}$) reduce EPS concentration [Boyd, 1978], thus the TMP/TMPnano could act on the biofilm indirectly, since high concentrations of F and Ca in the biofilm alter the bacterial metabolism and the biological activity, as well as the formation of EPS [van Loveren, 2001; Marquis et al., 2003]. Knowing that EPS formation is also dependent on the interaction of extracellular enzymes (i.e., glycosyltransferases and fructosyltransferases) with sucrose [Vacca-Smith et al., 1996; Zhang et al., 2015], we may suggest that TMP/TMPnano interferes with the action of these enzymes [Dennis et al., 1976] and consequently there is a reduction in the formation of extracellular polysaccharide. This reduction is of fundamental importance, since EPS provides binding sites and microbial adhesion, in addition to promoting structural changes in the matrix, facilitating acid diffusion and thus being a determinant virulence factor of cariogenic microorganisms [Koo et al., 2013]. Given that inorganic and organic phosphates have high affinity for hydroxyapatite, it is possible to assume that these substances interfere with the adsorption of organic materials and bacteria on

dental surfaces [Nordbö and Röllä, 1972]. This may change the formation of EPS; however, additional studies should be performed to confirm this hypothesis.

An important factor to be considered is that these data are based on an *in situ* protocol for dental demineralization and, although the basic chemical processes are the same for all individuals, the susceptibility to dental caries differs significantly [ten Cate, 2015]. Thus, the protocol used in the present study presents some limitations, since our model did not simulate different cariogenic challenges and the inorganic composition of saliva and biofilm fluid was not evaluated. Moreover, even though *in situ* models are currently a great improvement over *in vitro* caries models [Higham et al., 2005], performing an *in vivo* protocol would be extremely important to confirm our findings.

Considering the results obtained in this study, we conclude that 1100F/TMPnano promoted a greater protective effect against enamel demineralization and significantly affected the composition of biofilm formed *in situ* when compared to 1100F toothpaste. Therefore, it can be considered an effective alternative to improve the oral health of individuals, especially those at high risk to dental caries.

Acknowledgments

We thank the volunteers for their participation in the study, CAPES (Coordination of Higher Education Personnel) and FAPESP (The State of São Paulo Research Foundation, grant 2016/12581-6) for the concession of a scholarship to the first, four and seven authors, respectively.

References

- Alves KMRP, Pessan JP, Brighenti FL, Franco KS, Oliveira FAL, Buzalaf MAR, Sasaki KT, Delbem ACB. In vitro Evaluation of the Effectiveness of Acidic Fluoride Dentifrices. *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.
- Buzalaf MA, Pessan JP, Honório HM, ten Cate JM. Mechanisms of action of fluoride for caries control. *Monogr Oral Sci* 2011; 22:97-114.
- 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.
- Chow LC. Tooth-bound fluoride and dental caries. *J Dent Res* 1990;69:595-600.
- Cury JA, Rebello MA, Del Bel Cury AA. In situ relationship between sucrose exposure and the composition of dental plaque. *Caries Res* 1997; 31:356-360.
- Cury JA, Tenuta LM. Evidence-based recommendation on toothpaste use. *Braz Oral Res* 2014; 28:1-7.
- 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.
- Danelon M, Pessan JP, Souza-Neto FN, de Camargo ER, Delbem AC. Effect of fluoride toothpaste with nano-sized trimetaphosphate on enamel demineralization: An in vitro study. *Arch Oral Biol* 2017; 78:82-87.

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, Takeshita EM, Peixoto LC, Sasaki KT, Delbem AC. Effect of fluoride gels supplemented with sodium trimetaphosphate in reducing demineralization. *Clin Oral Investig* 2014; 18:1119-1127.

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.

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; 39:258-260.

Delbem AC, Sasaki 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.

Dennis DA, Gawronski TH, Cressey DE, Folke LE. Effects of sodium trimetaphosphate supplementation of a high sucrose diet on the microbial and biochemical composition of four-day plaque and on urine calcium and phosphorus levels. *J Dent Res* 1976; 55:787-796.

do Amaral JG, Sasaki KT, Martinhon CCR, Delbem ACB. Effect of low-fluoride dentifrices 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.

Ferreira SH, Béria JU, Kramer PF, Feldens EG, Feldens CA. Dental caries in 0- to 5-year-old Brazilian children: prevalence, severity, and associated factors. *Int J Paediatr Dent* 2007; 17:289-296.

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

Higham SM, Pretty IA, Edgar WM, Smith PW. The use of in situ models and QLF for the study of coronal caries. *J Dent* 2005; 33:235-241.

Koo H, Falsetta ML, Klein MI. The exopolysaccharide matrix: a virulence determinant of cariogenic biofilm. *J Dent Res* 2013; 92:1065-1073.

Marinho VC, Higgins JP, Logan S, Sheiham A. Topical fluoride (toothpastes, mouthrinses, gels or varnishes) for preventing dental caries in children and adolescents. *Cochrane Database Syst Rev* 2003:CD002782.

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

Marsh PD. Microbial ecology of dental plaque and its significance in health and disease. *Adv dent Res* 1994; 8:263-271.

Mattos-Graner Rde O, Rontani RM, Gavião MB, Bocatto HA. Caries prevalence in 6-36-month-old Brazilian children. *Community Dent Health* 1996; 13:96-98.

Moreno EC. Role of Ca-P-F in caries prevention: chemical aspects. *Int Dent J* 1993; 43:71-80.

