NAYARA GONÇALVES EMERENCIANO

DESENVOLVIMENTO, CARACTERIZAÇÃO E DETERMINAÇÃO DO EFEITO ANTICÁRIE DE DENTIFRÍCIOS CONTENDO MICROPARTÍCULAS E NANOPARTÍCULAS DE ISÔMERO β DE GLICEROFOSFATO DE CÁLCIO (CaGP): ESTUDOS *IN VITRO* E *IN SITU*

Araçatuba – SP
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Orientadora: Profª. Drª. Marcelle Danelon

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Dedicatória

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A Ele a Glória"

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Epígrafe

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 Geral

Emerenciano, NG. **Desenvolvimento, caracterização e determinação do efeito anticárie de dentifrícios contendo micropartículas e nanopartículas de isômero β de glicerofosfato de cálcio (CaGP): estudos *in vitro* e *in situ*** 2020 93f. Tese (Doutorado) - Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba 2020.

RESUMO

O objetivo geral deste estudo foi avaliar o efeito de diferentes concentrações de isômeros β de Glicerofosfato de cálcio (β-CaGP) microparticulado e nanoparticulado adicionados a dentifrícios convencionais (1100 ppm F) sobre o processo de desmineralização do esmalte *in vitro* e remineralização de lesões iniciais de cárie *in situ*. Para o ***Artigo 1 de desmineralização in vitro*** blocos bovinos (n = 120) foram selecionados pela dureza de superfície inicial (SHi) e divididos em 10 grupos de dentifrícios experimentais (n = 12): sem fluoreto/β-CaGPm/β-CaGPn (Placebo); 1100 ppm F (1100F); 1100F associado às concentrações de 0,125%; 0,25%; 0,5% e 1,0% de β-CaGPm e β-CaGPn. Os blocos foram tratados 2x/dia com os dentifrícios, sendo submetidos a 5 ciclagens de pH durante 7 dias. Após, determinou-se a porcentagem de perda de dureza de superfície (%SH), perda integrada de dureza de subsuperfície (ΔKHN) e análise do perfil e profundidade da lesão de subsuperfície pela microscopia de luz polarizada (MLP). Concentração de fluoreto (F), cálcio (Ca) e fósforo (P) no esmalte foram avaliadas. Os dados foram submetidos à análise de variância (ANOVA-1-critério) seguido pelo teste Student-Newman-Keuls ($p < 0,001$). O tratamento com 1100F-0,25%β-CaGPn levou à menores valores de %SH, ΔKHN e MLP ($p < 0,001$). A concentração de F do esmalte foi semelhante para todos os grupos fluoretados ($p > 0,001$). Com 1100F-0,5%β-CaGPm e 1100F-0,25%β-CaGPn, a concentração de Ca no esmalte aumentou ($p < 0,001$). A maior concentração de P foi observada para o grupo 1100F-0,25%β-CaGPn ($p < 0,001$). O ***Artigo 2 de remineralização in situ*** foi um estudo duplo-cego e cruzado, realizado em 4 fases experimentais com duração de 3 dias cada e washout de 7 dias. Voluntários (n=12) utilizaram dispositivos palatinos, contendo 4 blocos de esmalte bovino com lesão de cárie artificial. Os regimes de tratamentos com dentifrícios foram: 1) Placebo; 2) 1100F; 3) 1100F-0,5%β-CaGPm e 4) 1100F-0,25%β-CaGPn. Os voluntários foram orientados a escovar os dentes naturais com os dispositivos palatinos na cavidade bucal, sendo os blocos tratados com o slurry dos dentifrícios por 1 minuto (3x/dia). Após cada fase determinou-se a dureza de superfície final para o cálculo da porcentagem de recuperação de dureza de superfície (%SHR), recuperação da perda integrada de dureza

de subsuperfície (ΔIHR) e concentração de F, Ca e P no esmalte. Os dados foram analisados por ANOVA 1-critério de medidas repetidas seguida pelo teste de Student-Newman-Keuls ($p < 0,001$). O tratamento com 1100F-0,25% β-CaGPn, promoveu um aumento na %SHR. Além disso, a capacidade de reduzir o corpo da lesão (ΔIHR) foi maior com 1100F-0,25%β-CaGPn ($p < 0,001$). A adição de β-CaGPm e β-CaGPn ao dentifrício fluoretado não influenciou a concentração de F no esmalte ($p > 0,001$). O tratamento com 1100F-0,25%β-CaGPn promoveu um aumento na concentração de Ca e P no esmalte ($p < 0,001$). Conclui-se que a adição de 0,25%β-CaGPn a um dentifrício convencional promoveu um maior efeito protetor na inibição da desmineralização do esmalte *in vitro* e uma ação remineralizadora *in situ* significativamente mais elevada quando comparado ao dentifrício convencional (1100 ppm F).

Palavras-chave: Esmalte dentário, Fluoreto, Fosfato, Desmineralização, Remineralização.

General Abstract

Emerenciano, NG. **Development, characterization and determination of the anti-caries effect of toothpaste containing microparticles and nano-sizeds of β calcium glycerophosphate isomer (CaGP): *in vitro* and *in situ* studies** 2020 93f. Tese (Doutorado) - Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba 2020.

ABSTRACT

The aim of this study was to evaluate the effect of isomer micro and nano-sizeds of β calcium glycerophosphate (CaGP) in different concentrations added to conventional toothpastes (1100 ppm F) on the *in vitro* enamel demineralization process and remineralization of initial caries lesions *in situ*. For ***Manuscript 1 in vitro demineralization***, bovine blocks (n = 120) were selected for initial surface hardness (SHi) and divided into 10 groups of experimental toothpastes (n = 12): without fluoride/β-CaGPm/β-CaGPn (Placebo); 1100 ppm F (1100F); 1100F associated with concentrations of 0.125%; 0.25%; 0.5% and 1.0% of β-CaGPm and β-CaGPn. The blocks were treated 2x/day with the toothpastes, being submitted to 5 pH cycles during 7 days. Then, the percentage of surface hardness loss (%SH), integrated loss of subsurface hardness (ΔKHN) and analysis of the profile and depth of the subsurface lesion by polarized light microscopy (MLP) were determined. Fluoride (F), calcium (Ca) and phosphorus (P) concentration in the enamel were evaluated. The data were submitted to analysis of variance (ANOVA-1-criterion) followed by the Student-Newman-Keuls test ($p < 0.001$). Treatment with 1100F-0.25%β-CaGPn led to lower values of %SH, ΔKHN and MLP ($p < 0.001$). The enamel F concentration was similar for all fluoride groups ($p > 0.001$). With 1100F-0.5%β-CaGPm and 1100F-0.25%β-CaGPn, the concentration of Ca in the enamel increased ($p < 0.001$). The highest concentration of P was observed for the group 1100F-0.25%β-CaGPn ($p < 0.001$). **Manuscript 2 of *in situ remineralization*** was a double-blind, cross-over study, carried out in 4 experimental phases lasting 3 days each and washout of 7 days. Volunteers (n=12) used palatal devices, containing 4 blocks of bovine enamel with artificial caries lesion. The treatment regimens with toothpastes were: 1) without F/β-CaGPm/β-CaGPn (Placebo); 2) 1100 ppm F (1100F); 3) 1100F + 0.5%β-CaGPm (1100F-0.5%β-CaGPm) and 4) 1100F + 0.25%β-CaGPn (1100F-0.25%β-CaGPn). Volunteers were guided to brush their natural teeth with the palatal devices in the oral cavity, the blocks being treated with the toothpaste slurry for 1 minute (3x/day). After each phase the final surface hardness was determined for the calculation of the surface hardness recovery percentage (%SHR),

recovery of the integrated loss of subsurface hardness (ΔIHR) and concentration of F, Ca and P in the enamel. The data were analyzed by ANOVA 1-criterion of repeated measurements followed by Student-Newman-Keuls test ($p < 0.001$). The treatment with 1100F-0.25% β-CaGPn, promoted an increase in %SHR. In addition, the ability to reduce the body lesion (ΔIHR) was increased with 1100F-0.25%β-CaGPn ($p < 0.001$). The addition of β-CaGPm and β-CaGPn to the fluoride toothpaste did not influence the concentration of F in the enamel ($p > 0.001$). The treatment with 1100F-0.25%β-CaGPn promoted an increase in the concentration of Ca and P in the enamel ($p < 0.001$). It is concluded that the addition of 0.25%β-CaGPn to a conventional toothpaste promoted a greater protective effect in inhibiting *in vitro* enamel demineralization and a significantly higher remineralizing action *in situ* when compared to conventional toothpaste (1100 ppm F).

Keywords: Dental enamel, Fluoride, Phosphate, Demineralization, Remineralization.

Lista de Abreviaturas

LISTA DE ABREVIATURAS

ANOVA Analysis of Variance

ºC Degrees Celsius

Ca Calcium

CaF_2 Calcium fluoride ion

β-CaGPm β-Calcium glycerophosphate microparticle

β-CaGPn β-Calcium glycerophosphate nano-sized

Ca^{+2} Calcium ion

CT Conventional toothpaste

F Fluoride

F^- Fluoride ion

g Gram

h Hour

HMP Sodium hexametaphosphate

IF Ionic fluoride

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

PLM Polarized light microscopy

P_2O_5 Diphosphorus pentoxide

PO_4^{3-} Orthophosphate

pH Hydrogen potential

ppmF Parts per million of fluoride

s Seconds

SD Standard deviation

SHi Initial surface hardness

SHf Final surface hardness

%SH Surface hardness loss

%SHR Surface hardness recovery percentage

TF Total fluoride

TISAB Total ionic strength adjuster cap

TMP Sodium trimetaphosphate

µg Microgram

µg/mm^3 Microgram per cubic millimeter

μL/mg Microliters/milligram

μm Micrometer

ΔIHR Integrated hardness recuperation

ΔKHN Integrated loss of subsurface hardness

XRD X-ray diffraction

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SUMÁRIO

Introdução Geral

1 Introdução Geral

A cárie dentária é uma doença dinâmica multifatorial, modulada pela dieta e mediada pelo acúmulo de biofilme que resulta no desequilíbrio entre os processos de des e remineralização dos tecidos duros dentários [Machiulskiene et al., 2020]. Sua ocorrência é determinada por fatores biológicos, comportamentais e psicossociais relacionados ao meio do indivíduo [Pitts et al., 2019]. Ainda, é considerada o principal problema da Odontologia devido à alta prevalência entre a população mundial [Golubnitschaja et al., 2016], e causa um grande impacto na qualidade de vida dos indivíduos afetados. Diante disso, estudos veem buscando abordagens que visam reduzir a prevalência desta doença crônica. Sabendo-se que escovação regular com dentifrício fluoretado é a principal intervenção não profissional para prevenir a cárie dentária [Walsh et al., 2019], seria interessante aumentar a eficácia dos mesmos, proporcionando uma diminuição nos índices da doença.

A suplementação com cálcio (Ca) e fosfato (P) parece ser uma boa alternativa para potencializar os efeitos dos dentifrícios fluoretados, pois quando supersaturados no biofilme dentário durante os períodos de desafio cariogênico, tornam o F mais efetivo, podendo diminuir ou até mesmo inibir a dissolução do esmalte [Li et al., 2014]. A adição de fosfatos inorgânicos a dentifrícios com concentração reduzida de F e concentração convencional (1100 ppm F) mostrou aumentar o efeito anticárie [Takeshita et al., 2009; de Castro et al., 2015]. Estes fosfatos apresentam afinidade pelo esmalte reduzindo a perda mineral [da Camara et al., 2015; da Camara et al., 2016]. Dentre os fosfatos inorgânicos o trimetafosfato de sódio (TMP) e o hexametafosfato de sódio (HMP), fosfatos cíclicos, têm sido testados em diferentes concentrações de F e produtos, mostrando reduzir a perda mineral e aumentar o processo de remineralização, pois apresentam capacidade de modificar a superfície da hidroxiapatita, através de sua adsorção [Danelon et al., 2014; Takeshita et al., 2015]. Takeshita et al. [2009 e 2015] demonstraram *in vitro e in situ*, que a suplementação com 1%TMP em um dentifrício de reduzida concentração de F (500 ppm F) proporcionou ação semelhante à de um dentifrício padrão (1100 ppm F) no processo de desmineralização dentária do esmalte e biofilme. Já em relação ao HMP, da Camara et al. [2014] verificaram que a adição de HMP nas concentrações de 0,5 e 1,0% HMP em um dentifrício de baixa concentração de F (250 ppm F) apresentou perda mineral reduzida (%SH e ΔKHN) semelhante ao dentifrício convencional (1100 ppm F). Ainda, quando o sal está associado a dentifrícios convencionais, da Camara et al. [2015], em um estudo *in situ*, associaram 1,0% de HMP em dentifrícios com 1100 ppm F, mostrando que essa associação reduziu em

superioridade a perda mineral (%SH e ΔKHN) em relação ao dentifrício 1100 ppm F, aumentou a concentração de Ca no esmalte e biofilme, bem como reduziu a produção de EPS.

