Mariana Emi Nagata

CONCENTRAÇÃO DE FLÚOR E CÁLCIO NO FLUIDO DO BIOFILME ASSOCIADA AO USO DE DENTIFRÍCIOS FLUORETADOS SUPLEMENTADOS COM TRIMETAFOSFATO DE SÓDIO OU GLICEROFOSFATO DE CÁLCIO, SOB DESAFIO CARIOGÊNICO

ARAÇATUBA - SP

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Dissertação apresentada à Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, como parte dos requisitos para a obtenção do título de Mestre em Ciência Odontológica – Área Saúde Bucal da Criança.

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Dedicatória

Dedicatória

Dedico este trabalho

Aos meus pais, Hiromitsu e Clara

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"As palavras de amizade e conforto podem ser curtas e sucintas, mas o seu eco é infindável."

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Agradecimentos

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Epígrafe

"Não precisamos realizar grandes obras a fim de mostrarmos um grande amor por Deus e pelo próximo. É a intensidade do amor que colocamos em nossos gestos que os torna algo especial para Deus e para os homens."

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Resumo

NAGATA, M.E. **Concentração de flúor e cálcio no fluido do biofilme associada ao uso de dentifrícios fluoretados suplementados com Trimetafosfato de Sódio ou Glicerofosfato de Cálcio, sob desafio cariogênico**. 2015 72f. Dissertação (Mestrado em Ciência Odontológica, área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2015.

Estudos recentes demonstraram que dentifrícios com concentração reduzida de fluoreto (DCRF, 550 µg F/g) suplementados com cálcio ou fosfato apresentam efetividade clínica semelhante à de um dentifrício convencional (DC, 1100 µg F/g). Entretanto, o mecanismo pelo qual estes compostos atuam nos processos de des- e remineralização ainda é incerto. O presente estudo avaliou a concentração de F e Ca no fluido do biofilme formado *in situ* sob desafio cariogênico após o uso de dentifrícios fluoretados, suplementados ou não com trimetafosfato de sódio (TMP) ou glicerofosfato de cálcio (CaGP). Voluntários (n=12) foram aleatoriamente divididos em 5 grupos, de acordo com os seguintes dentifrícios: Placebo (sem F, TMP ou CaGP), DC, DCRF sem suplementação (550F) e DCRF suplementado com 1% TMP (550F-TMP) ou 0,25% CaGP (550F-CaGP). Em cada fase, os voluntários utilizaram um dispositivo palatino contendo 4 blocos de esmalte bovino. O desafio cariogênico foi realizado com solução de sacarose 30%, 6 vezes ao dia. Na manhã do 8º dia, o biofilme foi coletado 1h e 12h após a escovação e desafio cariogênico. As análises de F e Ca foram realizadas com eletrodo invertido após tamponamento com TISAB III e por espectrofotometria (Arsenazo III), respectivamente. Os dados foram submetidos a ANOVA a 2 critérios (medidas repetidas) e teste de Student-Newman-Keuls ($p<0,05$). Uma relação dose-resposta entre as concentrações de F nos dentifrícios e no fluido do biofilme foi verificada. Diferenças significativas foram observadas nas concentrações de F no fluido do biofilme apenas 1 hora após o uso dos dentifrícios Placebo, 550F e DC, sem diferenças significativas entre 550F, 550F-CaGP e 550F-TMP. Não houve um padrão definido para as concentrações de Ca no fluido do biofilme, sendo os maiores valores observados para o Placebo e 550F-CaGP. Conclui-se que o efeito anticárie de DCRFs suplementados com TMP ou CaGP não

pode ser relacionado a um aumento na disponibilidade de F e Ca no fluido do biofilme.

Abstract

NAGATA, M.E. **Fluoride and calcium concentration in the biofilm fluid associated with fluoridated dentifrices supplemented with Sodium Trimetaphosphate or Calcium Glycerophosphate, under cariogenic challenge**. 2015 72 f. Dissertação (Mestrado em Ciência Odontológica, área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2015.

Recent studies demonstrated that low fluoride dentifrices (LFD, 550 µg F/g) supplemented with calcium or phosphate have a similar effectiveness to a conventional dentifrice (CD, 1100 µg F/g). However, the mechanisms by which these compounds act on the de- and remineralization processes remain unclear. The present study evaluated fluoride (F) and calcium (Ca) concentrations in the biofilm fluid formed *in situ* under cariogenic challenge after using F dentifrices, supplemented or not with sodium trimetaphosphate (TMP) or calcium glycerophosphate (CaGP). Volunteers (n=12) were randomly divided into 5 groups according to the following toothpastes: Placebo (no F or CaGP, TMP), CD and LFD with no supplementation (550F) or supplemented with 1% TMP (550F-TMP) or 0.25% CaGP (550F-CaGP). In each phase, volunteers wore palatal appliances containing 4 bovine enamel blocks. The cariogenic challenge was produced using a 30% sucrose solution, 6 times a day. On the morning of the 8th day, biofilm samples were collected 1h and 12h after brushing and cariogenic challenge. F and Ca analyzes were performed with the inverted electrode after buffering with TISAB III and using the Arsenazo III method, respectively. Data were submitted to 2-way ANOVA (repeated measures) and Student-Newman-Keuls test ($p<0.05$). A dose-response relationship was verified between F concentrations in the dentifrices and those in the biofilm fluid. Significant differences were observed among Placebo, 550F and CD only 1 h after brushing, without statistical differences among 550F, 550F-TMP and 550F-CaGP. No defined trend was observed among the groups regarding Ca concentrations in the biofilm fluid, with the highest values found for Placebo and 500F-CaGP. It was concluded that the anticaries effects of LFDs supplemented with CaGP or TMP cannot be related to an increased availability of F and Ca in the biofilm fluid.

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LISTA DE ABREVIATURAS E SÍMBOLOS

ANOVA	Análise de Variância
am	Ante Meridiem
Ca	Cálcio
CAAE	Certificado de Apresentação para Apreciação Ética
CaGP	Calcium Gycerophosphate/*Glicerofosfato de Cálcio*
°C	Graus Celsius
CD	Conventional Dentifrice
DC	Dentifrício Convencional
DP	Desvio padrão
F	Fluoreto
FT	Fluoreto Total
FI	Fluoreto Iônico
g	Grama
g	Gravidade
h	Hora (s)
HCl	Ácido Clorídrico
H_2O	Água
KHN	Knoop Hardness Number/*Número de Dureza Knoop*
LFDs	Low fluoride dentifrices
Log_{10}	Logaritmo na base 10
mL	Mililitro
M	Molar
Mm	Milímetro
Min.	Minuto
Mg	Miligrama
mV	Milivoltagem/milivolt
NaOH	Hidróxido de Sódio
Nm	Nanômetro
µg	Micrograma
µg/g	Micrograma por grama