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

Nordbo H, Rolla G. Desorption of salivary proteins from hydroxyapatite by phytic acid and glycerophosphate and the plaque-inhibiting effect of the two compounds in vivo. *J Dent Res* 1972; 51: 800-811.

Paes Leme AF, Dalcico R, Tabchoury CP, Del Bel Cury AA, Rosalen PL, Cury JA. In situ effect of frequent sucrose exposure on enamel demineralization and on plaque composition after APF application and F dentifrice use. *J Dent Res* 2004; 83:71-75.

Paes Leme AF, Koo H, Bellato CM, Bedi G, Cury JA. The Role of Sucrose in Cariogenic Dental Biofilm Formation—new Insight. *J Dent Res* 2006; 85:878-887.

Rose RK, Shellis RP, Lee AR. The role of cation bridging in microbial fluoride binding. *Caries Res* 1996; 30: 458-464.

Sheiham A, James WP. Diet and Dental Caries: The Pivotal Role of Free Sugars Reemphasized. *J Dent Res* 2015; 94:1341-1347.

Souza JG, Tenuta LM, Del Bel Cury AA, Nóbrega DF, Budin RR, de Queiroz MX, Vogel GL, Cury JA. Calcium Prerinse before Fluoride Rinse Reduces Enamel Demineralization: An in situ Caries Study. *Caries Res* 2016; 50:372-377.

Souza MDB, Pessan JP, Lodi CS, Souza JAS, Camargo ER, Souza Neto FN, Delbem, ACB. Toothpaste with Nanosized Trimetaphosphate Reduces Enamel Demineralization. *JDR Clin & Trans Res* 2016; 2:233-240.

Takehita EM, Danelon M, Castro LP, Cunha RF, Delbem AC. Remineralizing Potential of a Low Fluoride Toothpaste with Sodium Trimetaphosphate: An in situ Study. *Caries Res* 2016; 50:571-578.

Takehita EM, Danelon M, Castro LP, Sasaki 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.

Takahashi N, Nyvad B. The Role of Bacteria in the Caries Process: Ecological Perspectives. *J Dent Res* 2011; 90:294-303.

ten Cate, JM. In vitro studies on the effects of fluoride on de-and remineralization. *J Dent Res* 1990; 69:614-619.

ten Cate, JM. Models and role models. *Caries Res* 2015; 49 Suppl 1:3-10.

Vacca-smith AM, Venkitaraman AR, Quivey RG, Bowen WH. Interactions of streptococcal glucosyltransferases with α -amylase and starch on the surface of saliva-coated hydroxyapatite. *Arch Oral Biol* 1996; 41:291-298

Vale GC, Tabchoury CP, Arthur RA, Del Bel Cury AA, Paes Leme AF, Cury JA. Temporal relationship between sucrose-associated changes in dental biofilm composition and enamel demineralization. *Caries Res* 2007; 41:406-412.

Van Houte J. Bacterial specificity in the etiology of dental caries. *Int dent J* 1980; 30:305-326.

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

Vanobbergen J, Martens L, Lesaffre E, Bogaerts K, Declerck D. Assessing risk indicators for dental caries in the primary dentition. *Community Dent Oral Epidemiol* 2001; 29:424-434.

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.

Vogel GL. Oral fluoride reservoirs and the prevention of dental caries. *Monogr Oral Sci* 2011; 22:146-157.

Walsh T, Worthington HV, Glenny AM, Appelbe P, Marinho VC, Shi X. Fluoride toothpastes of different concentrations for preventing dental caries in children and adolescents. *Cochrane Database Syst Rev* 2010; 20:CD007868.

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

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 *fff*. *Curr Microbiol* 2015; 70:716-723.

Contributions made by each author to the paper

N.G. Emerenciano: Accomplishment of experiments and participated in the manuscript writing; A.C.B. Delbem: Study's idea and design and participated in the manuscript writing; J.P. Pessan: Study's idea and design and participated in the manuscript writing; G.P. Nunes; Accomplishment of experiments and participated in the manuscript writing; E.R. Camargo: Synthesis, characterization of nano-sized TMP and participated in the manuscript writing; M. Danelon: Study's idea and design and participated in the manuscript writing.

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: **A** Enamel block preparation. **B** Hardness analysis. **C** Treatments. **D** Analysis of dental biofilm composition. **E** Hardness Analysis. **F** Cross-sectional. **G** Fluoride, calcium, and phosphorus in enamel. **H** Integrated loss of subsurface hardness.

Figure 2: X-ray patterns of the micrometric TMP and of the nano-sized TMP after milling for 48 h.

Figure 3: SEM images of the TMP powders (a) before milling and (b) after milling for 48 h.