Além dos fosfatos inorgânicos (TMP e HMP), os fosfatos orgânicos, como por exemplo o glicerofosfato de cálcio (CaGP), também vem sendo amplamente estudado. Segundo a literatura o CaGP apresenta uma ação protetora contra a cárie dentária [Winter et al., 1989]. Fedorov [1965] avaliou a adição de 2,5%CaGP em um dentifrício, o qual foi aplicado topicamente, observando uma redução nos incrementos de cárie em 48% em comparação a 15% de um dentifrício contendo 450 ppm F. Vários mecanismos são propostos para as propriedades cariostáticas do CaGP, estes incluem interação direta com esmalte, tamponamento do pH da placa, modificação do metabolismo e elevação dos níveis de Ca e/ou P na placa [Whitford, 2005; Lynch & ten Cate, 2006]. Desta forma, este fosfato age atuando no esmalte, reduzindo sua solubilidade, como demostrado por Grenby & Bull [1975].

Lynch & ten Cate [2006] mostraram uma diminuição na desmineralização do esmalte principalmente quando o CaGP foi aplicado antes do desafio cariogênico, nas concentrações de 0,10, 0,25 e 0,50% sem a presença de F. Tenuta et al. [2009] associaram 1500 MFP + 0,13% CaGP e não observaram efeito sobre a redução da desmineralização do esmalte. Isso pode ter ocorrido devido ao fato de que o MFP e o CaGP são fosfatos e apresentam afinidade pela HA, competindo entre si. do Amaral et al. [2013], em um estudo *in situ*, observaram que a associação de 500 ppm F associado à 0,25% CaGP mostrou eficácia semelhante a um dentifrício 1100 ppm F em reduzir a desmineralização do esmalte bovino, sendo o mesmo achados encontrados nos estudos de Zaze et al. [2014b], mas sobre a remineralização de lesões iniciais de cárie. Além disso, os autores notaram um aumento da concentração de íons de F, Ca e P tanto no esmalte como no biofilme [do Amaral et al., 2013; Zaze et al., 2014a; Zaze et al., 2014b], fato que pode estar ligado à concentração de F utilizado ou a adição de CaGP que disponibiliza Ca e P para o meio oral. Freire et al. [2016] em um estudo clínico, avaliaram dentifrícios de concentração reduzida de F (500 ppm F) associado ao CaGP (0,25%CaGP) na progressão de cárie dentária em dentição decídua, observando resultados similares ao dentifrício 1100 ppm F.

Além da adição de fosfatos, outra possibilidade de aumentar sua ação remineralizante ou o efeito inibidor da desmineralização seria utilizá-lo na forma de nanopartículas. O prefixo “nano” está relacionado a uma escala de medida em que um nanômetro representa um bilionésimo do metro ou um milionésimo do milímetro [Bayda,

2019]. Estruturas nessa escala apresentam propriedades funcionais únicas não encontradas na escala macro [Chau et al., 2007]. As nanopartículas apresentam propriedades físico-químicas únicas, como tamanho ultrapequeno, grande área de superfície em relação à massa e alta reatividade química [Oberdörster et al., 2005]. O desempenho de materiais nanométricos está relacionado a algumas propriedades termodinâmicas e cinéticas presentes apenas nas nanopartículas. Devido a essas propriedades, a nanotecnologia expandiu-se rapidamente em todas as áreas da ciência, uma vez que oferece formas alternativas significativas de resolver questões e problemas científicos e médicos [Abou Neel et al., 2015]. Na área odontológica, a ação de compósitos produzidos por meio de trituração de compostos de Ca e P junto com outros componentes em moinho de bolas, adicionadas a formulações dentifrícias sobre a remineralização *in vitro* do esmalte de dente bovino foi investigada, e os resultados se mostraram promissores [Karlinsey et al., 2009; Danelon et al., 2019]. Ainda, a adição de nanopartículas de fosfato em dentifrícios fluoretados reduziu o processo de desmineralização em esmalte em comparação a um dentifrício convencional [Dalpasquale et al., 2017]. Danelon et al. [2015 e 2017] avaliaram a ação de nanopartículas de TMP, sobre a remineralização de lesões iniciais de cárie e desmineralização em um estudo *in situ* e *in vitro*, observando que a associação de 3%TMP à um dentifrício 1100 ppm F aumentou em 20% a remineralização da superfície do esmalte, e reduziu em 43% o corpo da lesão. Emerenciano et al. [2018] demonstraram *in situ*, que a suplementação com 3%TMP nanopaticulado (TMPnano) a um dentifrício convencional (1100 ppm F) promoveu um maior efeito protetor contra a desmineralização do esmalte e afetou significativamente a composição do biofilme formado *in situ* quando comparado ao dentifrício 1100F.

Diante do exposto e da ausência de estudos avaliando a associação de diferentes concentrações de β-CaGP nas formas micrométricas e nanoparticuladas, bem como as associações com o F na prevenção da desmineralização dentária e remineralização, é notável a importância de avaliar quais as alterações que o uso de isômeros pode provocar na estrutura do esmalte.

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Objetivo Geral

Avaliar o efeito de diferentes concentrações de isômeros β de Glicerofosfato de cálcio (CaGP) microparticulado e nanoparticulado adicionados a dentifrícios convencionais (1100 ppm F) sobre o processo de desmineralização do esmalte *in vitro* e remineralização de lesões iniciais de cárie *in situ*.

Artigo 1

2.0 Effect of nano-sized β-calcium glycerophosphate supplementation in conventional toothpaste on enamel demineralization: An *in vitro* study

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Running Title: Effect of nano-sized β-CaGP in enamel demineralization

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Keywords: Enamel, Fluoride, Phosphate, Enamel caries, Toothpastes.

***Artigo formatado de acordo com as instruções aos autores do periódico Caries Research.**

2.1 Abstract

This study evaluated the effects of supplementing toothpastes containing 1100 ppm F with micrometric or nano-sized β-calcium glycerophosphate (β-CaGPm/β-CaGPn) on enamel demineralization *in vitro*, using a pH cycling model. Bovine enamel blocks (4 mm × 4 mm, n = 120) selected using initial surface hardness (SHi) were randomly allocated to ten toothpaste groups (n = 12): without fluoride/β-CaGPm/β-CaGPn (Placebo), 1100 ppm F (1100F), and 1100 ppm F plus 0.125%, 0.25%, 0.5%, and 1.0% of β-CaGPm or β-CaGPn. Blocks were treated two times per day with toothpaste slurry and subjected to five pH cycles (demineralizing/remineralizing solutions) at 37 °C. The final surface hardness (SHf), percentage of surface hardness loss (%SH), integrated loss of subsurface hardness (ΔKHN), and profile analysis and lesion depth subsurface were analyzed using polarized light microscopy (PLM). Fluoride (F), calcium (Ca), and phosphorus (P) concentrations were also evaluated. Data were analyzed using ANOVA and Student-Newman-Keuls tests ($p < 0.001$). Blocks treated with toothpaste containing 1100F and 0.5% β-CaGPm or 0.25% β-CaGPn presented with reduced %SH values when compared to those treated with 1100F ($p < 0.001$). Reduced lesion depths (ΔKHN and PLM) were also observed for the slurry made up of 1100F and 0.25% β-CaGPn ($p < 0.001$). The addition of β-CaGPm and βCaGPn did not influence the enamel F concentration, with the 1100F-0.25% β-CaGPn group exhibiting the highest Ca and P enamel concentrations ($p < 0.001$). Our results indicate that toothpastes supplemented with 0.25% β-CaGPn produce a greater protective effect, reducing enamel demineralization compared to that by conventional toothpastes (1100 ppm F) and may be an effective alternative to improve the oral health of individuals, especially those at high risk for dental caries.

Keywords: Dental enamel, Fluoride, Phosphate, Demineralization.

2.2 Introduction

Dental caries is a multifactorial disease impacted by biological, behavioral, psychosocial, and environmental factors [Pitts et al., 2017; Machiulskiene et al., 2020]. Biological factors involved in dental caries are modulated by the presence of a biofilm on the surface of the teeth, with this biofilm being closely linked to high sucrose diets [Sheiham, and James, 2015]. Recently there has been a significant reduction in dental caries incidence; this has been attributed to the use of fluoride-enriched topical agents, including fluoride toothpastes, which are the major nonprofessional intervention used for the prevention of dental caries [Walsh et al., 2019]. However, dental caries still affects approximately 600 million preschoolers worldwide, impacting their quality of life and that of their families [Pitts et al., 2017]. Therefore, studies designed to develop new strategies to increase the effectiveness of fluoride toothpastes and enhance their anticaries effect are widely supported. These studies have focused on calcium (Ca) and phosphate (P) supplementation, because when the dental biofilm is supersaturated with these elements, F-mediated protection is more effective during cariogenic challenge, decreasing the dissolution of the enamel substrate [Souza et al., 2016].

Formulations containing calcium glycerophosphate (CaGP) are an effective alternative for F-enriched toothpastes, as they have demonstrated some protective function mediated by de/remineralization [do Amaral, 2013; Zaze et al., 2014a; Zaze et al., 2014b]. CaGP is an organic source of calcium and phosphate and can be found in three isomeric forms: d (+)- and l (-)- α-glycerophosphate and β-calcium glycerophosphate. Some of the cariostatic properties of CaGP may be explained by various factors including its interaction with the enamel, buffering of the pH of the biofilm, modifying bacterial metabolism and increasing Ca/P levels in the biofilm [Whitford et al., 2005; Lynch, and ten Cate, 2006; Tenuta et al., 2009]. An *in situ* study evaluated the effect of addition of micrometric CaGP at a concentration of 0.25% (50% α-calcium glycerophosphate and 50% β-calcium glycerophosphate) in toothpaste containing 500 ppm F, supplemented as sodium fluoride (NaF) and sodium monofluorophosphate (SMFP), on the remineralization of initial caries. The presence of CaGP in the toothpaste formulation (NaF and SMFP) was shown to correlate with improvements in remineralization when compared with the 500 ppm F toothpaste, including demonstrable improvements in the surface (20% higher) and cross-sectional (60% higher) hardness of treated samples [Zaze et al., 2014a]. Freire et al. [2016], used an 18-month-randomized clinical trial to confirm that this same formulation exerts a similar anticaries effect to toothpaste supplemented with 1100 ppm F.

Another strategy aimed at increasing the effects of fluoride toothpastes on caries control includes the use of phosphates in the form of nano-sized [Danelon et al., 2017; Emerenciano et

al., 2018]. Active agents embedded in nano-sized particles exhibit greater area and surface chemistry reactivity when applied to the enamel [Danelon et al., 2017; Garcia et al., 2019]. Danelon et al. [2015] and Garcia et al. [2019] evaluated the *in situ* effects of nano-sized inorganic phosphates on dental enamel re and demineralization. The authors concluded that fluoride toothpastes (1100 ppm F) supplemented with nano-sized sodium Trimetaphosphate (TMP) and sodium hexametaphosphate (HMP) were more efficient in promoting remineralization and reducing dental demineralization than the 1100 ppm F toothpastes.

Given this and the absence of studies evaluating the effects of different concentrations of β-CaGP in toothpaste, the aim of this study was to evaluate the effect of 1100 ppm F toothpastes supplemented with micrometric or nano-sized β-calcium glycerophosphate (β-CaGPm/β-CaGPn) on enamel demineralization *in vitro*, using a pH cycling model. The null hypothesis of the study was that supplementing conventional fluoride toothpastes (1100 ppm F) with β-CaGPm or β-CaGPn would not have an additional protective effect on the enamel demineralization process when compared to the 1100 ppm F toothpaste.

2.3 Materials and methods

Experimental design

Enamel blocks (4 mm × 4 mm, n = 120) were obtained from bovine incisors, and the enamel surfaces were polished (outer enamel removed ~120 μm), evaluated for initial surface hardness (SHi; 320.0–380.0 KHN), and randomly divided into ten experimental toothpaste groups (n = 12 each) (without fluoride/β-CaGPm/β-CaGPn (Placebo), 1100 ppm F (1100F), 1100 ppm F plus 0.125%; 0.25%; 0.5% and 1.0% of β-CaGPm or β-CaGPn) based on 95% confidence interval values. The blocks were treated two times per day (2×/day) with the appropriate toothpaste slurry and subjected to five pH cycles (demineralizing/remineralizing solutions) at 37 °C. Next, pH cycling, the percentage of surface hardness loss (%SH), integrated loss of subsurface hardness (ΔKHN), profile analysis and lesion depth subsurface evaluations were conducted using polarized light microscopy (PLM). Enamel fluoride (F), calcium (Ca), and phosphorus (P) concentrations were also evaluated.

