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JOÃO GABRIEL SILVA SOUZA

EFEITO DO PRÉ-BOCHECHO COM CÁLCIO NA POTENCIALIZAÇÃO DA INIBIÇÃO DA DESMINERALIZAÇÃO DO ESMALTE POR BOCHECHO FLUORETADO

EFFECT OF CALCIUM PRERINSE ON THE ENHANCEMENT OF FLUORIDE MOUTHRINSE ON THE INHIBITION OF ENAMEL DEMINERALIZATION

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Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestre em Odontologia, na Área de Cariologia.

Dissertation presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Master in Dentistry, in Cariology area.

Orientadora: Profa. Dra. Livia Maria Andaló Tenuta

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A Ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

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RESUMO

Visando potencializar o efeito anticárie do fluoreto, aumentando sua retenção na cavidade bucal (saliva, biofilme dental) após o uso de dentifrícios ou bochechos, a utilização prévia de um bochecho contendo cálcio tem sido preconizada. Embora esse pré-bochecho, seguido do uso de solução fluoretada, esteja associado a um aumento significativo na concentração de fluoreto na saliva e biofilme dental, seu efeito anticárie ainda não foi comprovado experimentalmente. Assim, o objetivo deste trabalho foi verificar se um pré-bochecho contendo cálcio potencializa o efeito do fluoreto na redução da desmineralização do esmalte dentário. Para tal, foi realizado um experimento in situ, duplo cego e cruzado, conduzido durante 4 fases de 14 dias cada, no qual 10 voluntários utilizaram dispositivos intrabucais palatinos contendo blocos de esmalte bovino hígidos. Sacarose a 20% foi gotejada sobre os blocos na frequência de 8 vezes ao dia simulando um alto desafio cariogênico. Duas vezes por dia os voluntários realizaram bochechos com: (1) pré-bochecho placebo para cálcio (lactato de sódio) seguido de bochecho placebo para fluoreto (água purificada) (controle negativo); (2) pré-bochecho placebo para cálcio seguido de bochecho contendo 250 ppm F; (3) pré-bochecho placebo para cálcio seguido de bochecho contendo 1000 ppm F; (4) pré-bochecho com cálcio (150 mM de lactato de cálcio), seguido de bochecho contendo 250 ppm F. As variáveis-resposta estudadas foram: concentrações de fluoreto no fluido e na porção sólida do biofilme; nos blocos dentais: porcentagem de perda de dureza de superfície (%PDS), área de lesão de cárie e concentração de fluoreto fracamente e firmemente ligado. O grupo Ca + F não diferiu do grupo 1000 ppm F na perda mineral resultante (ANOVA, $p > 0{,}05$), mas ambos diferiram dos demais grupos ($p < 0{,}05$). A concentração de F na porção sólida do biofilme exposto ao tratamento Ca + F foi 22 vezes maior quando comparado ao uso de apenas F (250 ppm F), sendo significativamente mais alto do que todos os grupos ($p < 0{,}05$). No entanto, no fluido, o aumento de 2 vezes na concentração de F na comparação do grupo Ca + F e 250 ppm F não foi significativo ($p > 0{,}05$). Não houve efeito significativo do grupo Ca+F no aumento das concentrações de F no esmalte. Conclui-se que o pré-bochecho com cálcio potencializou o efeito do fluoreto na redução da desmineralização, o que parece estar ligado à sua capacidade de aumentar a concentração de fluoreto no biofilme.

Palavras-chave: Flúor. Fluoreto de Sódio. Biofilmes. Desmineralização do dente.