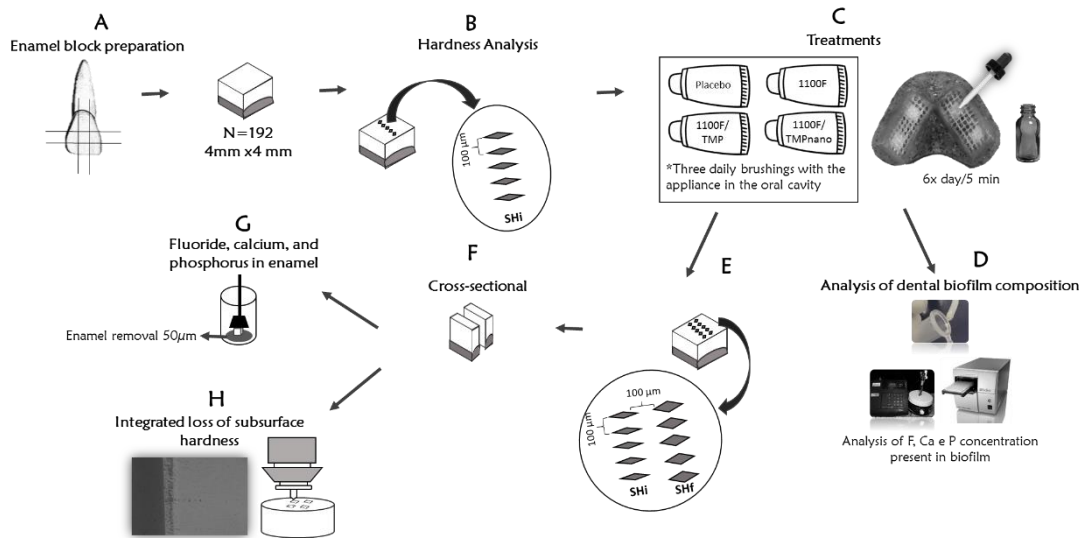


Figure 1: A Enamel block preparation. **B** Hardness analysis. **C** Treatments. **D** Analysis of dental biofilm composition. **E** Hardness Analysis. **F** Cross-sectional. **G** Fluoride, calcium, and phosphorus in enamel. **H** Integrated loss of subsurface hardness.

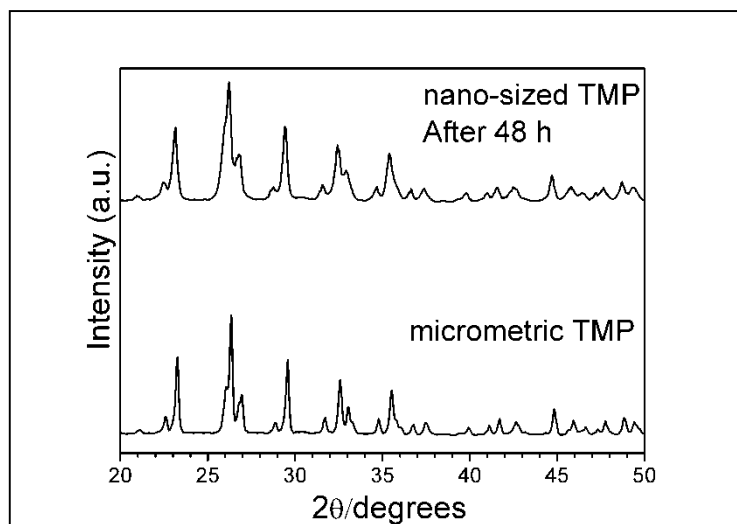


Figure 2: X-ray patterns of the micrometric TMP and of the nano-sized TMP after milling for 48 h.

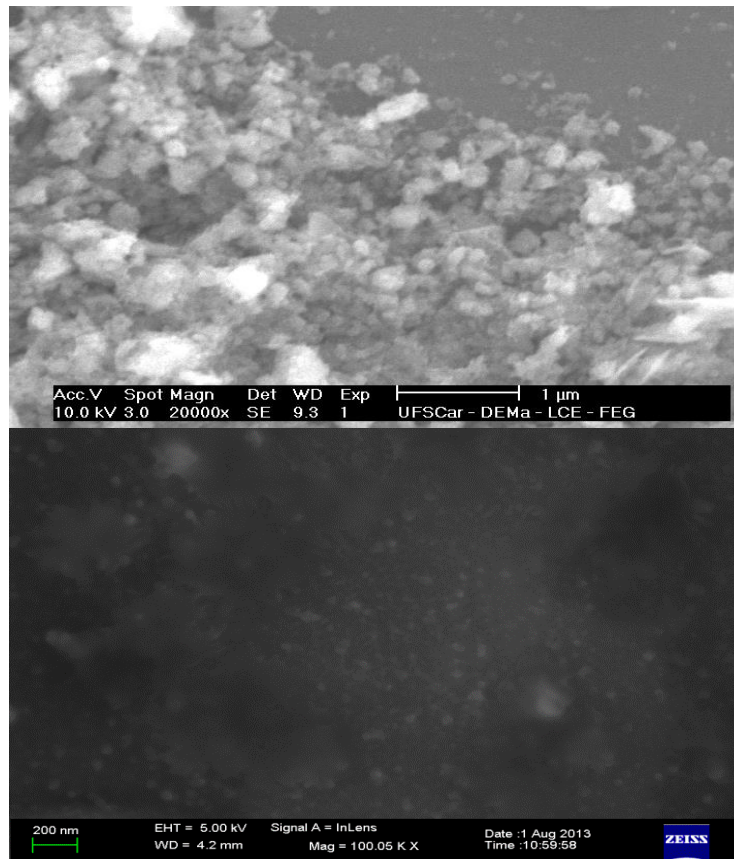


Figure 3: SEM images of the TMP powders (a) before milling and (b) after milling for 48 h.