Processing and characterization of nano-sized β-CaGP

Nano-sized β-CaGP (β-CaGPn) was processed using the methods described by Danelon et al. [2015 and 2017]. β-CaGPn was obtained from commercial CaGPm (80% β-isomer and 20% rac-α-isomer, CAS 58409-70-4, Sigma Chemical Co, St Louis, Missouri, USA) by ball-milling this compound with 500 g of 2 mm zirconia spheres. Commercial material (70 g) was then added to a polyethylene bottle containing sintered zirconia spheres (5 mm in diameter) in isopropyl alcohol (Merck KGaA, Darmstadt; Deutschland, Germany) at one third of its volume. The bottles were sealed and subjected to milling for 48 h in a ball mill and then filtered and sealed with aluminum foil. The vials were dried at 85 °C to evaporate the isopropyl alcohol (Merck KGaA, Darmstadt, Germany) and X-ray diffraction (XRD) was used to identify the crystalline structures and estimate the crystallographic coherency domains of the CaGP (β-CaGPn). X-ray diffractograms were obtained from samples in powder form using a Shimadzu XRD 6000 using a CuK radiation source (λ = 1.54056 Å) at a voltage of 30 kV and a current of 30 mA. Measurements were made continuously in the range of $10° \leq 2\theta \leq 80°$ with a 2° sweep speed. The structural identification of the samples was carried out by comparing the diffraction patterns obtained from our analysis with tabulated patterns available in various databases and reported in the BJoint Committee on Powder Diffraction Standards—Powder Diffraction File (JCPDS—PDF). The particle morphologies of the β-CaGPm and β-CaGPn suspensions were analyzed using SEM. The SEM images were collected using an MEV-PHILIPS (XL-30 FEG, Philips, Amsterdam, The Netherlands). To determine the nanoparticles size, the high-resolution trasmission electron microscopy (HRTEM) was performed, using a FEI TECNAI F20 microscope operating at 200 kV; for this, the samples were dripped on copper grids of 400 mesh (PELCO®), and the solvent evaporated at room temperature. After, histograms were constructed using the public domain ImageJ image processing software (version 1.50, National Institute of Health, Bethesda, MD, USA).

Assessment of the fluoride content and pH of toothpaste formulations

Toothpastes were produced using the following components: titanium dioxide, carboxymethyl cellulose, methyl p-hydroxybenzoate sodium, saccharin, mint oil, glycerin, abrasive silica, sodium lauryl sulfate, and deionized water. Toothpastes containing β-CaGPm and β-CaGPn (80% β-isomer and 3 20% rac-α-isomer, CAS 58409-70-4, Sigma Chemical Co, St Louis, Missouri, USA) were prepared using concentrations of 0.125%, 0.25%, 0.5%, and 1.0% and supplemented with F at a concentration of 1100 ppm in the form of NaF (Merck, CAS 7681-49-4, Germany). A toothpaste without F/β-CaGPm/β-CaGPn (Placebo) and a toothpaste with

1100 ppm F were also prepared as controls. The toothpastes used in this study were stored at room temperature and kept properly sealed to prevent any composition changes. Total and ionic fluoride concentrations were determined using a fluoride-specific electrode (Orion 9609-BN; Orion Research Inc., Beverly, MA, USA) connected to an ion analyzer (Orion 720 A^{+}; Orion Research Inc.) [Delbem et al., 2009]. Three toothpastes per group were analyzed, and the data are presented in micrograms of fluoride per gram of toothpaste. The pH level of each toothpaste was determined using a pH electrode (2A09E, Analyzer, São Paulo, Brazil) calibrated using solutions with pH 7.0 and 4.0 [Danelon et al., 2019].

Toothpaste treatment and pH cycling

Each of the enamel blocks was subjected to five pH cycles over a 7-day period at a constant temperature of 37 °C [Vieira et al., 2005]. These blocks were immersed, under constant agitation, 2×/day, for 1 min, in toothpaste suspensions in deionized water (1: 3-weight: weight); then, they were placed in a demineralizing solution (DE) for 6 hours (Ca and P 2.0 mmol L^{-1} in acetate buffer 0.075 mol L^{-1}, 0.04 μg F/mL at pH 4.7 - 2.2 mL/mm^2) and then in a remineralizing solution (RE) for 18 hours (Ca 1.5 mmol L^{-1}, P 0.9 mmol L^{-1}, 0.15 mol L^{-1} KCl in 0.02 mol L^{-1} sodium cacodylate buffer, 0.05 μg F/mL at pH 7.0 - 1.1 mL/mm^2) for each cycle. Deionized water rinses were performed between each step, and the blocks were placed in a fresh remineralizing solution for the final 2 days.

Analysis of enamel hardness

Surface hardness was determined using a Micromet 5114 hardness tester (Buehler, Lake Bluff, USA) and Buehler Omni Met software (Buehler, Lake Bluff, USA), with a Knoop diamond indenter under a 25 g load for 10 s. Five indentations spaced 100 μm apart were produced in the center of each of the enamel blocks (SHi) and then, after pH cycling, five more indentations at a distance of 100 μm from the impressions of SHi were created to allow for the analysis of the final hardness (SHf) of each block. The %SH (%SH = [(SHf-SHi)/SHi] *100) was then calculated. Blocks were halved, with one half being immersed in acrylic resin and gradually polished until the enamel was totally exposed. Fourteen indentations at different distances (5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220, and 330 μm) from the external enamel surface were then added to the central area of the blocks, and integrated hardness (KHN × μm) was calculated using 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 of the enamel, which is described in the results as the integrated loss of subsurface hardness (ΔKHN; KHN x μm) [Emerenciano et al., 2018].

Analysis of the profile and depth of subsurface lesions using polarized light microscopy

After determining the integrated loss of subsurface hardness (ΔKHN), the bases containing the enamel blocks were first sectioned to obtain slices of 300 μm; these slices were then worn to a thickness of ~ 100 μm. The initial wear of the slices was performed using 400 grit paper (Paper Discs CARBIMET, 30-5108-320, Buehler) in a polishing BETA - grinder polisher (Buehler, Lake Bluff, Illinois, USA) under constant refrigeration. Then the enamel slices were manually polished using 600, 800, and 1200 grit sandpaper. Enamel sections were mounted on slides with deionized water and covered with a cover glass, the edges of which were sealed with synthetic resin (Entellan, Merck, Darmstadt, Germany) to allow for the evaluation of any subsurface lesions. In addition, three areas in the central region of the slices were analyzed to determine the presence and thickness (micrometer) of the enamel surface layer (PLM superficial) and the demineralization depth (PLM depth) [Danelon et al., 2014; Medeiros et al., 2018]. The sections were examined using PLM (AxioPhot, Zeiss, Oberkochen, Germany) at ×40 magnification.

Analysis of F, Ca, and P concentrations in the enamel

The other half of the longitudinally sectioned blocks was sectioned again to obtain 2 mm × 2 mm blocks. One of these blocks was fixed with adhesive glue to a mandrel with a straight piece and an enamel layer (~ 50 μm) removed by micro abrasion [Weatherell et al., 1985]. A modified microscope base with a manometer (Pantec, São Paulo, Brazil) and self-adhesive sanding disc (13 mm in diameter, 400 grit-silicon-carbide, Buelher) in a crystal polystyrene flask (J- 10, Injeplast, Brazil). We then added 0.5 ml HCl 1.0 mol L^{-1} to the enamel powder and kept the flasks kept under constant stirring for 1 hour. For F analysis, a specific electrode 9409BN (Thermo Scientific, Beverly, USA) and microelectrode reference (Analyzer, São Paulo, Brazil) coupled to an ion analyzer (Orion 720A^{+}, Thermo Scientific, Beverly, USA) were utilized. The electrodes were calibrated using standards ranging from 0.25 to 4.00 ppm F (100 ppm F, Orion 940,907) the same conditions as the samples. Readings were conducted using 0.250 mL of the biopsy solution supplemented with the same volume of TISAB II modified NaOH [Akabane et al., 2018]. The data were obtained in mV and were converted to μg/mm^3 using an Excel spreadsheet. Ca analysis was performed using the Arsenazo III colorimetric method [Vogel and Chow, 1983]. Absorbance readings were recorded at 650 nm with a plate reader (PowerWave 340, Biotek, Winooski, USA) while P was measured using the Fiske and Subbarow [1925] method, with absorbance readings recorded at 660 nm. The results of these assays are expressed in μg/mm^3.

Statistical analysis

The statistical program Sigmaplot® version 12.0 (version 12.0, Systat Software Inc., San Jose, CA, USA) was used to evaluate all data, and a significance level of 5% was used in all analyses. The %SH, ΔKHN, PLM, F, Ca, and P values for each enamel sample were treated as variables, and the treatment type was the variation factor. The data had a normal (Shapiro-Wilk test) homogeneous distribution (Cochran test), allowing us to use the ANOVA and Student-Newman-Keuls tests for all evaluations.

2.4 Results

The milling process reduced the particle size of the β-CaGPn powder without affecting the crystalline structure of the material (Figure 1). The size of the β-CaGPn particles was confirmed following 48 h of grinding using SEM, as shown in Figure 1. The average size of the particles was observed to be 220.0 nm.

The mean concentration (SD) of total (FT) and ionic F (FI) (n = 3) for the groups were determined to be as follows: Placebo - 24.4 (5.0) and 17.0 (0.4); 1100F - 1168.8 (10.8) and 1061.0 (25.2); 1100F-0.125%β-CaGPm - 1202.5 (12.7) and 1149.3 (12.2); 1100F-0.25%β-CaGPm - 1178.4 (10.1) and 1176.0 (8.3); 1100F-0.5%β-CaGPm - 1005.5 (9.5) and 1004.9 (11.1); 1100F-1.0%β-CaGPm - 1137.7 (9.4) and 1058.1 (13.4); 1100F-0.125%β-CaGPn - 1050.7 (11.1) and 1056.0 (10.4); 1100F-0.25%β-CaGPn - 1070.2 (12.3) and 1041.8 (9.4); 1100F-0.5%β-CaGPn - 1110.0 (14.4) and 1071.8 (9.4); and 1100F-1.0%β-CaGPn - 1127.6 (8.2) and 1048.4 (9.6), respectively. The mean pH value (SD) of each of the experimental toothpastes was 7.4 (1), the pH values ranged from 7.3 to 7.6.

The mean (SD) SHi for the blocks was 368.4 (0.2) KHN, and no significant differences in SHi were observed between the groups after random allocation ($p = 0.104$). Treatment with toothpastes containing 1100F and 0.5%β-CaGPm or 0.25%β-CaGPn showed reduced %SH values compared to that with the 1100F control ($p < 0.001$), and the ΔKHN value for 1100F-0.25% β-CaGPn was ~ 43% and ~ 10% lower than those for 1100F and 1100F-0.5%β-CaGPm, respectively ($p < 0.001$) (Table 1 and Figure 2).

β-CaGPm and β-CaGPn supplementation did not influence the concentration of F in the enamel, making its effects similar to that of 1100F alone; only the Placebo group demonstrated a significant reduction in F content ($p < 0.001$). Treatment with 1100F-0.5%β-CaGPm and 1100F-0.25% β-CaGPn increased the enamel concentration of Ca by ~ 21% and ~ 54% compared to treatment with 1100F ($p < 0.001$), while the P content of the enamel in the 1100F-0.25% β-

CaGPn group was higher than that in the other treatment groups ($p < 0.001$) and ~ 29% higher than that for the 1100F control ($p < 0.001$) (Table 2).

2.5 Discussion/Conclusion

Several therapies designed to control dental caries have been proposed in the literature; amongst these, the use of F in association with Ca and P ions stands out, as the presence of these ions in the salivary and biofilm environments positively interferes with the processes of remineralization and demineralization [Cochrane and Reynolds, 2012; Rechmann et al., 2018; Garcia et al., 2019]. The results showed that β-CaGPn added at a concentration of 0.25% confers superior protection of dental enamel when compared with that provided by a conventional fluoride toothpaste, leading to the rejection of the null hypothesis.