ABSTRACT

A calcium (Ca) pre-rinse has been recommended to enhance the anticaries effect of fluoride by increasing its retention in the oral cavity (saliva, dental biofilm) after the use of toothpastes or mouthrinses. Although this pre-rinse, followed by the use of fluoride rinse, has been associated with a significant increase in the fluoride concentration in saliva and dental biofilm, its anticaries effect has not yet been demonstrated experimentally. Thus, the objective of this study was to verify if a calcium pre-rinse potentiates the effect of fluoride on the reduction of enamel demineralization. In a double-blind, crossover, in situ study in 4 phases of 14 days each, ten volunteers wore a palatal appliance containing enamel slabs. Sucrose solution (20%) was dripped onto the slabs 8 times/day to simulate a high cariogenic challenge. Twice/day volunteers rinsed with: (1) A Ca placebo pre-rinse (sodium lactate) followed by a distilled water rinse (negative control); (2) A placebo pre-rinse followed by a 250 ppm F rinse; (3) A placebo pre-rinse followed by a 1000 ppm F rinse; or (4) A Ca pre-rinse (150 mM Ca as Ca lactate), followed by a 250 ppm F rinse. Response variables were: in the biofilm: F concentrations in the fluid and solid; in enamel slabs: percentage of surface hardness loss (%SHL), cross-sectional hardness and F concentration in enamel (loosely-bound and firmly-bound). The Ca+ F group resulted in similar mineral loss than the 1000 ppm F group (ANOVA, $p>0.05$), but both differed significantly from the other groups ($p<0.05$). The F concentration in the solids of the biofilm treated with Ca + F was ≈22 times greater when compared to using only F (250 ppm F), being significantly higher than all other groups ($p<0.05$). However, in the biofilm fluid, the twice increase in F concentration caused by the Ca + F treatment when compared to the 250 ppm F group was not significant. The Ca pre-rinse did not increase F concentrations in the enamel. In conclusion, the Ca pre-rinse followed by an F rinse enhances the effect of F on the inhibition of enamel demineralization, which seems to be linked to its ability to increase the F concentration in the biofilm.

Keywords: Fluoride; Calcium fluoride; Biofilm; Demineralization.

SUMÁRIO

1 INTRODUÇÃO

A cárie dentária é conceituada como uma doença biofilme-açúcar dependente que resulta na perda mineral da estrutura dentária (Fejerskov, 2004; Fejerskov e Kidd, 2005). Em contrapartida a esses fatores negativos (biofilme e açúcar), o uso de fluoreto (F) tem sido indicado como fator determinante positivo para o controle da cárie dentária, reduzindo a desmineralização e potencializando a remineralização dental, culminando, portanto, com redução da progressão e aceleração da reversão de lesões de cárie (Ekstrand et al., 1990; Fejerskov e Kidd, 2005; Cury e Tenuta, 2008). O declínio de cárie observado nas últimas décadas tem sido atribuído ao amplo uso de fluoretos (Brathall et al., 1996). Sua eficácia anticárie tem sido demonstrada por revisões sistemáticas da literatura avaliando diferentes meios de utilização do F, como dentifrícios e bochechos fluoretados (Marinho et al., 2003a; 2003b).

O mecanismo pelo qual o fluoreto controla o desenvolvimento de lesões de cárie está baseado na sua manutenção nos fluidos bucais, principalmente no fluido do biofilme, onde ocorre o processo de perda mineral (Featherstone, 2000; Vogel, 2011). A concentração de fluoreto no fluido do biofilme aumenta logo após a exposição a agentes fluoretados (Cury et al., 2010; Vale et al., 2011), mas pode também ser mantida elevada pela liberação a partir de reservatórios de fluoreto formados durante a aplicação desses produtos. No biofilme, o fluoreto pode estar retido de duas formas: ligado a superfície bacteriana através de íons cálcio (chamado de reservatório biológico) (Rose et al., 1996); ou precipitado na forma de minerais, como fluoreto de cálcio ("CaF$_2$") e fluorapatita (Vogel, 2011). Quando na forma ligada a superfície bacteriana, a concentração de fluoreto será função da sua concentração no fluido do biofilme, liberando o íon mediante sua diminuição no biofilme (Vogel, 2011). No entanto, o fluoreto retido na forma de "CaF$_2$" pode ser um reservatório mais duradouro de F, liberando-o lentamente para agir nos processos de des- e remineralização dentária (Vogel, 2011). Porém, a formação de reservatórios do tipo "CaF$_2$" no biofilme dental não tem sido alcançada a partir do uso de veículos de uso diário, como os bochechos fluoretados (Vogel et al., 2010), devido a baixa concentração prévia de cálcio presente no fluido do biofilme (Vogel, 2011), que é inferior a concentração de fluoreto disponibilizada pelos bochechos.