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

Analysis	Toothpastes			
	Placebo	1100F	1100F/TMP	1100F/TMPnano
%SH	-52.24 ^a	-31.5 ^b	-24.7 ^c	-16.1 ^d
(KHN)	(8.2)	(6.4)	(8.8)	(4.3)
Δ KHN	6533.3 ^a	3274.5 ^b	2744.8 ^c	1321.1 ^d
(KHN x μ m)	(1008.8)	(464.4)	(362.4)	(159.2)
Fluoride-enamel	0.3 ^a	0.6 ^b	0.6 ^b	0.6 ^b
(μ g/mm ³)	(0.1)	(0.1)	(0.1)	(0.2)
Calcium-enamel	371.7 ^a	548.8 ^b	796.5 ^c	1290.2 ^c
(μ g/mm ³)	(80.3)	(118.0)	(289.7)	(450.7)
Phosphorus-enamel	162.4 ^a	195.7 ^b	218.2 ^b	343.9 ^c
(μ g/mm ³)	(44.5)	(26.0)	(68.4)	(116.9)
Fluoride-biofilm	5.87E-05 ^a	6.86E-04 ^b	8.94E-04 ^{b,c}	1.31E-03 ^c
(mol/kg)	(1.42E-05)	(4.44E-04)	(3.65E-04)	(9.091E-04)
Calcium-biofilm	1.08E-01 ^a	1.47E-01 ^a	1.90E-01 ^b	1.91E-01 ^b
(mol/kg)	(3.85E-05)	(4.36E-02)	(5.91E-02)	(5.97E-02)
Phosphorus-biofilm	7.97E-02 ^a	9.10E-02 ^{a,b}	1.12E-01 ^c	1.07E-01 ^{c,b}
(mol/kg)	(2.65E-02)	(2.60E-02)	(4.27E-02)	(4.14E-02)
ESP-biofilm	44.8 ^a	32.9 ^b	16.3 ^c	6.5 ^d
(mg/g)	(10.1)	(8.1)	(6.2)	(2.2)

Different superscript letters indicate significant differences among the treatments for each variable separately. (One-way ANOVA, repeated measures followed by Fisher LSD test; $p < 0.05$).

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

<i>Ion activity,</i> <i>mol/Kg</i>	Toothpastes			
	Placebo	1100F	1100F/TMP	1100F/TMPnano
Ca ²⁺	2.05E-02 ^a (5.42E-03)	2.46E-02 ^{a,b} (1.03E-02)	2.92E-02 ^b (8.66E-03)	3.00E-02 ^b (1.06E-02)
CaPO ₄ ⁻	1.27E-03 ^a (6.74E-04)	1.80E-03 ^b (4.99E-04)	2.36E-03 ^b (9.28E-04)	2.20E-03 ^b (9.39E-04)
CaHPO ₄ ⁰	4.32E-02 ^a (2.29E-02)	6.91E-02 ^b (1.70E-02)	8.03E-02 ^b (3.34E-02)	7.5E-02 ^b (3.19E-02)
CaH ₂ PO ₄ ⁺	3.06E-03 ^a (1.63E-03)	4.35E-03 ^b (1.20E-03)	5.69E-03 ^b (2.37E-03)	5.32E-03 ^b (2.26E-03)
PO ₄ ³⁻	1.82E-08 ^a (1.07E-08)	2.42E-08 ^a (1.06E-08)	2.35E-08 ^a (7.45E-09)	2.33E-08 ^a (1.14E-08)
HPO ₄ ²⁻	3.21E-03 ^a (1.89E-03)	4.26E-03 ^a (1.86E-03)	4.14E-03 ^a (1.31E-03)	4.10E-03 ^a (2.01E-03)
H ₂ PO ₄ ⁻	4.84E-03 ^a (2.85E-03)	6.43E-03 ^a (2.81E-03)	6.26E-03 ^a (1.98E-03)	6.19E-03 ^a (3.04E-03)
F ⁻	3.56E-05 ^a (9.96E-06)	3.85E-04 ^b (2.52E-04)	4.71E-04 ^{b,c} (1.90E-04)	7.19E-04 ^c (5.42E-04)
CaF ⁺	8.07E-06 ^a (2.21E-06)	1.06E-04 ^b (7.58E-05)	1.56E-04 ^{b,c} (7.57E-05)	2.10E-04 ^c (1.36E-04)
HF ⁰	6.66E-09 ^a (1.86E-09)	7.20E-08 ^b (4.72E-08)	8.81E-05 ^{b,c} (3.55E-05)	1.35E-04 ^c (1.01E-04)
<i>Degree of Saturation</i>				
HA	16.03 ^a (1.57)	16.99 ^b (0.46)	17.47 ^c (0.65)	17.39 ^c (0.59)
CaF ₂	-0.16 ^a (0.22)	1.79 ^b (0.72)	2.16 ^{b,c} (0.54)	2.39 ^c (0.61)

Distinct superscript letters indicate statistical significance among the toothpastes for each ions species or solid phase (One-way ANOVA, repeated measures followed by Fisher LSD test; $p < 0.05$). Values between parentheses indicate the standard deviation of the mean.

Anexo

ANEXO A

INSTRUÇÕES AOS AUTORES

Caries Research

Guidelines for Authors

www.karger.com/cre_guidelines

Aims and Scope

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

Submission

Manuscripts written in English should be submitted online:

Should you experience problems with your submission, please contact:

Prof. David Beighton

(Editor-in-Chief, Caries Research)

Department of Microbiology

The Henry Wellcome Laboratories for Microbiology and Salivary Research

KCL Dental Institute, Floor 17, Guys Tower

London Bridge SE1 9RT (UK)

Tel. +44 2071887465

Fax +44 2071887466

david.beighton@kcl.ac.uk

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

Plagiarism Policy

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

Conditions

All manuscripts are subject to editorial review. Manuscripts are received with the explicit understanding that the data they contain have not previously been

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

Types of Papers

Original papers or Short Communications are reports of original work (including systematic reviews and meta-analyses). Both have the structure outlined below but for Short Communications the abstract should be less than 100 words and the manuscript should not exceed 3 printed pages, equivalent to about 9 manuscript pages (including tables, illustrations and references).