µg F/g	Micrograma de fluoreto por grama
µg/mL	Micrograma por mililitro
µL	Microlitro
µM	Micro molar
p	Probabilidade
pH	Potencial Hidrogeniônico
Pm	Post Merediem
Ppm	Parte por milhão
Q.S.P	Quantidade Suficiente Para
R	Coeficiente de correlação
rpm	Rotações por minuto
SD	Standard Deviation
SH	Surface hardness
TISAB	Total Ionic Strenght Adjustment Buffer/*Tampão de Ajuste da Força Iônica Total*
TMP	Sodium Trimetaphosphate/*Trimetafosfato de sódio*
UNESP	Universidade Estadual Paulista
α	Alfa
B	Beta
ΔKHN	Integrated subsurface hardness/*Dureza integrada de subsuperfície*

Sumário

SUMÁRIO

Fluoride and calcium concentration in the biofilm fluid after the use of fluoridated dentifrices supplemented with sodium trimetaphosphate or calcium glycerophosphate, under cariogenic challenge

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Short tittle: Biofilm fluid F levels related to TMP or CaGP dentifrices

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

ABSTRACT

The present study evaluated fluoride (F) and calcium (Ca) concentrations in the biofilm fluid formed *in situ* under cariogenic challenge after using F dentifrices supplemented or not with sodium trimetaphosphate (TMP) or calcium glycerophosphate (CaGP). Volunteers (n=12) were randomly divided into 5 groups according to the toothpastes used: Placebo (no F, CaGP or TMP), conventional dentifrice (CD, 1100 ppm F) and low-fluoride dentifrice (550 ppm F) with no supplementation (550F) or supplemented with 1% TMP (550F-TMP) or 0.25% CaGP (550F-CaGP). In each phase, volunteers wore palatal appliances containing 4 bovine enamel blocks. Cariogenic challenge was performed with 30% sucrose solution, 6 times/day. On the morning of the 8th day, biofilm samples were collected 12h and 1h after brushing and cariogenic challenge. F and Ca analyses in the biofilm fluid were performed with the inverted electrode after buffering with TISAB III and using the Arsenazo III method, respectively. Data were submitted to 2-way ANOVA (repeated measures) and Student-Newman-Keuls test ($p<0.05$). A dose-response relationship was verified between F concentrations in the dentifrices and in the biofilm fluid. Significant differences were observed among Placebo, 550F and 1100F only 1 h after brushing, without statistical differences among 550F, 550F - TMP and 550CaGP. No defined trend was observed among the groups regarding Ca concentrations, with the highest values seen for Placebo and 550F - CaGP. It was concluded that the anticaries effects of LFDs supplemented with CaGP or TMP cannot be related to an increased availability of F and Ca in the biofilm fluid.

INTRODUCTION

The uncertainties surrounding the efficacy of low-fluoride dentifrices (LFD, 500-550 ppm F) against dental caries when compared to conventional dentifrices (CD, 1,000-1,100 ppm F) has prompted to several studies attempting to increase the anticaries effects of such formulations [Walsh et al., 2010; Wong et al., 2011]. Among the strategies available, the supplementation of LFDs with calcium and/or phosphate salts has been intensively studied in recent years [Pessan et al., 2011]. *In vitro* and *in situ* studies showed that LFDs supplemented with calcium glycerophosphate (CaGP) [Amaral et al., 2013; Zaze et al., 2014a] or sodium trimetaphosphate (TMP) [Takeshita et al., 2009] have a similar anticaries effect when compared to a CD. The effects of these formulations were further tested in a recent randomized clinical trial, in which the progression of caries lesions was assessed in children using a CD (1,100 ppm F) and LFDs containing CaGP or TMP [Amaral et al., 2014]. After a 18-month follow-up period, caries progression was shown to be significantly lower in children using the TMP-containing toothpaste in comparison to the conventional formulation, while the progression in the group using the CaGP-containing dentifrice was similar to the 1,100 ppm F toothpaste.

The addition of CaGP and TMP to other topically applied fluoridated products has also been shown to promote a synergistic protective effect against dental caries and erosive wear, using *in vitro* [Danelon et al., 2014; Pancote et al., 2014; Manarelli et al., 2014] and *in situ* models [Amaral et al., 2013; Moretto et al., 2013; Zaze et al., 2014a]. Little is known, however, about the mechanisms by which these salts interfere with the de- and remineralization processes of dental enamel. The use of the LFDs supplemented with CaGP or TMP was shown to promote an increase on enamel surface hardness, besides a marked effect on the depth of enamel subsurface lesions [Takeshita et al., 2009; Amaral et al., 2013; Zaze et al., 2014a, b]. Furthermore, F and calcium (Ca) concentrations in biofilm formed *in situ* in the presence of these toothpastes were significantly higher than their counterparts without calcium or phosphate salts, reaching levels similar to those attained by the use of the CD [Amaral et al., 2013; unpublished data].

Although this increase in biofilm F and Ca levels seems to explain the synergistic effect of TMP and CaGP when added to a fluoride dentifrice, the

availability of these ions in the fluid phase of the biofilm remains unknown. Previous studies demonstrated that F and Ca ions may be retained in biofilm through the formation of mineral deposits [Kaufman and Kleinberg 1973; Rose et al., 1996; Gao et al., 2001], which may be released from biofilm fluid during a cariogenic challenge. The literature also reports that even when biofilm is not completely removed, F retained in this reservoir can be released into the fluid of biofilm, reducing demineralization of enamel covered by biofilm [Tenuta et al., 2009b]. Based on the above, the assessment of the effects of LFDs containing TMP or CaGP on the mineral composition of the biofilm fluid could bring useful information for a better understanding of the mechanisms by which these salts interfere with the dynamics of dental caries.

Thus, the present study aimed to evaluate the concentration of F and Ca in the biofilm fluid formed *in situ* associated with the use of LFDs supplemented with TMP or CaGP, under cariogenic challenge. The study's hypothesis was that the supplementation of LFDs with TMP or CaGP would significantly increase F levels in the biofilm fluid when compared to their counterpart without TMP or CaGP.

MATERIALS AND METHODS

This study was approved by the Human Research Ethics Committee of Araçatuba Dental School (CAAE 20146313.7.0000.5420) and all participants signed an informed consent form (ANEXO B).

Experimental design

The study was carried out through an *in situ*, double-blind, crossover and randomized design comprising five experimental phases of 7 days each. A 7-day washout period was done prior to each experimental phase to eliminate possible residual effects from the previous treatments. Twelve volunteers, regardless of gender, aged 20-34 years-old and resident in Araçatuba participated in the study. The inclusion criteria comprised good general and oral health volunteers, without systemic drugs use that might interfere with biofilm formation or salivary flow. On the other hand, individuals with active caries lesions, who received fluoride applications two weeks before the experiment, water activities practitioners, environment polluted by low pH components

workers (industry), smokers and volunteers diagnosed with systemic diseases (xerostomia, diabetes, autoimmune diseases, malnutrition, gastroesophageal problems) were excluded. The volunteers wore acrylic palatal appliances (24 hours/day) containing four bovine enamel blocks (4 × 4 × 2 mm) placed 1 mm below the acrylic level and covered by a plastic mesh to allow dental biofilm accumulation. During each phase, the volunteers dripped a 30% sucrose solution on the enamel blocks (6 times/day), and brushed their teeth three times a day, for 7 days, using one of the following toothpastes: (1) placebo (fluoride-free), (2) 550 ppm F, (3) 1,100 ppm F, (4) 550 ppm F with 0.25% CaGP, (5) 550 ppm F with 1% TMP, hereafter abbreviated as Placebo, 550F, 1100F, 550F-aGP and 550F-TMP, respectively. Biofilm samples were collected in the morning of the 8th day after overnight fasting (2 enamel blocks), and sixty minutes after tooth brushing and exposure to sucrose. A sample of 11 volunteers was calculated based on a previous *in situ* study conducted with a similar protocol, assessing whole biofilm fluoride concentrations after the use of Placebo and 550 ppm F toothpastes (mean difference = 0.16 µM F/Kg, standard deviation = 0.1) [Amaral et al., 2013], considering α - error of 5% and β - error of 20% (SigmaPlot, version 12.0). Assuming a dropout rate of 20%, sample size was then determined as 14 volunteers (ANEXOS C, D e E).