Recentemente foi proposta na literatura uma forma de superar esta limitação e aumentar a formação desses reservatórios de fluoreto no biofilme dental (Vogel et al., 2008a, 2008b, 2014), principalmente na forma de "CaF$_2$" (Vogel et al., 2014). Este fluoreto de cálcio é formado no biofilme dental por um bochecho com solução contendo cálcio, visando aumentar a concentração de cálcio livre na cavidade bucal, previamente a utilização de um produto fluoretado, e consequentemente favorecer a formação de fluoreto de cálcio (Vogel et al., 2014). Estudos in vivo avaliando a capacidade de enriquecimento do biofilme dental com fluoreto utilizando esse protocolo (pré-bochecho com cálcio seguido de bochecho com solução fluoretada) demonstraram aumentos na concentração de fluoreto da ordem de 12 vezes no biofilme total e 5 vezes no fluido do biofilme (Vogel et al., 2008a; Vogel et al., 2014). Porém, o efeito anticárie deste protocolo no esmalte dental ainda não tem sido bem esclarecido na literatura.

Atualmente, os únicos estudos disponíveis que avaliaram o efeito de um pré-bochecho com cálcio na des- e remineralização do esmalte (Magalhães et al., 2007; Furlani et al., 2009) não comprovaram a superioridade desse tratamento em relação ao uso isolado do F. Estes estudos identificaram que a utilização prévia de produto contendo cálcio resulta em um aumento significativo da concentração de fluoreto no biofilme. No entanto, na avaliação de perda ou ganho de mineral, o grupo controle, sem F, não diferiu do grupo utilizando apenas fluoreto (Magalhães et al., 2007; Furlani et al., 2009), indicando falta de poder para diferenciar os grupos experimentais pela falta de dose-resposta ao fluoreto do modelo de estudo utilizado. Ressalta-se ainda que estes estudos utilizaram o fluoreto em forma de dentifrício, porém, estudos prévios evidenciaram o aumento dos reservatórios de fluoreto de cálcio no biofilme dental a partir da utilização de um pré-bochecho com cálcio seguido do uso de solução fluoretada em forma de bochecho (Vogel et al., 2008a, 2008b, 2014). Dentifrícios contém detergentes, como o lauryl sulfato de sódio, que diminuem a formação de "CaF$_2$"(Barkvoll et al., 1988), o que pode ter influenciado os resultados e impede a avaliação isolada do efeito da combinação entre cálcio e fluoreto.

Assim, o objetivo deste trabalho foi verificar o potencial de inibição de desmineralização do esmalte de um pré-bochecho com cálcio, seguido de um bochecho fluoretado, utilizando um modelo de estudo que apresente dose-resposta ao F, bem como investigar os mecanismos que explicam seu efeito.

2 ARTIGO*

Ca pre-rinse before F rinse reduces enamel demineralization – an in situ caries study

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Short Title: Ca pre-rinse enhances the fluoride anticaries potential

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Declaration of Interests

G.L.V. (ADA Foundation) holds a patent for the Ca pre-application technique: U.S. Patent No. 9,011,823 on April 21, 2015. There are no other conflicts of interest with respect to the authorship and/or publication of this article.