Reviews can have a freer format but should nevertheless commence with a Title page, an Abstract and an Introduction defining the scope. Current topics are concise articles that present critical discussion of a topic of current interest, or a fresh look at a problem, and should aim to stimulate discussion.

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

Preparation of Manuscripts

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

Manuscripts should be prepared as a text file plus separate files for illustrations. The text file should contain the following sequence of sections: Title page; Declaration of interests; Abstract; Introduction; Materials and Methods; Results; Discussion; Acknowledgements; References; Legends; Tables. Each section should start on a new page, except for the body of the paper (Introduction to Acknowledgements), which should be continuous. Lines in the manuscript must be numbered consecutively from the title page until the last page. Submissions which do not conform to these simple guidelines will be returned to the author.

Title page: The first page of each manuscript should show, in order:

- _ the title, which should be informative but concise;
- _ the authors' names and initials, without degrees or professional status, followed by their institutes;
- _ a short title, maximum length 60 characters and spaces, for use as a running head;
- _ a list of 3-10 key words;
- _ the name of the corresponding author and full contact details (postal address, telephone and fax numbers, and e-mail address).

Declaration of Interests: Potential conflicts of interest should be identified for each author or, if there are no such conflicts, this should be stated explicitly. Conflict of interest exists where an author has a personal or financial relationship that might

introduce bias or affect their judgement. Examples of situations where conflicts of interest might arise are restrictive conditions in the funding of the research, or if an author or their employer holds patente (s) on a product used in the study, or payment to an investigator from organisations with an interest in the study (including employment, consultancies, honoraria, ownership of shares, travel grant). Investigators should disclose potential conflicts to study participants and should state whether they have done so. The possible existence of a conflict of interest does not preclude consideration of a manuscript for publication, but the Editor might consider it appropriate to publish the disclosed information along with the paper.

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

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

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

work. The name and brief address of the manufacturer or supplier of major equipment should be given.

Statistical methods should be described with enough detail to enable a knowledgeable reader with access to the original data to verify the reported results. When possible, findings should be quantified and appropriate measures of error or uncertainty (such as confidence intervals) given. Sole reliance on statistical hypothesis testing, such as the use of P values, should be avoided. Details about eligibility criteria for subjects, randomization and the number of observations should be included. The computer software and the statistical methods used should be specified. See Altman et al.: Statistical guidelines for contributors to medical journals [Br Med J 1983;286:1489–93] for further information.

Manuscripts reporting studies on human subjects should include evidence that 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 purposes.

Results: Results should be presented without interpretation. The same data should not be presented in both tables and figures. The text should not repeat numerical data provided in tables or figures but should indicate the most important results and describe relevant trends and patterns.

Discussion: This section has the functions of describing any limitations of material or methods, of interpreting the data and of drawing inferences about the contribution of the study to the wider field of research. There should be no repetition of preceding sections, e.g. reiteration of results or the aim of the research. The discussion should end with a few sentences summarising the conclusions of the study. However, there should not be a separate 'Conclusions' section.

Acknowledgements: Acknowledge the contribution of colleagues (for technical assistance, statistical advice, critical comment etc.) and provide the position(s) of author(s) employed by commercial firms. This section should describe the source(s) of funding that have supported the work including relevant grant numbers. Please also include this sentence: "The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript." If this statement is not correct, you must describe the role of any

sponsors or funders, and amend the sentence as needed. Additionally, the roles of all authors must be described (For example: Conceived and designed the experiments: AA, BB. Performed the clinical examination: AA, CC. Performed the experiments: DD, FF. Analyzed the data: BB, FF. Wrote the paper: AA, CC, FF, EE). Legends: The table headings should be listed first, followed by the legends for the illustrations. Tables: Tables should be numbered in Arabic numerals. Each table should be placed on a separate page. Tables should not be constructed using tabs but by utilising the table facilities of the word-processing software.

Illustrations:

_ Illustrations should be numbered in Arabic numerals in the sequence of citation. Figure numbers must be clearly indicated on the figures themselves, outside the image area.

Black and white half-tone illustrations must have a final resolution of 300 dpi after scaling, line drawings one of 800-1200 dpi.

_ Figures with a screen background should not be submitted.

_ When possible, group several illustrations in one block for reproduction (max. size 180 x 223 mm).

Color Illustrations

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

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

at CHF 800.00 per page.

References

Reference to other publications should give due acknowledgement to previous work; provide the reader with accurate and up-to-date guidance on the field of research under discussion; and provide evidence to support lines of argument. Authors should select references carefully to fulfil these aims without attempting to be comprehensive. Cited work should already be published or officially accepted for publication. Material submitted for publication but not yet accepted should be cited as 'unpublished results', while unpublished observations communicated to the authors by another should be cited as 'personal communication', with credit in both cases being given to the source of the information. Neither unpublished nor personally communicated material should be included in the list of references. Abstracts more than 2 years old and theses should not be cited without a good reason, which should be explained in the covering letter accompanying the paper.

References should be cited by naming the author(s) and year. Where references are cited in parenthesis, both names and date are enclosed in square brackets. Where the author is the subject or object of the sentence, only the year is enclosed in brackets.

One author: [Frostell, 1984] or Frostell [1984].

Two authors: [Dawes and ten Cate, 1990] or Dawes and ten Cate [1990].

More than two authors: [Trahan et al., 1985] or Trahan et al. [1985].