Fluoride dosage in the experimental dentifrices

The experimental dentifrices were produced in the laboratory of Pediatric Dentistry from Araçatuba Dental School, using the same basic formulation (except for F, CaGP and TMP concentrations) with the following components: titanium dioxide, carboxymethyl cellulose, methyl p-hydroxybenzoate, sodium saccharine, oil peppermint, glycerin, silica abrasive, sodium lauryl sulfate and water. Formulations without F (Placebo) and containing F (NaF - Merck®, Germany) in the concentrations of 550 and 1,100 mg F/g were obtained. Also, CaGP and TMP (Sigma® - Aldrich, USA) were added to the 550 ppm F dentifrice at concentrations of 0.25% and 1%, respectively. The CaGP concentration was determined based on studies of Amaral et al. [2013] and Amaral et al. [2014], which used *in situ* and *in vivo* protocols, respectively. TMP concentration was determined based on the study of Amaral et al. [2014]. Fluoride concentrations in the toothpastes were determined using an ion-

specific electrode (9409 BN) connected with an ion analyzer (Orion 720 Aplus), previously calibrated with 5 standards (0.125, 0.25, 0, 5, 1.0 and 2.2 mg F/mL) [Delbem et al., 2002] (ANEXO F).

Enamel blocks and appliance preparation

Two hundred eighty enamel blocks measuring 4 × 4 x 2 mm were obtained from bovine incisors previously stored in 2% formaldehyde solution (pH 7.0) for 1 month. Enamel blocks were serially polished and selected according to their surface hardness (SH, 369.0 ± 3.1 KHN). Each acrylic palatal appliance had 4 enamel blocks, which were randomly assigned into the 5 experimental groups (p=0.97). A 4.0 mm-deep space was created in the appliances, leaving 1.0 mm space for dental biofilm accumulation on the enamel blocks. A plastic mesh was fixed in acrylic resin to avoid mechanical disturbance and to induce dental biofilm formation [Amaral *et al.*, 2013].

Intraoral procedures

The cariogenic challenge was produced by the use of a 30% sucrose solution (Synth, Brazil), which was replaced every 48 h. The volunteers were instructed to remove the appliance from the oral cavity before dropping two drops of sucrose solution on each block (sufficient amount to fill out the space of 1.0 mm), 6 times a day, in predetermined times (8:00 am, 11:00 am, 02:00 pm, 05:00 pm, 07:00 pm, 09:00 pm). After dripping, the appliances were left to rest for 5 minutes before being returned to the oral cavity, in order to allow the diffusion of the sucrose in the biofilm. Treatment with dentifrices was performed 3 times a day, for 7 days. The volunteers brushed their natural teeth with the device in the oral cavity, therefore producing natural dentifrice/saliva slurry, which was later squished in the oral cavity during 30 seconds. The volunteers were instructed to use the appliances 24 hours a day and remove only during meals. They were restricted to use antimicrobials and fluoride products throughout the entire experiment (ANEXO C e D).

Sampling procedures

Biofilm samples were collected in the morning of the 8th day of each experimental phase, at 2 moments (biofilm from 2 enamel blocks each time),

with volunteers fasting overnight. The first sample was collected about 12 hours after the last treatment with the toothpastes done on the previous night. Following, the volunteers brushed their teeth with the provided dentifrice and the cariogenic challenge was performed five minutes later. The second sample was then collected 60 minutes after tooth brushing. The biofilm was collected with a plastic spatula and weighed in preweighed microcentrifuge cap tubes filled with mineral oil. Tubes were then centrifuged (21023 *g*, 5 minutes, 4 ºC) in order to separate the biomass from the fluid phase. After centrifugation, a small fraction of the fluid was collected with a micropipette, also filled with mineral oil. (ANEXOS G, H e I)

Fluoride Analysis in the Biofilm Fluid

After biofilm fluid separation from its solid phase, it was transferred to the surface of an inverted ion-specific electrode, immersed in mineral oil. Through this microanalysis technique, multiple samples were placed simultaneously on the electrode [Vogel *et al.*, 1997]. The samples were placed on drops of TISAB III (Orion) previously placed on the electrode membrane, in a ratio of 10:1 (sample:TISAB) and were read by the positioning of the reference microelectrode within each sample in order to close the circuit. This electrode was calibrated with standard solutions of known fluoride concentrations (ANEXO I).

Calcium Analysis in the Biofilm Fluid

Calcium analysis of biofilm fluid samples were performed by spectrophotometry. A quartz nanopipette of approximately 1 µL was used, allowing standardized volumes of Calcium standards and samples. Arsenazo III was used as colorimetric reagent [Tenuta *et al.*, 2006] and samples readings were performed on a microplate reader (Biotek Eon).

Fifty microliters of deionized water and 1 µL of the samples/standard were added in each microplate well. Then, 50 µL of the colorimetric reagent were added in each plate well and the plates were shaken during 60 seconds in the microplate reader, allowing the reaction between sample and Arsenazo III prior to obtaining the resulting absorbance. The absorbance reader was performed in 610 nm (ANEXO J)

Statistical Analysis

Statistical analysis was performed on the software SigmaPlot version 12.0, at a significance level of 5%. Data analysis considered the types of experimental toothpastes and the time of sample collection. Fluoride (Log_{10} transformed) and calcium (raw) data passed normality (Shapiro-Wilk) and homogeneity tests (Bartlett), and were submitted to two-way, repeated-measures ANOVA, followed by the Student-Newman-Keuls test.

RESULTS

Table 1 shows mean F concentrations in the experimental dentifrices, which presented a maximum variation within 10% according to the allowed for dentifrices.