Agents containing Ca and P, such as β-calcium glycerophosphate (β-CaGP), have been shown to provide protective effects against enamel demineralization [Grenby and Bull, 1975; do Amaral et al., 2013; Nagata et al., 2017]. This may be the result of various mechanisms of action, including synergic interactions with F resulting in increased resistance of hydroxyapatite during acid challenge, pH buffering of the biofilm covering tooth enamel, elevation of the Ca and P levels in this biofilm [Lynch, 2004; do Amaral et al., 2013], and varied functions altering the demineralization process [Lynch, 2004; Zaze et al., 2014b]. Additionally, the addition of micrometric CaGP to topical fluoridated products, especially toothpastes, has been shown to significantly improve their effect against enamel demineralization [do Amaral et al., 2013; Nagata et al., 2017]. For toothpastes with a low concentration of F (500 ppm F), CaGP in concentrations of up to 0.25% increase their ability to reduce enamel demineralization as demonstrated by the Zaze et al. [2014b] study. In this study, toothpastes containing 1100F and supplemented with 0.5% β-CaGPm or 0.25% β-CaGPn reduced %SH compared to conventional toothpaste (1100F) with the ΔKHN for 1100F-0.25%β-CaGPn being reduced by ~ 43% compared to 1100F and ~ 10% compared to 1100F-0.5%β-CaGPm (Table 1 and Figure 2). The link between the Ca and P groups of the β-CaGP structure is considered to be the most unstable part of the molecule [Inoue, 1992], suggesting that this instability favors the release of Ca, leaving the anionic phosphate groups free. This explains the increase in Ca levels in the treated samples described in this and other studies [do Amaral et al., 2013; Zaze et al., 2014b]. The β-CaGP layer adsorbs to the enamel and functions as a source of calcium, favoring the reduction of mineral loss in the caries processes, as observed in both *in vitro* [Zaze et al., 2014b] and *in situ* studies [do Amaral et al., 2013; Zaze et al., 2014a]. Moreover, the bond between glycerol and phosphate is the most stable part of the molecule [Inoue et al., 1992]; thus, CaGP does not donate phosphate

ions to the medium [Sidi and Wilson, 1991]. Therefore, the increase in the P content in the enamel may be related to the greater availability of Ca in the medium, the CaGP adsorption capacity of the enamel and the interaction of the enamel with F when applied at an appropriate molar ratio. This reduces the mineral loss and, consequently, there is a greater presence of Ca and P in the enamel, as observed in the 1100F-0.25%β-CaGPn treatment group and not in the 1100F samples. In addition to the above-mentioned mechanisms, another factor that can interfere with the properties of the active agent is its dimensions; the use of nano-sized phosphate has emerged as an innovative method to optimize the effect of fluoride toothpastes in the control of dental caries.

Nano-sized particles have unique physicochemical properties, including an ultra-small controllable size, large surface area in relation to mass, high reactivity, and a functionalizable structure. There is a consensus that nano-sized materials exhibit different and enhanced properties from those of their micro-sized counterparts. The performance of nano-sized materials is related to some thermodynamic and kinetic properties only possessed by nanoparticles. For this reason, a direct comparison of these two materials in terms of size is not accurate. For instance, toothpastes formulated with the same amount (in mass) of micro - and nano-sized particles can show quite different rheological properties that affect their general performance, which obviously cannot be analyzed exclusively in terms of particle size. In fact, microparticles cannot be used as a reliable reference material for nano-sized particles. In the case of nano-sized β-CaGP, there are only two mechanisms that can recrystallize nanoparticles into bigger particles: i) Ostwald ripening and ii) oriented attachment. The first occurs only in saturated solutions, and the second requires appropriate kinetic conditions. These two requirements are not fulfilled by toothpastes, which thus guarantee the size stability of the nanoparticles. Moreover, nano-sized particles exhibit extra colloidal stability that is not present in microparticles, which also contributes to their size stability over time. This colloidal nature of nano-sized materials is also responsible for their chemical activity and homogeneous distribution in toothpaste media.

XRD patterns of the β-CaGPn samples demonstrated that the material was still crystalline after processing in a ball mill for 48 h (Figure 1). Peak broadening resulted from the size effect and was evaluated using the Scherrer equation; however, the crystalline structure was preserved. On the contrary, it is well known that particle size affects the solubility and dissolution kinetics of nano-sized materials. Although the presence of water in toothpaste can promote dissolution, it affects nano-sized particles and microparticles in different ways, thereby enhancing the nano-size effect. This means that the velocity of dissolution is different for β-CaGPm and β-CaGPn, which in turn should affect the amount of active phosphate available and therefore, improve the effectiveness of the supplemented toothpaste. It is of fundamental importance to point out that

toothpaste is not a system under thermodynamic equilibrium, and, for this reason, aspects of kinetics affect the reactivity of β-CaGPm and β-CaGPn. When the size of β-CaGP is reduced to the nano-scale, its contact area is significantly increased, profoundly affecting its physical and mechanical properties, including surface area and intensity of chemical reactivity with enamel. Thus, the procedure used to synthesize the β-CaGPn promotes more reactive particles with higher enamel adsorption through the reduction in size and increase in surface area (in proportion to its volume), which leads to a larger number of particles on the surface, increasing reactivity, as evidenced by Danelon et al. [2015, 2017]. However, reduction in particle size does not necessarily contribute to improved protection against demineralization. Here we demonstrate that 0.25%β-CaGPn is more effective than 0.125%β-CaGPn and 1.0%β-CaGPn. Thus, illustrating the importance of establishing a correct molar ratio between micro/nano CaGP and F, since inadequate CaGP: F ratios result in different reactions with the target tooth enamel. This was validated in the study completed by Zaze et al. [2014a]. It is also important to note that the pH cycling model used to evaluate the anticaries effect of the experimental toothpastes in this *in vitro* study produce more subsurface lesions (Figure 2), with a higher diameter and greater number of pores. This type of lesion has greater superficial enamel permeability, which facilitates ionic changes. When these pores are obliterated, exchanges cannot occur, leading to a reduction in the remineralization of demineralized areas with depth [Lynch et al., 2007]. Here we found that, at concentrations above 0.5% for β-CaGPm and 0.25% for β-CaGPn, there was no improvement in mineral gain; this was also observed in the Zaze et al. [2014a] study.

It is known that maintaining the solubility of F in toothpaste formulations can be difficult. This is primarily due to the undesirable reactions between Ca, F, and P [Shen et al., 2018]. Ca reacts with F, forming poorly soluble phases reducing the bioavailability of both ions. Therefore, toothpastes containing a soluble calcium source are formulated in combination with sodium monofluorophosphate [Tenuta et al., 2009]. However, the study by do Amaral et al. [2013] demonstrated that toothpaste formulations using 500 ppm F (NaF) + 0.25% CaGP resulted in increased F, Ca, and P concentrations within the enamel and biofilms, similar to those described for 1100F (NaF) toothpaste. In a study by Zaze et al. [2014b], which evaluated the same toothpaste as that evaluated by the authors mentioned above, it was observed that 0.25% CaGP, when associated with toothpastes containing NaF and SMFP at a concentration of 500 ppm F, resulted in similar Ca and F enamel concentrations to those for samples treated with 1100F. Then, a randomized clinical trial with an 18-month follow-up confirmed these *in vitro* findings. Toothpastes containing 500 ppm F (NaF) + 0.25%CaGP were shown to exert a similar anticaries effect to 1100F (NaF) toothpaste [Freire et al., 2016]. This suggests that NaF and CaGP form a

novel complex F-CaGP which is responsible for the previously reported protective effects (*in vitro*, *in situ*, and *in vivo*). Therefore, another advantage of using nano-sized β-CaGP is the addition of less CaGP, which facilitates improved solubility and thus ease of manipulation for β-CaGP. Even assuming that there is some interaction between F and CaGP during the 15 min need to prepare the toothpaste suspensions, this did not interfere with the uptake of fluoride into the enamel, regardless of the amount of β-CaGP added (Table 2). Increased β-CaGP content did not lead to a greater presence of calcium and phosphate in the enamel. Thus, even with the possibility of reduced fluoride solubility in the presence of CaGP, the creation of the F-β-CaGP complex and its adsorption to the enamel is responsible for reducing demineralization and the depth of the enamel lesions (Table 1, Figure 2). As a result, we are able to definitively characterize the F/β-CaGP ratio as a critical factor in the development of functional products. Moreover, conventional toothpaste formulations (without the addition of Ca and P) have a greater capacity to interact with the surface layers of the enamel, leading to the formation of CaF_2 with smaller representation in areas of the subsurface, as evidenced by the data (ΔKHN and PLM depth) for the 1100F group when compared to 0.5% β-CaGPm and 0.25% β-CaGPn. Thus, it is possible that most F present in enamel treated with 1100F is restricted to the enamel surface.

Although it is known that pH cycling models are suitable for assessing the impact of new active agents in fluoridated toothpastes as well as their association with other anticaries treatments, *in vitro* protocols have limitations. They do not simulate all the complexities of an oral cariogenic environment *in vivo*, forcing researchers to disregard the impact of the saliva/biofilm on the enamel. Therefore, the results obtained in this study are only an initial evidence designed to support the further evaluation of the potential interaction mechanisms proposed here and evaluate their application in preventing dental caries. Some examples of points that future studies should focus on include: 1) activity in the presence of human saliva, 2) activity in the presence of a biofilm, which contributes to a state of supersaturation of both Ca and P ions and 3) the evaluation of the inorganic composition of saliva and biofilm fluids both *in vitro* and *in vivo*. Based on the findings of this *in vitro* study, we can conclude that the addition of β-CaGP to a 1100 ppm F toothpaste produces a synergistic effect, improving the inhibition of enamel demineralization. This effect was further enhanced by supplementation with nano-sized particles.

2.6 Acknowledgments

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2.7 Disclosure Statement

Only the author Marcelle Danelon, Alberto Carlos Botazzo Delbem, Juliano Pelim Pessan and Emerson Rodrigues de Camargo has a patent for a product used in the study, by the National Institute of Industrial Property - INPI/SP, on 06/16/2010 under number C1 0801811-1, and granted on April 11, 2017.

2.8 Author Contributions

Conceived and designed the experiments: MD, ACBD, ERC, ERC, FNSN, LFG and YTCSS. Performed the experiments: NGE, MD, FMCG, ERC, FNSN, FLG, and YTCSS. Analyzed the data: NGE, MD, ACBD and JPP. Wrote/revised the paper: NGE, MD, ACBD, FMCG, JPP, ERC, FNSN, LFG and YTCSS. For all the experiments performed there is no laboratory technician to support either of us. It is the authors who perform each of the study stages. This way we justify the number of authors.

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Figure legend

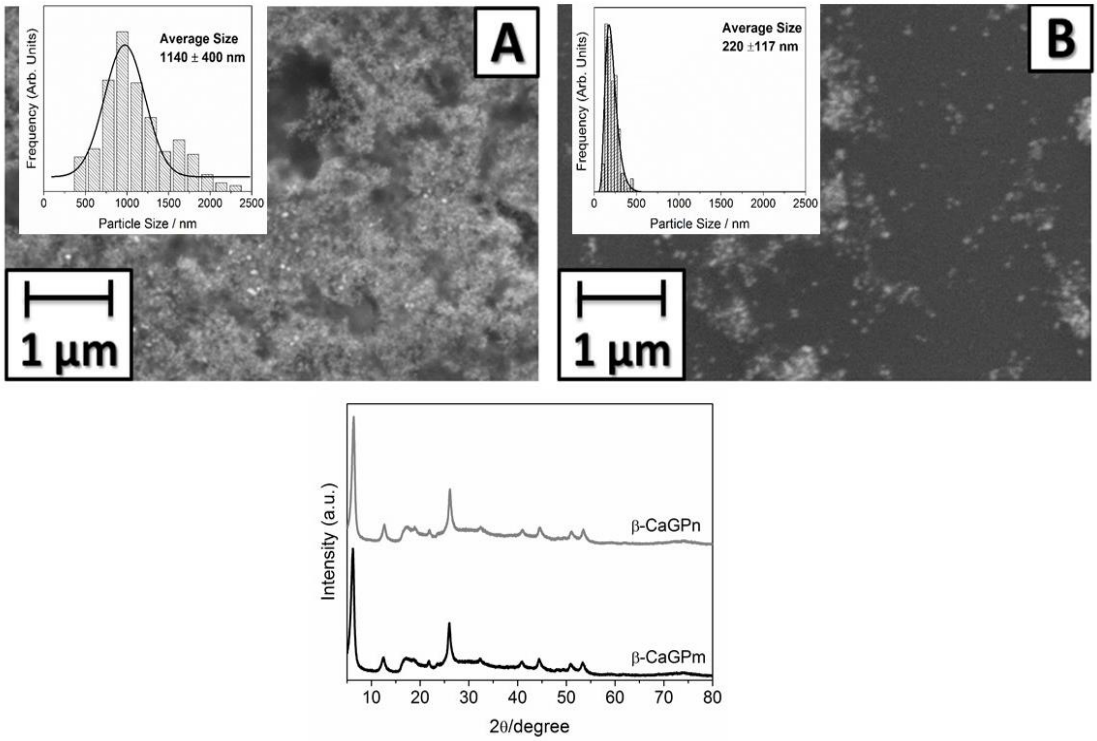
Figure 1: Scanning electron microscopy images and average size (histograms) and X-ray patterns of the (A) β-CaGPm and (B) β-CaGPn after milling for 48h. Magnification: 20,000 x.