Several references cited in parenthesis should be in date order and separated by semi-colons: [Frostell, 1984; Trahan et al., 1985; Dawes and ten Cate, 1990].

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

Examples

(a) *Papers published in periodicals:* Lussi A, Longbottom C, Gyax M, Braig F: Influence of professional cleaning and drying of occlusal surfaces on laser fluorescence in vivo. *Caries Res* 2005;39:284-286.

(b) *Papers published only with DOI numbers:* Theoharides TC, Boucher W, Spear K: Serum interleukin-6 reflects disease severity and osteoporosis in mastocytosis patients. *Int Arch Allergy Immunol* DOI: 10.1159/000063858.

(c) *Monographs:* Matthews DE, Farewell VT: *Using and Understanding Medical Statistics*. Basel, Karger, 1985.

(d) *Edited books*: DuBois RN: Cyclooxygenase-2 and colorectal cancer; in Dannenberg AJ, DuBois RN (eds): COX-2. Prog Exp Tum Res. Basel, Karger, 2003, vol 37, pp 124-137.

(e) *Patents*: Diggins AA, Ross JW: Determining ionic species electrochemically. UK Patent Application GB 2 064 131 A, 1980.

(f) *World Wide Web*: Chaplin M: Water structure and behavior. www.lsbu.ac.uk/water, 2004.

Supplementary Material

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

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

Digital Object Identifier (DOI) S. Karger Publishers supports DOIs as unique identifiers for articles. A DOI number will be printed on the title page of each article. DOIs can be useful in the future for identifying and citing articles published online without volume or issue information. More information can be found at www.doi.org *Author's Choice*TM Karger's *Author's Choice*TM service broadens the reach of your article and gives all users worldwide free and full access for reading, downloading and printing at www.Karger.com. The option is available for a one-time fee of CHF 3,000.00, which is a permissible cost in grant allocation. More information can be found at www.karger.com/authors_choice. NIH-Funded Research.

The U.S. National Institutes of Health (NIH) mandates under the NIH Public Access Policy that final, peer-reviewed manuscripts appear in its digital database within 12 months of the official publication date. As a service to authors, Karger submits your manuscript on your behalf to PubMed Central (PMC) immediately upon publication. It usually receives a PMCID within approximately a month and will appear in PMC after 12 months. For those selecting our premium *Author's Choice*TM service, the usual embargo will be overridden, accelerating the accessibility of your work. More details on NIH's Public Access Policy are available [here](#) Self-Archiving Karger permits authors to archive their pre-prints (i.e. pre-refereeing) or postprints (i.e. final draft post-refereeing) on their personal or institution's servers, provided the following conditions are met: Articles may not be used for commercial purposes, must be linked to the publisher's version, and must acknowledge the publisher's copyright. Authors selecting Karger's *Author's Choice*TM feature, however, are also permitted to archive the final, published version of their article, which includes copyediting and design improvements as

well as citation links.

Page Charges

There are no page charges for papers of seven or fewer printed pages (including tables, illustrations and references). A charge of CHF 650.00 will be levied for each page in excess of the allotted seven printed pages. The allotted size of a paper is equal to approximately 21 typescript pages (including tables, illustrations and references).

Proofs

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

Reprints

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

ANEXO B

COMITÊ DE ÉTICA

UNESP - FACULDADE DE
ODONTOLOGIA-CAMPUS DE
ARAÇATUBA/ UNIVERSIDADE



PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Efeito anticárie de um dentífrico fluoretado e suplementado com nanopartículas de trimetafosfato de sódio sobre a desmineralização do esmalte e biofilme: estudo *in situ*.

Pesquisador: Marcelle Danelon

Área Temática:

Versão: 1

CAAE: 61591416.1.0000.5420

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

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.807.683

Apresentação do Projeto:

Avaliação *in situ* da capacidade de dentífricos fluoretados e suplementados com trimetafosfato de sódio nanoparticulado (TMPnano), em reduzir da desmineralização do esmalte bovino e sua ação sobre o biofilme.

Objetivo da Pesquisa:

Objetivo Primário:

O objetivo projeto será avaliar, *in situ*, a capacidade de dentífricos fluoretados e suplementados com trimetafosfato de sódio nanoparticulado (TMPnano), em reduzir da desmineralização do esmalte bovino e sua ação sobre o biofilme.

Objetivo Secundário:

O objetivo projeto será avaliar, *in situ*, a capacidade de dentífricos fluoretados e suplementados com trimetafosfato de sódio nanoparticulado (TMPnano), em reduzir da desmineralização do esmalte bovino e sua ação sobre o biofilme.

Avaliação dos Riscos e Benefícios:

Riscos:

Endereço: JOSE BONFACIO 1193
 Bairro: VILA MENDONÇA CEP: 16.015-060
 UF: SP Município: ARACATUBA
 Telefone: (18)3636-3200 Fax: (18)3636-3332 E-mail: andrabartou@foa.unesp.br

UNESP - FACULDADE DE
ODONTOLOGIA-CAMPUS DE
ARAÇATUBA UNIVERSIDADE



Continuação do Parecer: 1.837.965

O Risco será mínimo, uma vez que o voluntário poderá apresentar um leve desconforto ao utilizar o dispositivo palatino.

Benefícios:

Espera-se, com este projeto desenvolver uma formulação dentífrica com 1100 ppm F e eficácia superior à de um dentífrico padrão/comercial (1100 ppm F) mantendo a estabilidade do fluoreto no dentífrico e entender o mecanismo de ação da associação F/TMPiano no processo da cárie dentária e sua ação no biofilme.