A dose-response relationship was observed between fluoride concentrations in the dentifrices and the resulting levels in the biofilm fluid (Table 2). Significant differences were observed among the dentifrices ($F=16.7$, $p<0.001$) and time after brushing ($F=38.0$, $p<0.001$), with a significant interaction between the two variables ($F=4.2$, $p=0.003$). For samples collected 12 hours after brushing, no significant differences were observed among the dentifrices, with mean values of 5.8 ± 3.1, 14.5 ± 12.9, 8.3 ± 6.5, 11.8 ± 7.5, 13.5 ± 10.0 µM for placebo, 550F, 550F-CaGP, 550F-TMP and 1,100F, respectively. For samples obtained 1 hour after brushing, the highest F concentration (µM) was observed for 1,100F dentifrice (45.4 ± 22.8) and significant differences were observed among Placebo (5.1 ± 3.3), 550 (31.2 ± 26.4) and 1,100F (45.4 ± 22.8) toothpastes. No significant differences were verified among 550F (31.2 ± 26.4), 550F-CaGP (23.5 ± 11.5) and 550F-TMP (24.2 ± 22.7).

As for Ca concentrations in the biofilm fluid, significant differences were observed only among the dentifrices ($F=3.59$, $p<0.013$) with no significant differences for time after brushing ($F=2.37$, $p<0.151$) and no interaction between the two variables ($F=1.90$, $p=0123$), as shown in Table 3. No defined trend was observed among the groups, with the highest values seen for Placebo and 550F-CaGP. Significant differences were observed among

Placebo and 1,100F and Placebo and 550F-TMP, with no differences among 550F, 1,100F, 550 TMPF and 550F-CaGP.

DISCUSSION

The addition of calcium and/or phosphate salts to fluoridated toothpastes has been proposed to reduce F concentration in the products without compromising their anticaries effect when compared to a conventional dentifrice, in order to minimize F intake from this source, and consequently reducing the risk of dental fluorosis. There is strong evidence on the effects of dental products supplemented with TMP [Takeshita et al., 2009; Favretto et al., 2013; Danelon et al., 2014] and CaGP [Amaral et al., 2013; Zaze et al., 2014a, b] on the dynamics of dental caries using different methods, but information about the mechanism of action of these compounds is still lacking. The present study showed that the addition of TMP or CaGP to low-fluoride toothpastes did not increase the availability of F and Ca in the fluid phase of biofilms formed *in situ* under cariogenic challenge, leading to the rejection of the study's hypothesis.

Literature has shown that the supplementation of low-fluoride toothpastes with TMP or CaGP has a marked effect on enamel mineral composition, leading to a higher degree of surface hardness and decreased loss of integrated subsurface hardness. The use of a 500 µg F/g toothpaste associated with TMP at concentrations higher than 0.5% increased surface hardness and decreased ΔKHN when compared to the negative control, with the greatest effect observed for the low-F toothpaste containing 3% TMP (190% higher) [Takeshita et al., 2009]. As for CaGP, the addition of 0.25% of this salt to a low-fluoride dentifrice decreased mineral loss by 132%, reaching greater protective effect than that seen for the positive control *in vitro* [Zaze et al., 2014b]. The same toothpaste resulted in a 44% decrease in enamel surface hardness change *in situ* in comparison with its counterpart without CaGP, having a similar effect than a 1,100 ppm F toothpaste [Amaral et al., 2013].

The information above is in line with a mechanism recently proposed, according to which TMP seems to act as a partial barrier to acids, by binding to enamel and forming a "network" able to retain fluoride compounds that are released during subsequent cariogenic challenges [Manarelli et al., 2014]. The

authors demonstrated that while the effects of TMP alone are negligible, it has a synergistic effect with fluoride, which gives support to the above-mentioned hypothesis. The same trend was later confirmed by another investigation showing that gels containing 1% NaF and 5% TMP were able to inhibit enamel demineralization similarly to a 2% NaF gel, while TMP alone did not reduce demineralization [Danelon et al., 2014]. Regarding CaGP, the possible anticaries mechanism has been suggested to be related to interactions with enamel during the de/remineralizing process in a similar fashion as TMP [Amaral et al., 2013; Zaze et al., 2014a, b]. It has been also suggested that the availability of Ca and F in the enamel from the fluoridated dentifrices associated with CaGP was the main factor to improve the ability of remineralization [Zaze et al., 2014a].

Despite the growing body of evidence on the synergistic effects of fluoride and TMP or CaGP when added to toothpastes on enamel mineral composition, little is known about the effects of these salts on the dental biofilm. The use of low-fluoride formulations containing these salts was shown to significantly reduce the formation of extracellular polysaccharides, as well as to significantly raise F and Ca concentrations in biofilms formed *in situ*, in comparison to the 550 ppm F without TMP or CaGP [Amaral et al., 2013; unpublished data]. These positive results led to the assumption that the increased levels of F and Ca in the biofilm would be reflected in a higher availability of these ions in the fluid phase of the biofilm, but such effect was not confirmed in the present study. Although significant differences were seen in F levels in the biofilm fluid 1 h after brushing with Placebo, 550 and 1,100 dentifrices (what validates the method used), no significant differences were observed among toothpastes containing 550 ppm F, regardless the addition or not of CaGP or TMP. These findings are in line with previous data on the effects of CaGP when added to a conventional dentifrice (1,500 µg F/g), showing no significant differences between toothpastes with or without CaGP at 0.13% regarding F, Ca and inorganic P concentrations in the biofilm fluid [Tenuta et al., 2009a]. Although literature reports an increase in the total biofilm mineral ions after the addition of Ca, Pi and F supplements [Pearce et al., 1999], this increase was not observed for the biofilm fluid, which might suggest the existence of equilibrium or a homeostatic mechanism that maintains ion

concentration in the biofilm fluid, regardless of its concentration in the whole biofilm, as previously hypothesized [Tenuta et al., 2006].

For samples collected 12 hours after brushing, no significant differences were noted among the dentifrices, indicating that all fluoride retained in the biofilm after brushing was slowly released over time, returning to baseline levels afterwards. This result is consistent with previous data showing higher F concentrations in whole biofilm samples one hour after brushing with F dentifrices, which returned to baseline (placebo) values 12 h after the use of a conventional toothpaste [Whitford et al., 2002]. Also, fluoride concentrations in saliva and dental biofilm collected 8 h after the last use of fluoride products (dentifrices and fluoride solution) did not differ among treatments (placebo, 1,100 ppm F and 1,100 ppm F + fluoride solution), suggesting that F products for home-use have no long-term effect on fluoride concentrations in saliva and in dental biofilm, mainly in residents of an area with a fluoridated water supply [Souza et al., 2014].

Although it is not possible to make a direct comparison between whole biofilm F concentrations after the use of LFDs supplemented with CaGP [Amaral et al., 2013] or TMP [unpublished data] with the present results in the biofilm fluid, the discrepancies between the present results and the above-mentioned studies are evident. The only methodological difference in the study protocol was the mode of application of the dentifrices. While a dentifrice:water slurry (1:3) was dripped *ex vivo* directly on enamel blocks during 1 minute [Amaral et al., 2013], in the present study volunteers were instructed to brush their teeth with the device in the oral cavity in order to produce a natural dentifrice:saliva slurry, which was later squished in the oral cavity during 30 seconds. Given that penetration of fluoride into biofilms is highly dependent on biofilm's thickness [Watson et al., 2005] and that fluoride is mainly restricted to the biofilm/saliva interface after brushing with conventional or low-fluoride toothpastes in thick biofilms (as in the present study) [Pessan et al., 2014], it is likely that the mode of application (dentifrice foam *versus* dentifrice slurry) and exposure time (30 *versus* 60 sec) might have played a major role in the results obtained in the present study. This aspect is of outermost relevance and raises questions on the *in situ* models currently used to assess the effects of fluoride

toothpastes on the dynamics of dental caries, and therefore should be better investigated in future studies.