Figure 2: Photomicrograph with polarized light of the lesion formed after treatment with (A) Placebo; (B) 1100F; (C) 1100F-0.125%β-CaGPm; (D) 1100F-0.25%β-CaGPm; (E) 1100F-0.5%β-CaGPm; (F) 1100F-1.0%β-CaGPm; (G) 1100F-0.125%β-CaGPn; (H) 1100F-0.25%β-CaGPn; (I) 1100F-0.5%β-CaGPn; (J) 1100F-1.0%β-CaGPn. (× 40).

Table legend

Table 1: Mean (SD) of the variables analyzed in the enamel according to the toothpastes

Table 2: Mean (SD) of the variables analyzed in the enamel according to the toothpastes



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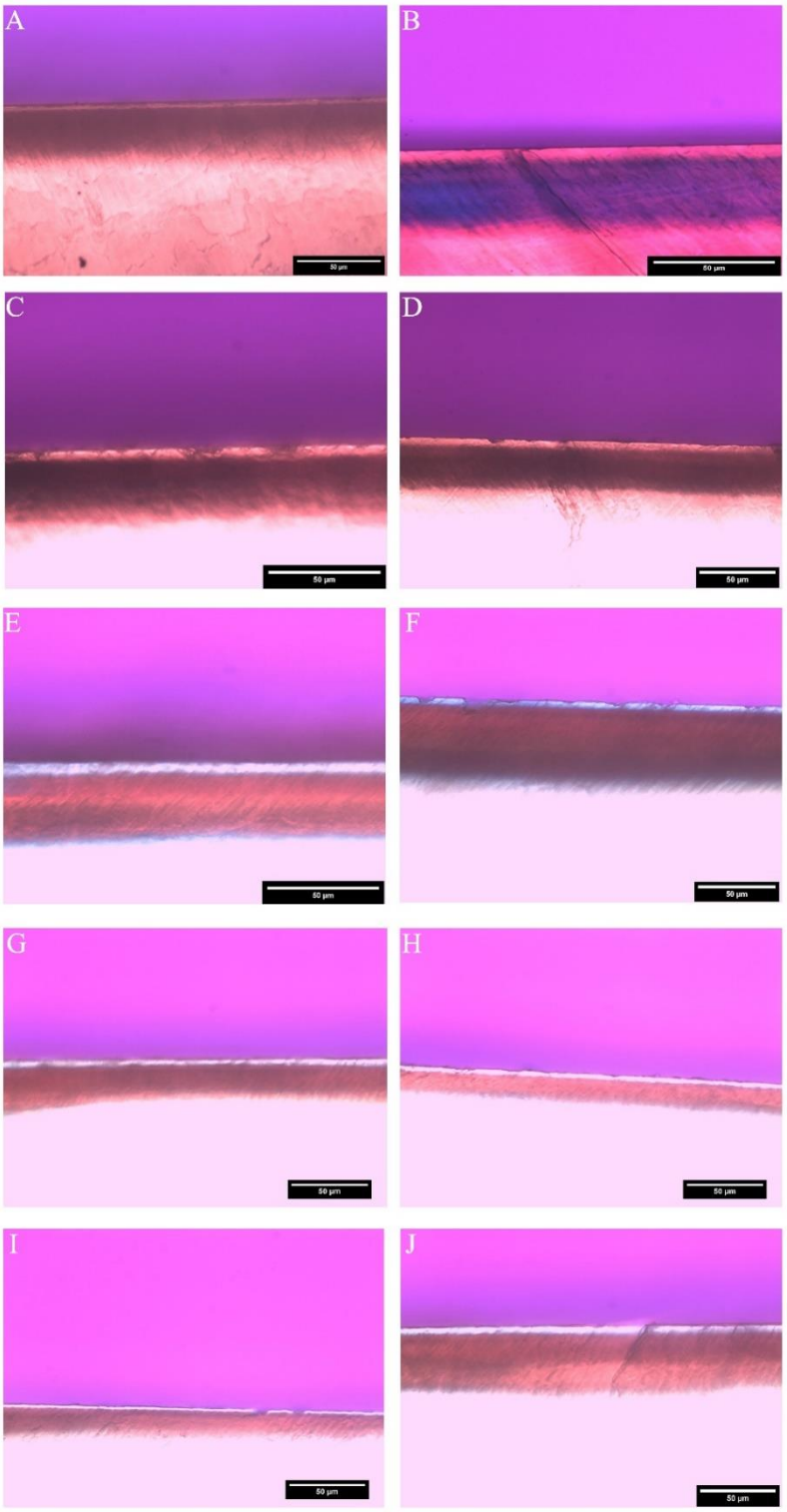

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Table 1: Mean (SD) of the variables analyzed in the enamel according to the toothpastes

Toothpastes	%SH (KHN)	ΔKHN (KHN × µm)	PLM Surface (µm)	PLM Depth (µm)
Placebo	-84.4[a] (5.6)	7,183.9[a] (1,167.5)	0.6[a] (0,3)	44.6[a] (3.6)
1100F	-44.6[b] (3.2)	4,545.4[b] (682.5)	4.9[b] (0.5)	22.4[b] (5.5)
1100F-0.125%β-CaGPm	-46.8[b] (4.7)	5,577.5[c] (504.9)	5.0[b] (0.3)	21.4[b] (4.6)
1100F-0.25%β-CaGPm	-46.0[b] (5.5)	5,508.7[c] (641.6)	5.7 [b] (1.0)	13.3[c] (2.2)
1100F-0.5%β-CaGPm	-31.4[c] (1.9)	2,893.7[d] (492.9)	6.9[c] (0.2)	9.5[d] (5.5)
1100F-1.0%β-CaGPm	-53.0[d] (4.9)	4,740.6[e,b] (635.6)	3.2[d] (1.1)	17.2[c] (5.5)
1100F-0.125%β-CaGPn	-53.7[e] (4.8)	5,337.2[c,e] (502.0)	3.4[d] (0.8)	17.7[c] (1.5)
1100F-0.25%β-CaGPn	-30.3[c] (4.0)	2,578.8[f] (372.1)	11.4[e] (1.4)	6.0[d] (1,7)
1100F-0.5%β-CaGPn	-58.2[d] (3.5)	5,027.3[b,c,e] (653.6)	1.9[f] (0.5)	17.6[c] (0.6)
1100F-1.0%β-CaGPn	-61.7[d] (4.8)	5,589.8[e] (975.1)	2.5[f] (0.7)	16.4[c] (1.1)

Distinct superscript letters indicate statistical significance among the treatment in each analysis (One-way ANOVA, followed Student–Newman–Keuls' test). Values represent means (standard deviations). %SH: percentage of surface hardness loss. ΔKHN: integrated subsurface hardness loss. PLM surface: thickness of the enamel surface layer. PLM depth: thickness of demineralization depth. KHN: Knoop hardness. µm: micrometers.

Table 2: Mean (SD) of F, Ca and P in enamel according to toothpastes

Toothpastes	F (µg/mm³)	Ca (µg/mm³)	P (µg/mm³)
Placebo	1.5[a] (0.2)	126.4[a] (21.0)	159.2[a] (32.2)
1100F	2.5[b] (0.5)	305.5[b] (64.0)	248.2[b] (45.1)
1100F-0.125%β-CaGPm	2.5[b] (1.0)	217.7[b,d] (76.9)	240.1[b] (56.9)
1100F-0.25%β-CaGPm	2.1[b] (0.6)	276.8[b,d] (85.2)	229.5[b,c] (46.5)
1100F-0.5%β-CaGPm	2.3[b] (0.5)	368.5[c] (70.4)	268.2[b] (50.6)
1100F-1.0%β-CaGPm	2.3[b] (0.7)	224.2[d] (60.5)	176.7[a,c] (50.8)
1100F-0.125%β-CaGPn	2.4[b] (0.8)	243.9[b,d] (46.4)	197.9[a,c] (47.4)
1100F-0.25%β-CaGPn	2.5[b] (0.8)	469.4[e] (78.2)	319.1[d] (86.1)
1100F-0.5%β-CaGPn	2.1[b] (0.5)	227.5[d] (63.4)	216.5[b,c] (54.9)
1100F-1.0%β-CaGPn	2.4[b] (0.6)	245.5[b,d] (49.6)	224.8[b,c] (48.9)

Distinct superscript letters indicate statistical significance among the treatment in each analysis (One-way ANOVA, followed Student–Newman–Keuls' test). Values represent means (standard deviations). F: fluoride in the enamel. Ca: calcium in the enamel. P: phosphorus in the enamel.

Artigo 2

3.0 Effect of the association of microparticles and nano-sizeds β-calcium glycerophosphate in conventional toothpaste on enamel remineralization: *in situ* study

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Short Title: Effect of nano-sized β-CaGP in enamel remineralization

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3.1 Abstract

The aim of this study was, therefore, to investigate, whether the addition of β-calcium glycerophosphate in micro (β-CaGPm) and nano-sized forms (β-CaGPn) to conventional toothpastes (1100 ppm F) could increase the remineralizing effect of initial caries lesions *in situ* when compared to fluoride alone. This double-blinded crossed study was performed in 4 phases of 3 days each. Twelve subjects used palatal appliances containing 4 bovine enamel blocks with artificial caries lesions. Volunteers were randomly assigned into the following toothpastes groups: no F-CaGPm-CaGPn (Placebo); 1,100 ppm F (1100F); 1100F plus 0.5% micrometric β-CaGP (1100F-0.5%β-CaGPm); and 1100F plus 0.25% nano-sized β-CaGP (1100F-0.25%β-CaGPn). Volunteers were instructed to brush their natural teeth with the palatal appliances in the mouth for 1 min (3 times/day), so that blocks were treated with natural slurries of toothpastes. After 3 days of the remineralization period, surface hardness post-remineralization was again applied, and integrated recovery of subsurface hardness (ΔIHR), fluoride (F), calcium (Ca) and phosphorus (P) concentration in enamel was also determined. Data were analyzed by 1-way, repeated-measures ANOVA, followed by the Student-Newman-Keuls test ($p < 0.001$). The treatment with 1100F-0.25%β-CaGPn, led to %SHR ~ 69% and ~ 40% higher when compared to 1100F and 1100F-0.5%β-CaGPm toothpastes groups ($p < 0.001$), respectively. In addition, the capacity to reduce the lesion body (ΔIHR) was ~ 40% and ~ 17% higher with 1100F-0.25%β-CaGPn and 1100F-0.5%β-CaGPm ($p < 0.001$) when compared to 1100F. The addition of β-CaGPm and β-CaGPn to the F toothpaste did not influence enamel F concentration, so its effect was similar to 1100F ($p > 0.001$). The treatment with 1100F-0.25%β-CaGPn promoted an increase in the concentration of Ca in the enamel by ~ 77% and ~ 28% when compared to the 1100F and 1100F-0.5%β-CaGPm group ($p < 0.001$). The treatment with 1100F-0.25%β-CaGPn promoted an increase in the concentration of P in the enamel by ~ 64% and ~ 28% when compared to the 1100F and 1100F-0.5%β-CaGPm groups ($p < 0.001$), respectively ($p < 0.001$). The addition of 0.25%β-CaGPn to a conventional toothpaste increased the bioavailability of calcium and phosphate promoting a significantly higher remineralizing effect when compared to 1100 ppm F toothpaste.

Keywords: Dental enamel, Fluoride, Phosphate, Remineralization.

3.2 Introduction

Fluoride has been used for many decades to prevent, reduce or reverse caries lesions [Walsh et al., 2019; Whelton et al., 2019]. Appropriate fluoride policies have radically improved the oral health of individuals. After insertion at population level of water and fluoride toothpastes there has been a remarkable reduction in caries in many industrialized countries [Whelton, 2019]. However, there is a concern about the percentage of children still affected by the disease [Pitts et al., 2019]. In view of this, research has been ceaselessly seeking alternatives that aim to enhance the effect of fluoridated products.