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:

Os termos obrigatórios 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 468 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 07/05/2017. 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.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_809794.pdf	25/10/2016 17:46:13		Aceito

Endereço: JOSE BONIFACIO 1193
Bairro: VILA MENDONÇA CEP: 16.015-050
UF: SP Município: ARACATUBA
Telefone: (16)3036-3200 Fax: (16)3036-3332 E-mail: anelrebertos@fob.unesp.br

Página 52 de 53

UNESP - FACULDADE DE
ODONTOLOGIA-CAMPUS DE
ARAÇATUBA/ UNIVERSIDADE



Continuação do Parecer: 1.807/683

Folha de Rosto	FR.pdf	25/10/2016 17:45:46	Marcelle Danelon	Aceito
Projeto Detalhado / Brochura Investigador	Projeto.pdf	13/10/2016 15:50:39	Marcelle Danelon	Aceito
TCE / Termos de Assentimento / Justificativa de Ausência	TCE.pdf	13/10/2016 15:49:49	Marcelle Danelon	Aceito


Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

ARACATUBA, 07 de Novembro de 2016


Assinado por:
André Pinheiro de Magalhães Bertoz
(Coordenador)

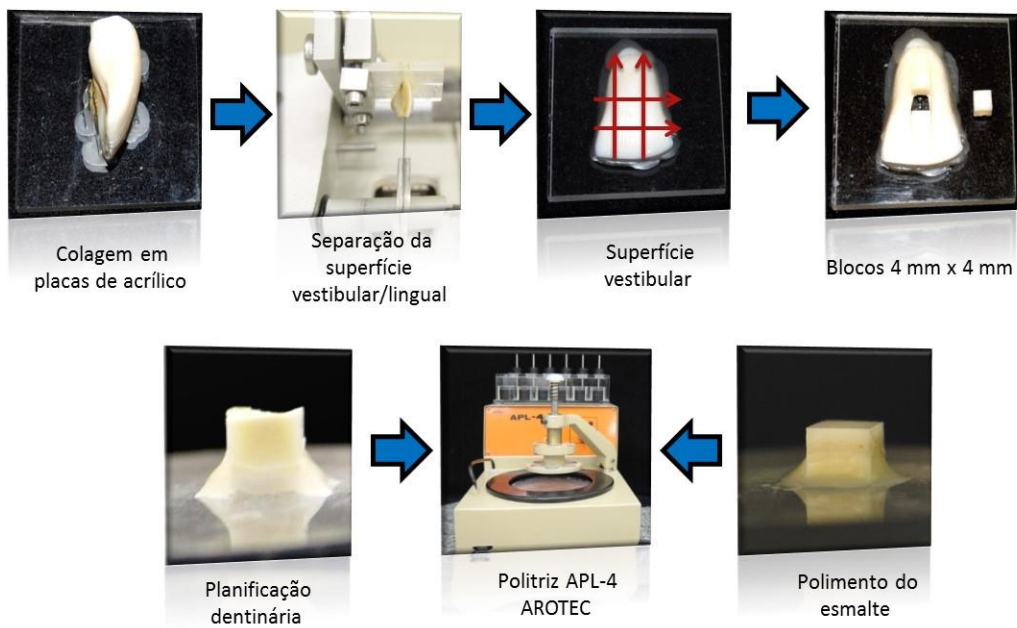
Assinado por	Assinado em
Assinado por	Assinado em
Assinado por	Assinado em

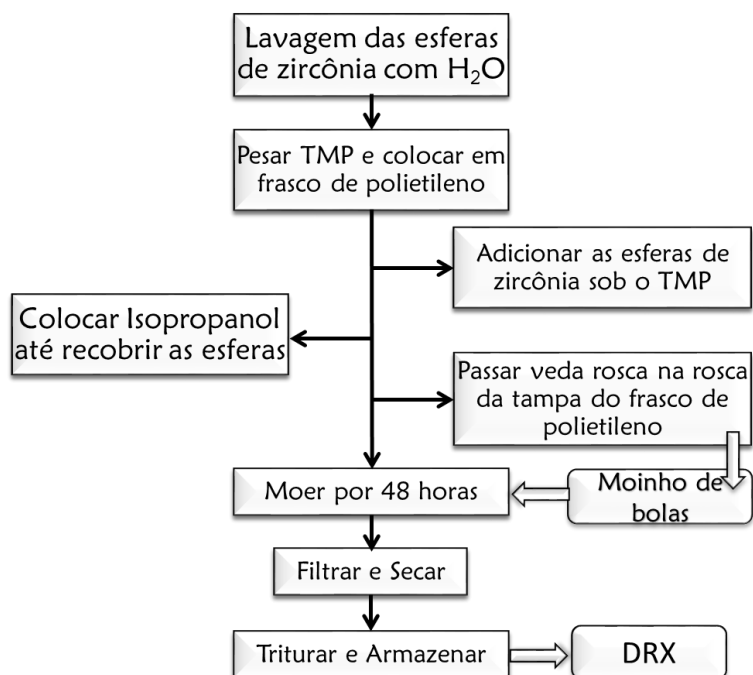
Endereço: JOSE BONIFACIO 1193
Bairro: VILA MENDONÇA CEP: 16.015-060
UF: SP Município: ARACATUBA
Telefone: (16)3636-3200 Fax: (16)3636-3332 E-mail: andrebortoz@fob.unesp.br