As for Ca concentrations in the biofilm fluid, no significant differences were observed for time after brushing. Surprisingly, the highest values of Ca concentration were obtained for placebo and 550 CaGP, lowest were found for 550 F – TMP and 1100. The reasons for this discrepancy are not known. However, considering that F is retained on the biofilm mediated by Ca bindings on bacteria walls [Rose et al., 1996], it is possible that brushing the teeth with a conventional dentifrice (1100 ppm) allowed F binding to Ca present in the biomass (mainly bacterial surfaces), further allowing ionic Ca present in biofilm fluid to bind to the biomass as well, which would, in turn, reduce Ca concentrations in the biofilm fluid. On the other hand, as Placebo has no fluoride, the above-mentioned mechanism would not occur, so that ionic Ca would remain free in the fluid phase of the biofilm. Moreover, the high values found for the 550CaGP dentifrice suggest that CaGP can be considered as a source of free calcium [Lynch, 2004], what may help to explain the anticaries effect of this toophaste in caries progression in children [Amaral et al., 2014].

Although the study's hypothesis was rejected, this investigation provided additional information for better understanding of the mechanisms of F and Ca uptake by biofilm fluid. In this sense, the lack of synergistic effect between F and TMP or CaGP in the biofilm fluid seen in the present study along with previous *in vitro* and *in situ* data clearly indicate that the anticaries effects of LFDs supplemented with these salts are more related to the interaction of these salts with tooth enamel than with an increased availability of F and Ca ions in the biofilm fluid. Further studies should be carried out to complement this *in situ* investigation, in order to clearly verify the clinical benefits provided by the supplementation of LFDs with phosphate salts, as well as to provide stronger evidence on the mechanisms of action of toothpastes containing TMP or CaGP.

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TABLE LEGENDS

Table 1. Mean (SD) concentrations (µg/g) in the experimental Fluoride toothpastes

Table 2. Fluoride concentrations in the biofilm fluid formed *in situ* 12 hours after brushing and 1 hour after brushing and cariogenic challenge (30% sucrose solution), according to the dentifrices used in each experimental phase

Table 3. Calcium concentrations in the biofilm fluid formed *in situ* 12 hours after brushing and 1 hour after brushing and cariogenic challenge (30% sucrose solution), according to the dentifrices used in each experimental phase

Table 1. Mean (SD) concentrations (µg/g) in the experimental Fluoride toothpastes

Dentifrices	Concentration (µg/g)	
	Total fluoride	Ionic fluoride
Placebo	11.6 (1.5)	11.1 (1.2)
550F	521.3 (27.7)	550.0 (12.6)
550F - CaGP	523.1 (21,8)	549.5 (5.3)
550F - TMP	553.1 (4.4)	558.6 (11.3)
1,100F	1100 (48.6)	1119.5 (19.6)

Table 2. Fluoride concentrations (µM) in the biofilm fluid formed *in situ* 12 hours after brushing and 1 hour after brushing and cariogenic challenge (30% sucrose solution), according to the dentifrices used in each experimental phase

Dentifrices	**Placebo**		**550F**		**550F-CaGP**		**550F-TMP**		**1,100**	
Time after brushing	*12h*	*1h*	*12h*	*1h*	*12h*	*1h*	*12h*	*1h*	*12h*	*1h*
Mean	5.8	5.1[a]	14.5	31.2[b]	8.3	23.5[b]	11.8	24.2[b]	13.5	45.4[c]
SD	3.1	3.3	12.9	26.4	6.5	11.5	7.5	22.7	10.0	22.8

Lowercase superscript letters indicate significant differences among the dentifrices 1 h after brushing. No significant differences were observed among the groups 12 h after brushing. Two-way ANOVA (data log transformed) and Student-Newman-Keuls test ($p<0.05$), n=12.

Table 3. Calcium concentrations (mM Ca) in the biofilm fluid formed *in situ* 12 hours after brushing and 1 hour after brushing and cariogenic challenge (30% sucrose solution), according to the dentifrices used in each experimental phase

Dentifrices	**Placebo**[a]		**550F**[a,b]		**550F-CaGP**[a,b]		**550F-TMP**[b]		**1,100**[b]	
Time after brushing	*12h*	*1h*	*12h*	*1h*	*12h*	*1h*	*12h*	*1h*	*12h*	*1h*
Mean	123.1	148.7	112.6	93.4	119.9	135.0	76.2	106.4	86.6	100.5
SD	63.0	58.0	68.5	34.0	42.3	34.1	31.5	27.4	32.3	35.4

Lowercase superscript letters indicate significant differences among the dentifrices. Two-way ANOVA and Student-Newman-Keuls test ($p<0.05$), n=12.

Anexos

ANEXO A

INSTRUÇÕES AOS AUTORES

Caries Research

Guidelines for Authors
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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
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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).

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

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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:

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- 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;
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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 inlcuding relevant grant numbers. Please also include this sentence: "The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript." If this statement is not correct, you must describe the role of any sponsors or funders, and amend the sentence as needed. Additionally, the roles of all authors must be described (For example: Conceived and designed the experiments: AA, BB. Performed the clinical examination: AA, CC. Performed the experiments: DD, FF. Analyzed the data: BB, FF. Wrote the paper: AA, CC, FF, EE).

Legends: The table headings should be listed first, followed by the legends for the illustrations.

Tables: Tables should be numbered in Arabic numerals. Each table should be placed on a separate page. Tables should not be constructed using tabs but by utilising the table facilities of the word-processing software.

Illustrations:

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

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

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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].

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The reference list should include all the publications cited in the text, and only those publications. References, formatted as in the examples below, should be arranged in strict alphabetical order. All authors should be listed. For papers by

the same authors, references should be listed according to year. Papers published by the same authors in the same year should be distinguished by the letters a, b, c, ... immediately following the year, in both the text citation and the reference list. For abbreviation of journal names, use the Index Medicus system. For journals, provide only the year, volume number and inclusive page numbers.

Examples

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

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

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

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

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

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

Supplementary Material

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

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ANEXO B

COMITÊ DE ÉTICA

FACULDADE DE ODONTOLOGIA - CÂMPUS DE ARAÇATUBA - JÚLIO DE

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Concentração de flúor e cálcio no biofilme dentário e fluido do biofilme associado ao uso de dentifrícios fluoretados suplementados com Trimetafosfato de Sódio ou Glicerofosfato de cálcio sob desafio cariogênico.

Pesquisador: Mariana Emi Nagata

Área Temática:

Versão: 4

CAAE: 20146313.7.0000.5420

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

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 612.688

Data da Relatoria: 28/03/2014

Apresentação do Projeto:

O projeto apresenta-se adequadamente.