Fluoride ion promotes the remineralization of caries lesions from the formation of fluoridroxiaptatite [$Ca_{10}(PO_4)_6(OH)_{2-2x}F_{2x}$] [Buzalaf et al., 2011]. The formation of this mineral depends on the presence of calcium ions (Ca^{2+}) and phosphate (PO_4^{3-}) bioavailable in saliva [Dai et al., 2019]. The addition of calcium phosphate technologies, e.g.: tricalcium phosphate (TCP), calcium carbonate/dicalcium phosphate with arginine (Pro Argin), amorphous calcium phosphate technology (ACP), casein phosphopeptide-amorphous calcium phosphate nanocomplexes (CPP-ACP) to fluoride toothpastes significantly increases the ability to remineralize lesions of enamel subsurface to fluoride alone. Stabilized calcium phosphate produces higher levels of bioavailable calcium and inorganic phosphate, whenever fluoride has been associate occurs a higher level of remineralization [Shen et al., 2018].

In addition to inorganic calcium phosphates, calcium glycerophosphate (CaGP), an organic phosphate salt associated with fluoride, has been tested in several studies. According to the literature, CaGP presents a protective action against dental caries [Freire et al., 2016]. Due to the ability of CaGP to interact with dental tissues, it has been inserted in dental products, such as fluoride toothpaste and glass-ionomer cementer [Zaze, 2014a; Zaze et al., 2014b; Santos, 2019]. *In situ* [do Amaral, 2013] and *in vivo* studies [Freire et al.,, 2016] demonstrated effectiveness in reducing the progression of caries lesions when 0.25% of micrometric CaGP (50% α-calcium glycerophosphate and 50% β-calcium glycerophosphate) was associated with low fluoride toothpaste (500 ppm F).

On the other hand, it is known that the use of toothpaste with a concentration above 1000 ppm of F has greater effectiveness in controlling dental caries [Walsh et al., 2019]. Thus, in an attempt to intensify the fluoride effect an alternative would be the supplementation of conventional (1100 ppm F) toothpaste with CaGP. Another possibility to increase its remineralizing action or the inhibiting effect of demineralization would be to use it in the form of nano-sizeds (CaGPn). Since studies show that phosphates in their nano-sized form exhibit greater area and surface of high chemical reactivity to enamel, promoting greater absorption and

consequently increasing the process of dental de-remineralization [Emerenciano, 2018; Danelon, 2019].

The aim of this study was, therefore, to investigate, whether the addition of β-calcium glycerophosphate in micro (β-CaGPm) and nano-sized forms (β-CaGPn) to conventional toothpastes (1100 ppm F) could increase the remineralizing effect of initial caries lesions *in situ* when compared to fluoride alone. The null hypothesis is that supplementation of conventional fluoride toothpastes with β-CaGPm and β-CaGPn will not promote an additional effect on *in situ* bovine tooth enamel remineralization processes when compared to 1100 ppm F toothpaste.

3.3 Material and Methods

Study design and Subjects

This study was approved by the Human Ethical Committee (Protocol: 10329419.2.0000.5420), and all participants read and signed informed consent statements prior to study initiation. This was a double blinded crossed *in situ* study performed in 4 phases of 3 days each [Danelon et al., 2015; Danelon et al., 2019]. The sample size of volunteers was based on a previous study [do Amaral et al., 2013; Danelon et al., 2015] considering primary outcomes from surface and cross-sectional hardness analysis, and the mean difference among groups (30 and 1,300, respectively), standard deviation (20 and 900, respectively), an α-error of 5% and a β-error of 20%. Volunteers aged 20– 30 years, who were in good general and oral health [Delbem et al., 2005] and did not violate the exclusion criteria (use of any form of medication likely to interfere with salivary secretion, use of fixed or removable orthodontic appliances, being an active smoker or having systemic illness) were included in the study. Enamel blocks (4 × 4 mm, n = 192) from bovine incisor teeth were sequentially polished and selected by surface hardness test (SH). Blocks were demineralized and submitted to post-demineralization surface hardness (SH1) testing. Based on the percentage of SH loss (post-demineralization) blocks were divided into 4 toothpastes groups: no F-β-CaGPm- β-CaGPm (Placebo); 1,100 ppm F (1100F); 1100F plus 0.5% micrometric β-CaGP (1100F-β-CaGPm); and 1100F plus 0.25% nano-sized β-CaGP (1100F-β-CaGPn). After 3 days of the remineralization period, surface hardness post-remineralization was again applied, and integrated recovery of subsurface hardness (ΔIHR), fluoride (F), calcium (Ca) and phosphorus (P) concentration in enamel was also determined.

Processing and characterization of nano-sized CaGP

The processing and characterization of CaGP nano-sizeds were performed at the Department of Chemistry, Federal University of São Carlos (UFSCar). Nano-sized β-CaGP (β-

CaGPn) was processed using the methods described by Danelon et al. [2015 and 2017]. β-CaGPn was obtained from commercial CaGPm (80% β-isomer and 20% rac-α-isomer, CAS 58409-70-4, Sigma Chemical Co, St Louis, Missouri, USA) by ball-milling this compound with 500 g of 2 mm zirconia spheres. Commercial material (70 g) was then added to a polyethylene bottle containing sintered zirconia spheres (5 mm in diameter) in isopropyl alcohol (Merck KGaA, Darmstadt; Deutschland, Germany) at one third of its volume. The bottles were sealed and subjected to milling for 48 h in a ball mill and then filtered and sealed with aluminum foil. The vials were dried at 85 °C to evaporate the isopropyl alcohol (Merck KGaA, Darmstadt, Germany) and X-ray diffraction (XRD) was used to identify the crystalline structures and estimate the crystallographic coherency domains of the CaGP (β-CaGPn). X-ray diffractograms were obtained from samples in powder form using a Shimadzu XRD 6000 using a CuK radiation source ($\lambda = 1.54056$ Å) at a voltage of 30 kV and a current of 30 mA. Measurements were made continuously in the range of $10° \leq 2\theta \leq 80°$ with a 2° sweep speed. The structural identification of the samples was carried out by comparing the diffraction patterns obtained from our analysis with tabulated patterns available in various databases and reported in the BJoint Committee on Powder Diffraction Standards—Powder Diffraction File (JCPDS—PDF). The particle morphologies of the β-CaGPm and β-CaGPn suspensions were analyzed using SEM. The SEM images were collected using an MEV-PHILIPS (XL-30 FEG, Philips, Amsterdam, The Netherlands). To determine the nanoparticles size, the high-resolution trasmission electron microscopy (HRTEM) was performed, using a FEI TECNAI F20 microscope operating at 200 kV; for this, the samples were dripped on copper grids of 400 mesh (PELCO®), and the solvent evaporated at room temperature. After, histograms were constructed using the public domain ImageJ image processing software (version 1.50, National Institute of Health, Bethesda, MD, USA).

Toothpaste formulation and fluoride and pH assessment

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* (*totalizing the final weight of 100 g of each toothpaste). Toothpastes containing micrometric or nano-sized β- CaGP were prepared (80% β-isomer and 20% rac-α-isomer, CAS 58409-70-4, Sigma Chemical Co, St Louis, Missouri, USA) at a concentration of 0.5%β-CaGPm and 0.25%β-CaGPn (*concentrations determined in previous pilot study). To these toothpastes, NaF (Merck, CAS 7681-49-4, and Germany) was added to reach a concentration of 1100 ppm F. In addition, toothpaste without β-CaGPm/β-CaGPn and F (Placebo) as well as with 1100 ppm F (without β-CaGPm/β-CaGPn)

were prepared. The toothpastes used in this study were stored at room temperature and kept properly closed to prevent any change of the samples.

The total fluoride (TF) and ionic fluoride (IF) amounts were determined [Danelon et al., 2015] with a F-specific electrode (Orion 9609- BN; Orion Research Inc., Beverly, USA) connected to an ion analyzer (Orion 720 A+; Orion Research Inc.). The pH levels of the toothpaste slurries were determined via a pH electrode (2A09E, Analyser, São Paulo, Brazil) calibrated with standard pH levels of 7.0 and 4.0.

Subsurface Enamel Demineralization

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

Study Design and Treatments

Four dental specimens were mounted in each acrylic resin palatal appliance. Treatment with toothpastes was performed 3 times per day with the palatal devices inside the volunteers' mouths during their habitual oral hygiene routine (for 1 min). They were instructed to initially brush their natural teeth and conduct 3 brushing strokes in each row of enamel blocks on the oral appliance with the natural slurry (saliva/toothpaste) formed. Palatal appliances were employed at all times during each experimental phase (including during sleep) and were to be removed only during the main meals. During the 7-day pre-experimental period and washout periods, volunteers brushed their teeth with an F-free toothpaste. Volunteers received verbal and written instructions prior to the beginning of the study [Danelon et al., 2015].

Hardness Analysis

Enamel hardness surface (SH) was determined before and after the induction of subsurface lesions (SH1) as well as after each experimental phase (SH2) using a Shimadzu HMV-2000 microhardness tester (Shimadzu Corp., Kyoto, Japan) under a 25-g load for 10s [Danelon et al., 2015]. Five indentations, spaced 100 μm from each other, were made at the center of the enamel surface. Indentations for (SH1) and (SH2) were spaced 100 μm from each other and from the baseline. For the cross-sectional hardness measurements, enamel blocks were longitudinally sectioned through their center and embedded in acrylic resin with the cut face exposed and gradually polished. One sequence of 14 indentations was created 100 μm apart at different

distances (5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220, and 330 μm) from the outer enamel surface using a Micromet 5114 hardness tester (Buehler, Lake Bluff, IL, USA) and the software Buehler OmniMet (Buehler) with a Knoop diamond indenter under a 5-g load for 10 s [Danelon et al., 2019]. The integrated area above the curve (cross-sectional profiles of hardness into the enamel), using the hardness values (KHN), was calculated by trapezoidal rule (GraphPad Prism, version 3.02) in each depth (μm) from the lesion up to sound enamel and subtracted from the integrated area of the sound enamel. The values obtained were subtracted from the integrated hardness area of the post-demineralized enamel, resulting in the integrated recovery of subsurface hardness (ΔIHR).

Analysis of the F, Ca e P Concentration Present in Enamel

The other half of each blocks was used for was used to determine fluoride (F), calcium (Ca) and phosphorus (P) present in the enamel. F in enamel was determined as described by Weatherell et al. (1985) and as modified by Alves et al. [2007]. A digital caliper (Mitutoyo CD-15B, Mitutoyo Corporation, Japan) was used to measure the surface area of the enamel blocks [Akabane et al., 2018]. Next, the blocks were fixed to a mandrel coupled to a modified microscope with a micrometer (Micrometer 733 MEXFLZ-50, Starret, Athol, MA, USA) to measure enamel wear. Self-adhesive polishing discs (diameter: 13 mm) and 400- grit silicon carbide (Buehler) were fixed to the bottom of polystyrene crystal tubes (J-10; Injeplast, Sao Paulo, Brazil). One layer of enamel (50.9 ± 0.2 μm) 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 h. For F analysis, specific electrode 9409BN (Thermo Scientific, Beverly, USA) and microelectrode reference (Analyser, São Paulo, Brazil) coupled to an ion analyzer (Orion 720A+, Thermo Scientific, Beverly, USA) was utilized. The electrodes were calibrated with standards ranging from 0.25 to 4.00 ppm F (100 ppm F, Orion 940,907) under the same conditions as the samples. The readings were conducted in 0.300 mL of the biopsy solution with the same volume of TISAB II modified NaOH [Gonçalves et al., 2018]. The data obtained in mV were converted to $\mu g/mm^3$ using Excel spreadsheet. Ca analysis was performed using the Arsenazo III colorimetric method [Fiske and Subbarow, 1925]. The absorbance readings were recorded at 650 nm with a plate reader (PowerWave 340, Biotek, Winooski, USA). P was measured according to Fiske and Subbarow [Fiske and Subbarow, 1925], and the absorbance readings were recorded at 660 nm. The results were expressed as $\mu g/mm^3$.

Statistical Analysis

The analysis was performed using SigmaPlot software (version 12.0, Systat Software Inc., San Jose, Calif., USA) at a significance level of 5%. The variables %SHR, ΔIHR, F, Ca and P in enamel exhibited normal (Shapiro-Wilk test) and homogeneous (Cochran test) distributions and submitted to one-way, repeated measures analysis of variance (ANOVA), followed by Student-Newman-Keuls' test.