Folha 02 de 02

ANEXO C

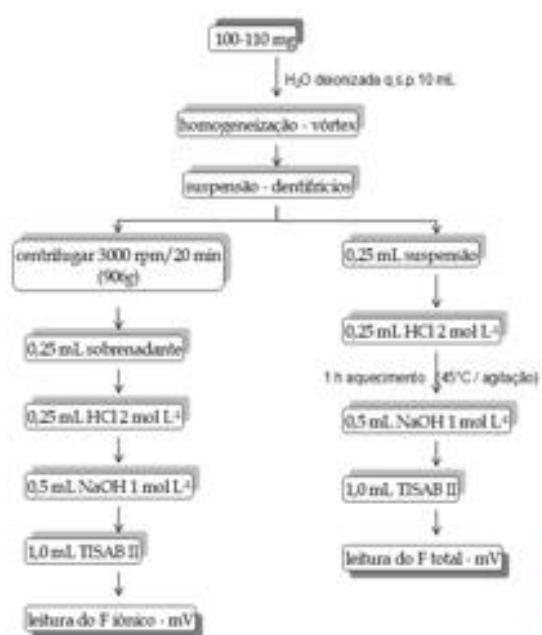
PREPARO DOS BLOCOS DE ESMALTE



*ANEXO D**SÍNTESE DO TRIMETAFOSFATO DE SÓDIO
NANOPARTICULADO*

ANEXO E

DOSAGEM DE FLUORETO NOS DENTÍFRÍCIOS EXPERIMENTAIS



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

ANEXO F

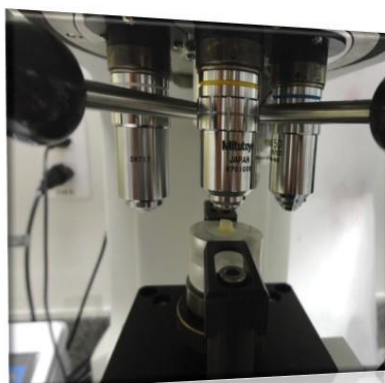
DISPOSITIVO PALATINO



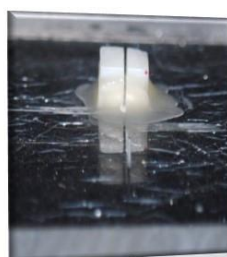
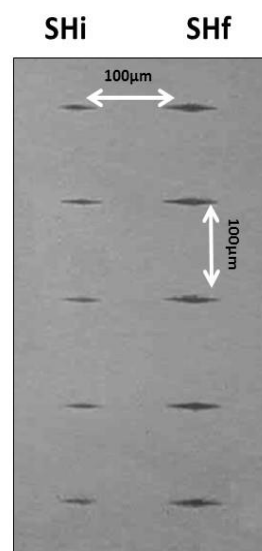
Kit fornecido ao voluntário a cada período experimental.

ANEXO G

ANÁLISE DA DUREZA SUPERFICIAL E LONGITUDINAL DO ESMALTE



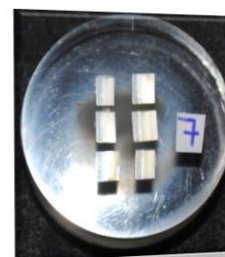
Microdurômetro Buehler
Carga 25 gramas
Tempo 10 segundos



Secção dos blocos de esmalte no sentido longitudinal



Embutimento dos blocos de esmalte com resina acrílica



Aspecto final dos blocos de esmalte



Microduromêtro Buehler
Carga 5g; Tempo 10 segundos



Análise da Lesão em Profundidade

ANEXO H

ANÁLISE DE FLUORETO, CÁLCIO E FÓSFORO NO ESMALTE



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



Desgaste 50 µm Lixa 400 (CARBIMET – BUEHLER)



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

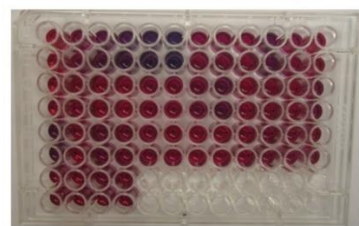
Agitação por 1 hora



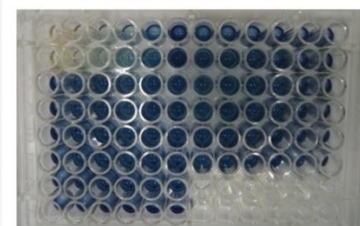
0,3 mL da amostra + 0,3 mL de Tisab II
Modificado com NaOH



Espectrofotômetro de microplaca
EONC, Biotek, USA



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



Fósforo - Método colorimétrico Fiske e Subbarow, 1925.

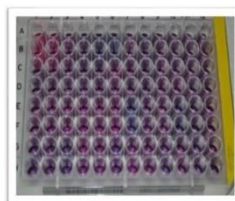
ANEXO I

ANÁLISE DA COMPOSIÇÃO DO BIOFILME DENTÁRIO

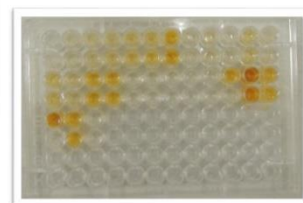
Biofilme Coletado



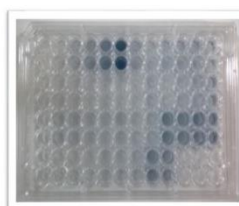
Fluoreto- Eletrodo específico (Orion 9409) - Eletrodo de referência (Orion 900200) - Analisador de íons (Orion 720A+).



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



EPS - Método Fenol-Sulfúrico
Dubois, 1956.



Fósforo - Método colorimétrico
Fiske e Subbarow, 1925.