Objetivo da Pesquisa:

O presente estudo tem por objetivo avaliar in situ a concentração de F e Ca no biofilme dentário e no fluido do biofilme formados sob desafio cariogênico após o uso de dentifrícios contendo F, suplementados ou não com trimetafosfato de sódio (TMP) ou glicerofosfato de cálcio (CaGP).

Avaliação dos Riscos e Benefícios:

Riscos: Os voluntários utilizarão dentifrícios convencionais e aparelhos intrabucais durante um total de 35 dias. Este dispositivo palatino é semelhante a um aparelho utilizado com finalidade ortodôntica, sendo o mesmo construído com os mesmos materiais e critérios de biossegurança para tal. Os riscos podem ser considerados como mínimos, sendo garantido ao voluntário o direito de desistir do estudo a qualquer momento.

Benefícios: Será possível observar se o dentifrício com reduzida concentração de flúor e suplementação com TMP ou CaGP é tão efetivo quanto um dentifrício de alta concentração de flúor.

Endereço: JOSE BONIFACIO 1193
Bairro: VILA MENDONCA **CEP:** 16.015-050
UF: SP **Município:** ARACATUBA
Telefone: (18)3636-3200 **Fax:** (18)3636-3332 **E-mail:** anacmsn@foa.unesp.br

FACULDADE DE ODONTOLOGIA - CÂMPUS DE ARAÇATUBA - JÚLIO DE

Comentários e Considerações sobre a Pesquisa:

Através da realização desta pesquisa, será possível observar se o dentifrício com reduzida concentração de flúor e suplementação com TMP ou CaGP é tão efetivo quanto um dentifrício.

Considerações sobre os Termos de apresentação obrigatória:

Os termos de apresentação obrigatória foram apresentados adequadamente.

Recomendações:

Nenhuma.

Conclusões ou Pendências e Lista de Inadequações:

A pesquisadora responsável acatou as sugestões do CEP, corrigindo o que lhe foi solicitado, portanto não havendo mais pendências,somos favorável à APROVAÇÃO deste projeto.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

O CEP acata o parecer do relator e salienta a necessidade de apresentação de relatórios semestrais, a partir desta data.

ARACATUBA, 11 de Abril de 2014

Assinador por:
Ana Claudia de Melo Stevanato Nakamune
(Coordenador)

Endereço: JOSE BONIFACIO 1193
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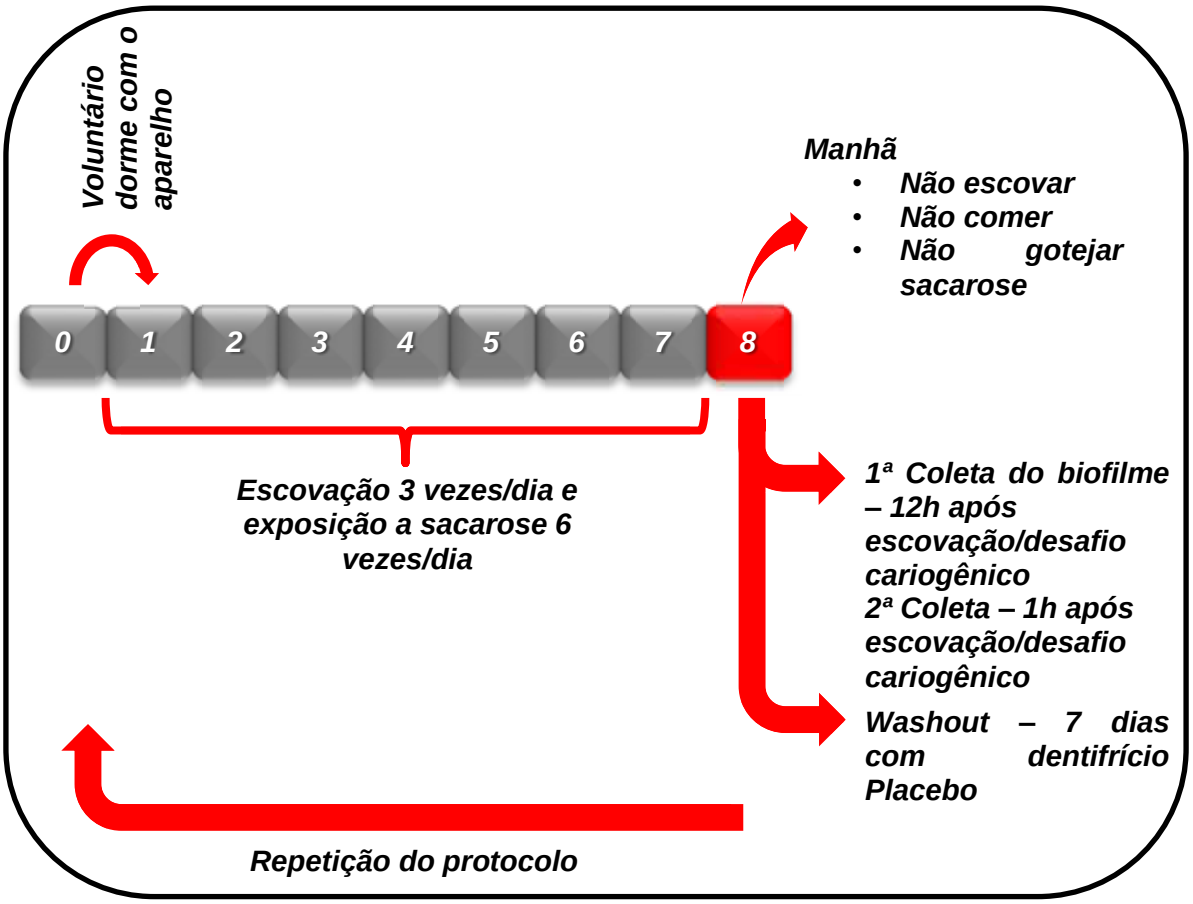
Telefone: (18)3636-3200 **Fax:** (18)3636-3332 **E-mail:** anacmsn@foa.unesp.br

ANEXO C

ESQUEMA REPRESENTATIVO DO PROTOCOLO DE TRATAMENTO



ANEXO D

INSTRUÇÕES AOS VOLUNTÁRIOS

1) O dispositivo intrabucal deve ser utilizado durante todo o dia e à noite, sendo removido da boca apenas durante as refeições ou quando o voluntário for ingerir algo, inclusive água.
2) Quando estiver fora da boca, em nenhum momento o dispositivo deve ser deixado a seco. Guarde-o na caixinha com uma gaze umedecida com água deionizada.
3) Procure evitar que o dispositivo fique fora da boca por um período prolongado, restringindo-se ao tempo necessário para a alimentação (máximo de 1 hora).
4) Durante toda a fase experimental, utilize o dentifrício fornecido pela pesquisadora.
5) Realize a higiene bucal (3x ao dia) com o mesmo dentifrício da fase experimental e com o dispositivo palatino dentro da boca.
6) Escove apenas a porção do dispositivo que fica em contato com o palato (nunca a parte em contato com a língua), para isso utilize o dentifrício e enxágue com a água.
7) Não utilize produtos para bochecho ou outros agentes tópicos de qualquer natureza na cavidade bucal durante a fase experimental.
8) Não utilize vitaminas ou suplementos sistêmicos que contenham flúor durante a fase experimental.
9) Quando o dentifrício, gaze ou água deionizada estiver acabando, entre em contato com a pesquisadora responsável para que sejam repostos.