3.4 Results

The milling process reduced the particle size of the β-CaGPn powder without affecting the crystalline structure of the material (Figure 1). The size of the β-CaGPn particles was confirmed following 48 h of grinding using SEM, as shown in Figure 1. The average size of the particles was observed to be 220.0 nm.

The mean concentration (SD) of total F (FT) and ionic (FI) (n = 3) was: Placebo - 24.4 (5.0) and 17.0 (0.4); 1100F - 1168.8 (10.8) and 1061.0 (25.2); 1100F-0.5%β-CaGPm - 1005.5 (9.5) and 1004.9 (11.1); 1100F-0.25%β-CaGPn - 1070.2 (12.3) and 1041.8 (9.4); The mean value (SD) of the pH of the experimental toothpastes was 7.4 (0.1), ranging from 7.3 to 7.6.

The mean (SD) initial SH for all blocks was 374.0 (1.0), and the means varied between 372.4 (1.2) up 376.6 (2.5) KHN, with no significant differences among the groups after random allocation ($p = 0.974$). The mean (SD) of SH after demineralization (SH1) was 58.2 KHN (3.7), ranging between 42.5 and 72.6 ($p = 0.441$). The addition of 0.5%β-CaGPm to fluoride toothpaste increased the %SHR to approximately 20% when compared with the 1100F group ($p < 0.001$). The treatment with 1100F-0.25%β-CaGPn, led to %SHR ~ 69% and ~ 40% higher when compared to 1100F and 1100F-0.5%β-CaGPm toothpastes groups ($p < 0.001$), respectively. In addition, the capacity to reduce the lesion body (ΔIHR) was ~ 40% and ~ 17% higher with 1100F-0.25%β-CaGPn and 1100F-0.5%β-CaGPm ($p < 0.001$) when compared to 1100F. The addition of 0.25%β-CaGPn in 1100F toothpaste reduced the body lesion in 27% when compared to 1100F-0.5%β-CaGPm toothpaste ($p < 0.001$) (Table 1).

The addition of β-CaGPm and β-CaGPn to the F toothpaste did not influence enamel F concentration, so its effect was similar to 1100F except for the Placebo that featured the lowest concentration ($p < 0.001$) (Table 1). The treatment with 1100F-0.25%β-CaGPn promoted an increase in the concentration of Ca in the enamel by ~ 77% and ~ 28% when compared to the 1100F and 1100F-0.5%β-CaGPm groups ($p < 0.001$), respectively. The treatment with 1100F-0.25%β-CaGPn promoted an increase in the concentration of P in the enamel by ~ 64% and ~

28% when compared to the 1100F and 1100F-0.5%β-CaGPm groups ($p < 0.001$), respectively ($p < 0.001$).

3.5 Discussion/Conclusion

The association of nano-sized phosphates to fluoride has been shown to significantly increase the process of re-demineralization of tooth enamel [Dalpasquale et al., 2017; Emerenciano et al., 2018; Danelon et al., 2019]. The results of present study demonstrated that the addition of β-CaGP to fluoride toothpastes increased the level of remineralization of subsurface enamel lesions *in situ* when compared to conventional toothpaste (1100 ppm F). Thus, the null hypothesis of the study that supplementation of conventional fluoride toothpastes with β-CaGPm and β-CaGPn would not promote an additional effect on the process of remineralization of bovine enamel *in situ* when compared to toothpaste with 1100F was rejected.

The association of CaGP to fluoride toothpastes is justified by the fact that it is a source of Ca and P. In the CaGP molecule, phosphate is covalently bound to glycerol and is released in the presence of salivary phosphates [Barbosa, 2012]. It is known that calcium and phosphate ions in association fluoride has a greater ability to remineralize initial caries lesions when compared to fluoride alone. In the present study, the treatments with toothpaste supplemented with β-CaGP showed higher values in %SHR compared to the toothpaste that contained only fluoride. Furthermore, the ability to remineralize the body of the lesion (ΔIHR) was ~ 40% and ~ 17% higher with 1100F-0.25%β-CaGPn and 1100F-0.5%β-CaGPm ($p < 0.001$) when compared to 1100F. The results of the present study are according to Zaze et al. [2014b], who evaluated the effect of low fluoride toothpastes with CaGP on the remineralization of enamel *in situ* and, observed that the addition of 0.25%CaGP improved the remineralization potential of low fluoride toothpastes. The above results can be explained by the direct interaction that CaGP presents with enamel [Grenby and Bull, 1980], when used in an adequate molar proportion. The determination of the ideal concentration of β-CaGPm and β-CaGPn was based on the results obtained in a previous study [Emerenciano et al., 2019].

Besides the use of phosphates to enhance the effect of fluoride products, the literature has shown that if these are associated in nano-sized form with fluoride there is a significant increase in their effect on demineralization and remineralization of enamel [Emerenciano et al., 2018; Danelon et al., 2019]. One of the aims of using nano-sized structures is to provide the development of structures with a greater stability and efficiency than in their original form. Nano-sized phosphates present unique physical-chemical properties; their ultra-small size promotes an increase in surface area in relation to mass and high chemical reactivity. The

performance of nano-sized materials is related to some thermodynamic and kinetic properties present only in the nano-sizeds [Abou Neel et al., 2015; Danelon et al., 2015; Danelon et al., 2017; Garcia et al., 2019]. XRD patterns of the β-CaGPn samples demonstrated that the material was still crystalline after processing in a ball mill for 48 h (Figure 1). Danelon et al. [2019], in an *in situ* study observed that treatment with conventional toothpaste associated with sodium hexametaphosphate (HMP) nano-sized resulted in a remineralizing capacity ∼42 and ∼41% higher when compared with 1100F/HMP and 1100F toothpastes. Our results have shown an increase in efficacy of 40% when compared to treatment with β-CaGPn when compared to β-CaGPm. As the nano-sized is more reactive than the micrometric particles, the concentration of β-CaGP in the product can be halved without reducing its remineralizing action, this is because β-CaGP facilitates the diffusion of calcium and phosphate ions in the enamel.

Additional extrinsic sources of stabilized Ca^{2+} and PO_4^{3-} ions can increase the potential for natural saliva remineralization by increasing diffusion gradients, increasing F^- ion-mediated remineralization, and thus favoring faster and deeper subsurface remineralization [Philip, 2019]. When evaluating the subsurface lesion we observed that the formulation containing β-CaGPn showed a 40% greater ability to reduce the body of the lesion when compared to fluoride toothpaste. These data are in line with previous studies, which showed that the association of phosphate salts with conventional toothpastes was more effective in remineralizing subsurface enamel lesions [Danelon et al., 2015; Danelon et al., 2019]. This ability to remineralize the lesion at the subsurface level is of fundamental importance, since within the body the subsurface lesion may have a loss of up to 50% of its original mineral content and are often covered by an apparently intact surface layer [Featherstone, 2000]. The lesion used in the *in situ* model of the present study is superficial (∼50 μm) with a 3-day remineralization protocol, therefore, 3-day experiments were sufficient to evaluate the differences between the groups, confirming data from studies showing that this *in situ* model was able to show a dose response when teeth with different fluoride concentrations were used [Afonso et al., 2013; Danelon et al., 2015; Danelon et al., 2019].

The synergistic effect of β-CaGP and F on toothpastes was also evaluated from the concentrations of F, Ca and P ions retained on enamel. The increase in remineralization by products containing β-CaGP can be attributed to the high level of bioavailable calcium and phosphate ions released by these products. In the present study, it was observed a significant increase in the calcium and phosphorus retained in the enamel in the groups that present β-CaGP when compared to the fluoride group alone. The link between Ca and the phosphate group of the structure β-CaGP is considered the unstable part of the molecule [Inoue, 1992], suggesting that

this instability favors the release of Ca to the medium, leaving the anionic phosphate groups free. A concern are unwanted reactions between calcium, fluoride and phosphate to form insoluble phases in the paste during storage and/or delivery, thus reducing the bioavailability of not only the fluoride ion but also the calcium [Sullivan, 2001]. Therefore, it is of fundamental importance to compare these new toothpastes with conventional toothpastes in order to evaluate the bioavailability of fluoride ions, as well as their ability to remineralize subsurface enamel lesions *in situ*. The addition of β-CaGPm and β-CaGPn to toothpaste F did not influence the concentration of F in the enamel, so its effect was similar to 1100F, demonstrating that the addition of β-CaGP to toothpastes did not influence the bioavailability of fluoride [Zaze et al., 2014a; Zaze et al., 2014b]. Thus, it is possible to suggest that a complex will form between NaF and β-CaGP (F-β-CaGP), being responsible for the remineralizing effect as demonstrated in this study. Therefore, another advantage of using nano-sized β-CaGP is the addition of less β-CaGP, which facilitates the manipulation of formulations, since the solubilization of large quantities of β-CaGP is difficult in toothpaste formulations. Even assuming the F-β-CaGP link this did not interfere in the incorporation of fluoride for the enamel, regardless of the amount of β-CaGP.

Although the remineralizing effect of β-CaGPn tested shows promise, the process of remineralization *in vivo* depend on several factors. Among these factors, the type of substrate and the depth of the artificial caries lesion seem to be the most important. The bovine enamel has higher reactivity and porosity, which leads to a faster remineralization when compared to human enamel [Lynch, 2006]. As for the substrate, the depth of enamel demineralization can also interfere with remineralization time. It is known that the remineralization process is slower in deep lesions (100 μm) due to a greater distance of ionic diffusion when compared to the lesion in the present study [Mellberg, 1991]. Furthermore, the protocol used in the present study did not simulate the controlling caries lesion progression, different cariogenic challenges and the inorganic composition of saliva and biofilm fluid was not evaluated. Therefore, further studies should be performed in order to provide a greater understand the effect of nano-sized β-CaGP on re/demineralization processes. Based on these results, it is concluded that the addition of 0.25%β-CaGPn to a conventional toothpaste increased the bioavailability of calcium and phosphate promoting a significantly higher remineralizing effect when compared to 1100 ppm F toothpaste. This formulation could be a safe alternative to increase the anticaries effects of conventional toothpaste (1100 ppm F) and would be useful for further reducing caries incidence in the general population, but mainly in patients at high risk of caries.

3.6 Acknowledgments

The authors are grateful to Coordination for the Improvement of Higher Education Personnel – Brasil (CAPES – Finance Code 001) for the concession of a scholarship to the first author, The São Paulo Research Foundation (FAPESP – Process: 2019/16106-9) for the concession of a scholarship to the fourth author, National Council for Scientific and Technological Development (CNPq – Process: 408681/2018-7) for the concession of financial assistance to the ninth author.

3.7 Disclosure Statement

Only the author Marcelle Danelon, Alberto Carlos Botazzo Delbem, Juliano Pelim Pessan and Emerson Rodrigues de Camargo has a patent for a product used in the study, by the National Institute of Industrial Property - INPI/SP, on 06/16/2010 under number C1 0801811-1, and granted on April 11, 2017.

3.8 Author Contributions

Conceived and designed the experiments: MD, ACBD, ERC, ERC, FNSN, LFG and YTCSS. Performed the experiments: NGE, MD, FMCG, JPQ, ERC, FNSN, LFG and YTCSS. Analyzed the data: NGE, MD, JPQ, ACBD and JPP. Wrote/revised the paper: NGE, MD, ACBD, FMCG, JPP, ERC, FNSN, LFG and YTCSS. For all the experiments performed there is no laboratory technician to support either of us. It is the authors who perform each of the study stages. This way we justify the number of authors.

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Figure legend

Figure 1: Scanning electron microscopy images and average size (histograms) and X-ray patterns of the (A) β-CaGPm and (B) β-CaGPnano after milling for 48h. Magnification: 20,000 x.