Fase de desmineralização

1) Gotejar a sacarose seguindo os horários descritos, totalizando 6x ao dia. (8:00/ 11:00/14:00/17:00/19:00/21:00)
2) Para fazê-lo, remova o dispositivo da boca, coloque sobre a caixinha do aparelho e goteje duas gotas da solução sobre cada bloco de esmalte, sem tocar a ponta do conta-gotas no dispositivo para evitar a contaminação da solução. Aguarde 5 minutos, para que a sacarose se difunda pela placa bacteriana, e retorne à cavidade bucal.
3) Para realizar o tratamento com dentifrício, fazer a escovação normalmente com o dispositivo palatino na cavidade oral, por 1 minuto. Durante a escovação haverá a formação do “slurry” (mistura de dentifrício e saliva). Deixe o mesmo em contado com o aparelho por 30 segundos (fazendo bochechos) e a seguir enxágue delicadamente a cavidade bucal. **O dispositivo não pode ser escovado na parte que contém os blocos de esmalte e a tela para acúmulo de biofilme.** (Tratamento/escovação - 8:00/ 13:00/21:30). Após esta etapa, o voluntário poderá completar sua higiene bucal sem o aparelho na boca.
4) Quando o horário de gotejamento coincidir com um período em que o dispositivo estiver fora da boca, realize o gotejamento 5 minutos antes de retornar o dispositivo para a boca. Os gotejamentos seguintes devem ser

realizados no horário pré-determinado. **Não goteje a sacarose e deixe por mais de 5 minutos sem colocar o dispositivo na boca.**

5) A solução de sacarose deverá permanecer em geladeira na maior parte do tempo possível. Retirar da geladeira 5 minutos antes de aplicar sobre o bloco, pois este procedimento deve ser realizado a temperatura ambiente (nunca colocar a solução resfriada sobre o biofilme!).
6) O acúmulo de biofilme sob a tela plástica nesta fase é desejável; **não** tente removê-la de forma alguma.
7) Qualquer dúvida entrar em contato com a pesquisadora responsável (Mariana – (18) 41413169/ (43) 9958-8373).

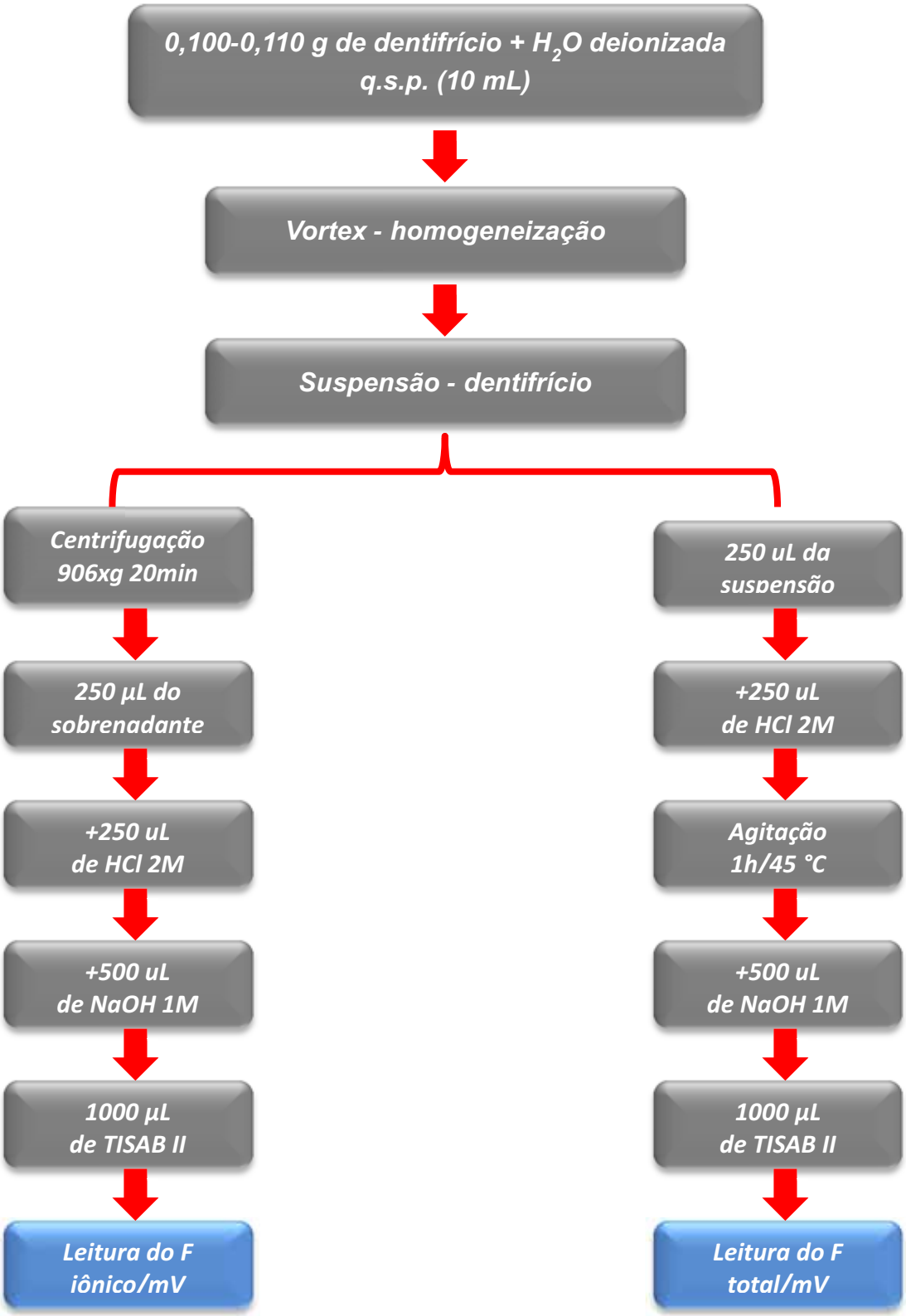
ANEXO E

1. Dentifrícios experimentais codificados por pesquisador não envolvido no experimento– Placebo, 550 µg F/g, 1100 µg F/g, 550 µg F/g + CaGP 0,25%, 550 µg F/g + TMP 1%

2. Kit de tratamento contendo escova dental, dentifrício experimental, solução de sacarose, água deionizada, gaze e dispositivo palatino

ANEXO F

ESQUEMA REPRESENTATIVO DA DOSAGEM DO F NOS DENTIFRÍCIOS



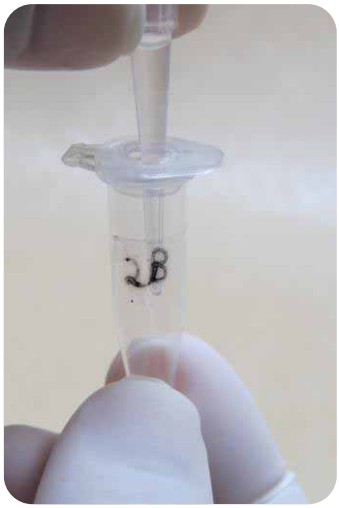
ANEXO G

PREPARO DO CONJUNTO ACONDICIONADOR DE BIOFILME

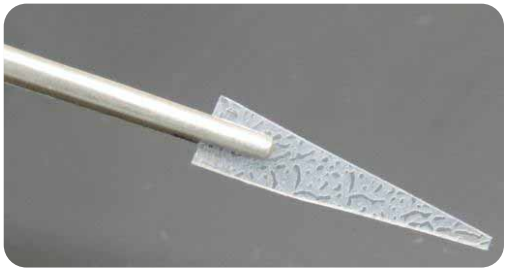
3. Confecção do orifício na tampa de um eppendorf com o auxílio de um soldador

4. Selamento da ponta da ponteira de 10 µL com chama de lamparina

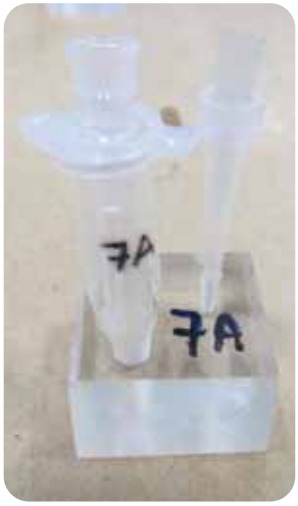
5. Inserção de óleo mineral na ponteira selada



6. Inserção da ponteira com óleo mineral no eppendorf perfurado



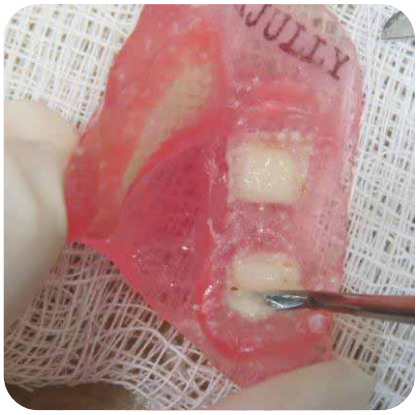
7. Espátula plástica para transferência do biofilme



8. Conjunto acondicionador do biofilme

ANEXO H

COLETA DO BIOFILME



9. Coleta do biofilme com auxílio de uma espátula Hollemback

10. Transferência do biofilme do Hollemback para a espátula plástica

11. Inserção da espátula com biofilme em óleo mineral

12. Conjunto para auxiliar a pesagem do biofilme

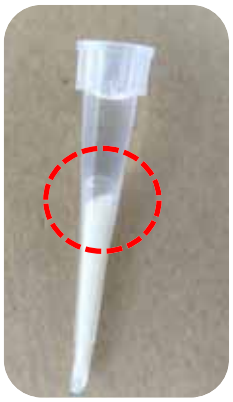
13. Pesagem do conjunto após a coleta do biofilme na balança de 5 casas (Shimadzu)

ANEXO I

PROCESSAMENTO E LEITURA DO F DO FLUIDO DO BIOFILME

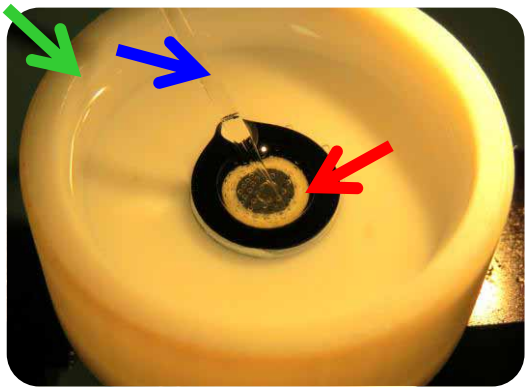
14. Centrífuga Combi 514-R

15. Centrifugação para obtenção do fluido do biofilme: 21023 g/ 4 ºC/ 5 minutos

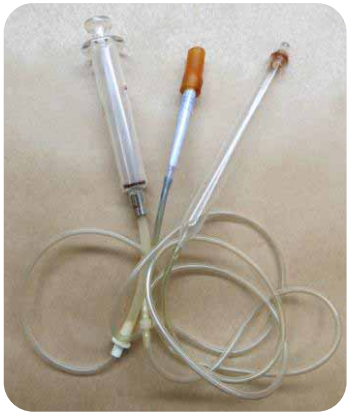


16. Aspecto após centrifugação com separação do fluido do biofilme da massa sólida do biofilme total

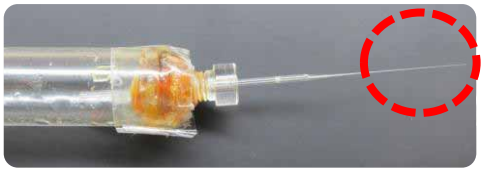
17. Aparelho utilizado para leitura de flúor + analisador de íons



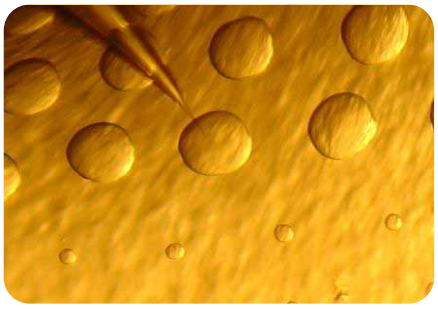
*18. Cubeta de teflon **(seta verde)** + eletrodo de referência **(seta azul)** + eletrodo invertido e sua respectiva membrana **(seta vermelha)** no centro da cubeta*



19. Aparato para utilização da micropipeta de vidro



20. Suporte e micropipeta de vidro

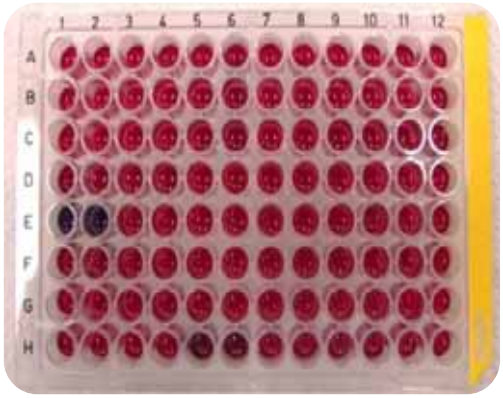


21. Vista aproximada do eletrodo de referência no interior do fluido do biofilme, tamponado com TISAB III sobre a superfície do eletrodo Orion 9409, sob óleo mineral

ANEXO J

ANÁLISE DE CÁLCIO NO FLUIDO - ESPECTROFOTOMETRIA

22. Leitor de microplacas



23. Placa de 96 poços com água deionizada + amostra + Arsenazo III