Table legend

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

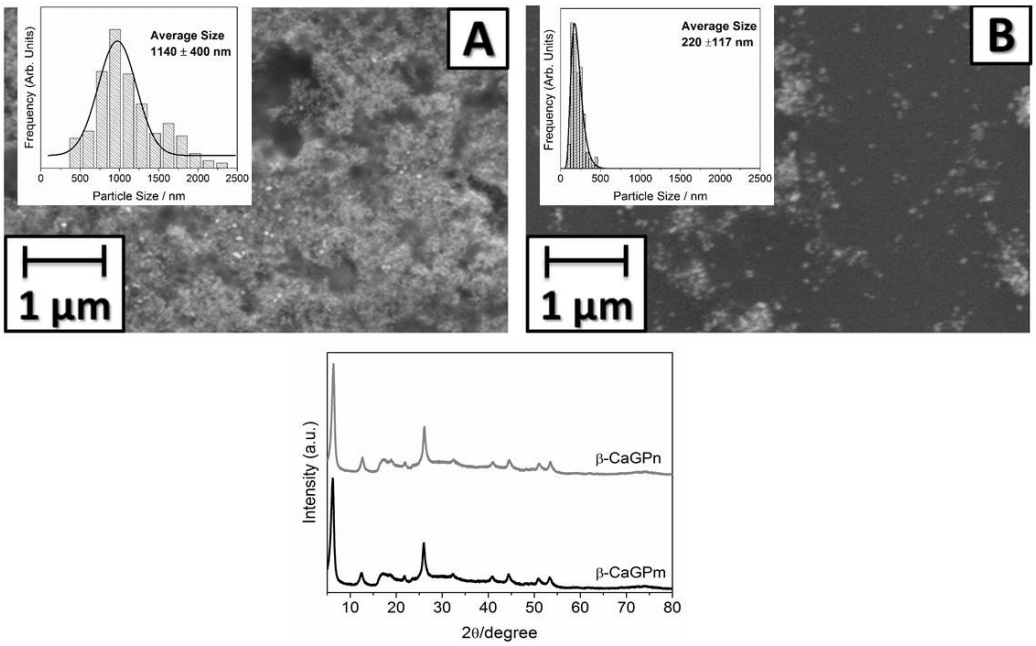


Figure 1: Scanning electron microscopy images and average size (histograms) and X-ray patterns of the (A) β-CaGPm and (B) β-CaGPnano after milling for 48h. Magnification: 20,000 x.

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

Analysis	Toothpastes			
	Placebo	**1100F**	**1100F-0.5%βCaGPm**	**1100F-0.25%β-CaGPn**
%SHR, KHN	16.3[a] (2.1)	29.6[b] (4.7)	35.7[c] (4.2)	50.0[d] (3.6)
ΔIHR, KHN x µm	7,983.5[a] (495.1)	5,427.8[b] (322.7)	4,486.2[c] (623.4)	3.264.4[d] (415.4)
Enamel, µg/mm^3				
Fluoride	1.6[a] (0.2)	2.3[b] (0.7)	2.4[b] (0.6)	2.5[b] (0.6)
Calcium	467.7[a] (177.8)	732.6[b] (317.6)	1019.1[c] (215.7)	1301.5[d] (234.3)
Phosphorus	313.0[a] (112.4)	483.6[b] (171.9)	618.2[c] (158.9)	792.8[d] (108.9)

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

Conclusão Geral

4. Conclusão geral

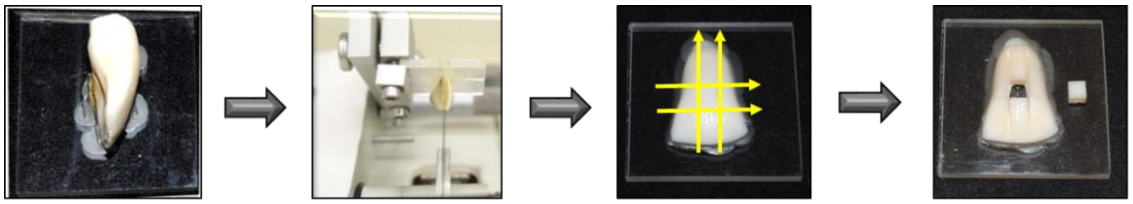
Conclui-se que a adição de 0,25%β-CaGPn a um dentifrício convencional promoveu um maior efeito protetor na inibição da desmineralização do esmalte *in vitro* e uma ação remineralizadora *in situ* significativamente mais elevada quando comparado ao dentifrício convencional (1100 ppm F), podendo ser uma alternativa efetiva para melhorar a saúde bucal dos indivíduos, especialmente aqueles com alto risco e atividade de cárie dentária.

Anexos

5.1 ANEXOS

ANEXO A

PREPARO DOS BLOCOS DE ESMALTE



Colagem em placa de acrílico

Separação da superfície vestibular/lingual

Superfície vestibular

Blocos de 4mm x 4mm

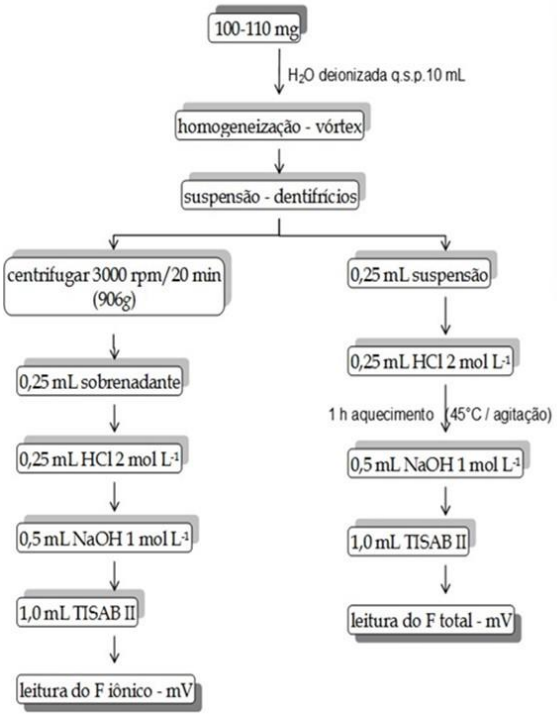
Planificação da dentina

Politriz BUEHLER

Polimento do esmalte

ANEXO B

DOSAGEM DE FLUORETO NOS DENTIFRÍCIOS EXPERIMENTAIS



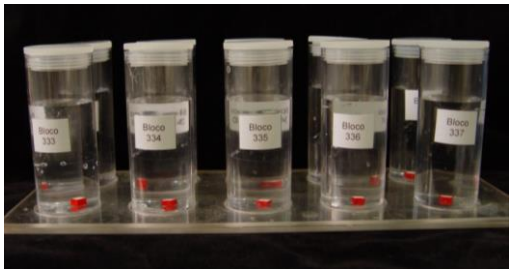
Eletrodo específico para F; Orion 9409-BN

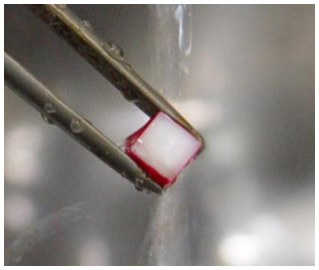
Microeletrodo de referência

Analisador de íons

ANEXO C

INDUÇÃO DE LESÃO DE CÁRIE ARTIFICIAL

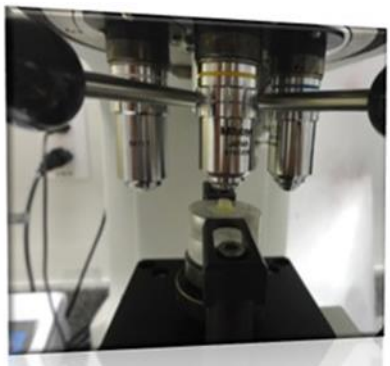




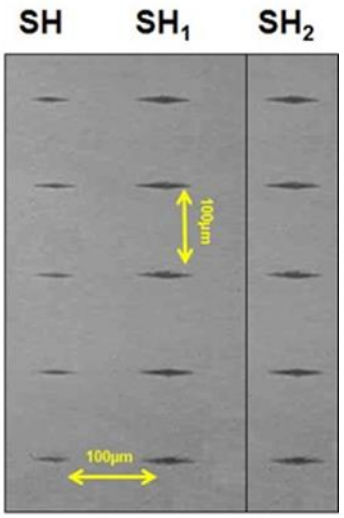
16 horas

ANEXO D

ANÁLISE DA DUREZA INICIAL, PÓS-DESMINERALIZAÇÃO E PÓS-REMINERALIZAÇÃO

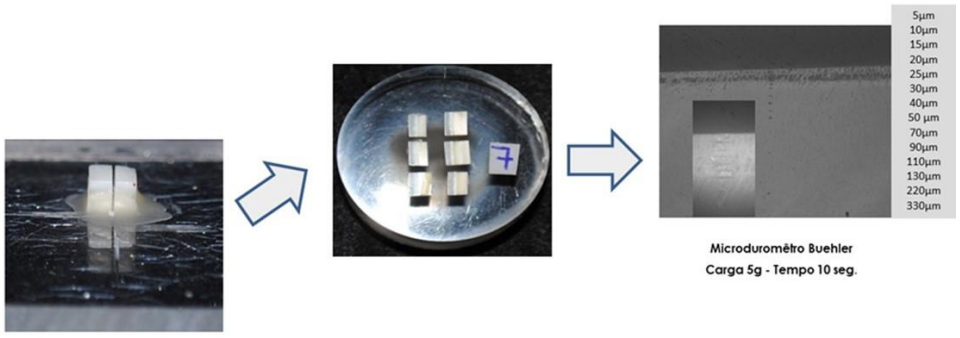


Microdurômetro Buehler
Carga 25 gramas, Tempo 10 segundos



ANEXO E

ANÁLISE DA PERDA INTEGRADA DE DUREZA DE SUBSUPERFÍCIE

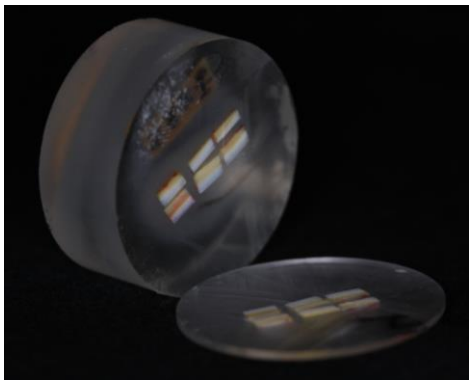


ANEXO F

CICLAGEM DE pH

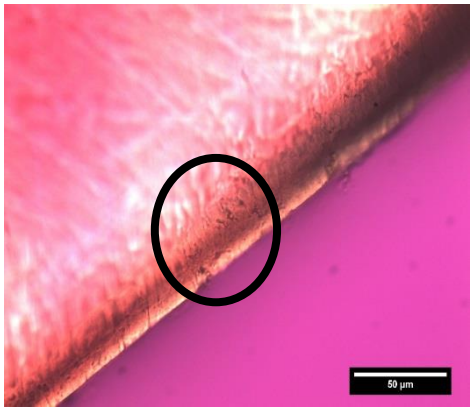
ANEXO G

MICROSCOPIA DE LUZ POLARIZADA



300 µm

Microscópio de Luz
Polarizada (AxioPhot, Zeiss)



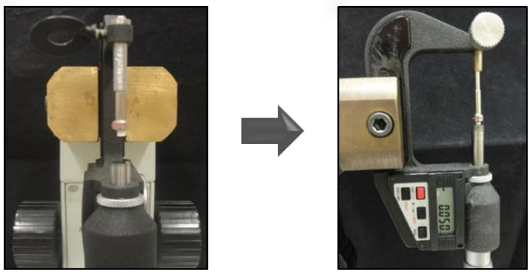
ANEXO H

DISPOSITIVO PALATINO (Artigo 2)

Kit fornecido ao voluntário a cada período experimental.

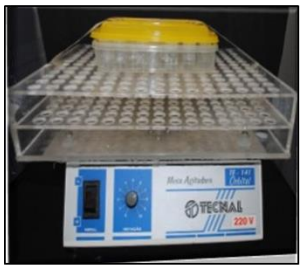
ANEXO H

ANÁLISE DE FLUORETO, CÁLCIO E FÓSFORO NO ESMALTE



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

Desgaste ~50µm Lixa 400 (CARBIMET - BUEHLER)



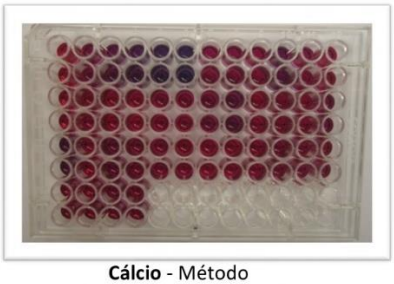
0,5 ml de HCl 1 mol/L

Agitação por 1 hora

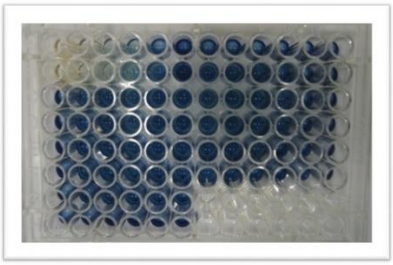
0,25 mL da amostra + 0,25 mL TISAB ll modificado com NaOH.

Akabane et al., 2018.

Espectrofotômetro de microplaca EONC, Biotek, USA



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



Fósforo - Método colorimétrico Fiske e Subbarow, 1925.