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ABSTRACT

A calcium (Ca) pre-rinse before a fluoride (F) rinse has been shown to increase oral F levels. We tested the anti-caries effect of this combination in a dose-response in situ caries model. In a double-blind, crossover experiment, 10 volunteers carried enamel slabs in palatal appliances for 14 days, during which they rinsed twice/day with one of the four rinse combinations: (1) A placebo pre-rinse (sodium lactate) followed by a distilled water rinse (negative control); (2) A placebo pre-rinse followed by a 250 ppm F rinse; (3) A placebo pre-rinse followed by a 1000 ppm F rinse; (4) A Ca pre-rinse (150 mM Ca, as calcium lactate), followed by a 250 ppm F rinse. Sucrose solution was dripped onto the slabs 8x/day to simulate a high cariogenic challenge. The reduction of surface hardness loss (SHL) caused by the Ca pre-rinse used with the 250 ppm F rinse (%SHL=38.0±21.0) was significantly higher than that caused by the F rinse alone (%SHL=59.5±24.1) and similar to the 1000 ppm F rinse (%SHL=42.0±18.3). Compared to the 250 ppm F rinse, the Ca pre-rinse increased biofilm fluid F only twice (non-significant), however, in accordance with previous studies, it greatly increased F in biofilm solids (≈22x). The Ca pre-rinse had little effect on loosely- or firmly-bound enamel F. The results showed an increased level of protection against demineralization by the use of a Ca pre-rinse, which seems to be caused by the enhancement of F concentration in the biofilm.

Introduction

The worldwide decline in dental caries of recent decades [Nadanosvsky and Sheiham, 1995; Marthaler, 2004; Narvai et al., 2006] has been primarily attributed to widespread fluoride (F) use [Bratthall et al., 1996]. However, caries still remains as an important oral health problem [Kassebaum et al., 2015].

The anti-caries effect of F is primarily based on obtaining an elevated F concentration in oral fluids, especially in the fluid phase of the biofilm (biofilm fluid) [Vogel et al., 1990; Margolis and Moreno, 1990]. These increased fluid F levels are able to reduce dental demineralization and enhance remineralization. However, the long-term maintenance of such elevated oral F levels is problematic given the poor oral retention (about 2-3 h) of F following topical administration [Zero et al., 1988].

A Ca pre-application followed by topical F treatment has been shown to produce significantly, persistent increases in F levels in saliva and biofilm [Vogel et al., 2006, 2008a, 2008b, 2014; Chen et al., 2014]. Specifically, when compared to F rinse used alone, the Ca pre-application has been shown to increase F concentration by 12 times in the whole biofilm and 5 times in the biofilm fluid or salivary samples 1 h after the use [Vogel et al., 2006; 2008a]. In samples collected in the morning after evening application, the Ca pre-rinse increased salivary F concentration by 5 times [Vogel et al., 2008b]. Surprisingly, in view of these large increases in oral levels of F, previous in situ studies on the inhibition of enamel demineralization or enhancement of remineralization [Magalhães et al., 2007; Furlani et al., 2009] failed to demonstrate a significant effect of the Ca pre-rinse when used before a F dentifrice. The lack of effect can be explained by the fact that the in situ model used by these authors did not show a dose-response effect for different F concentrations. Furthermore the dentifrices used in these studies contain high concentration of sodium lauryl sulfate that may have precipitated or inactivated the Ca applied by the pre-rinse [Vogel et al., 2006].

The "Piracicaba in situ model" [Benelli et al., 1993; Cury et al., 1997; 2000] has been successfully used to test the anticaries effect of F toothpastes [Cury et al., 2001; Paes Leme et al., 2004; Cury et al., 2010] and products for professional F use [Vale et al., 2011; Calvo et al., 2012]. Unfortunately, the sensitivity of this model to high strength products [Proskin, 1995] has not been evaluated. Therefore, the aims of this study were to test the anticaries effect of the Ca pre-rinse using the

"Piracicaba in situ model" in a "dose response" experimental design that also demonstrates that his model was sensitive to the increased levels of oral fluoride anticipated with this procedure. We hypothesized that the use of a Ca pre-rinse followed by an F rinse would be able to increase the F concentrations in the biofilm fluid and solids, and would also reduce enamel demineralization.

Materials and Methods

Ethical aspects and volunteers

The study was approved by the Piracicaba Dental School (UNICAMP) Research and Ethics Committee (protocol 020/2014). All volunteers signed an informed consent, according to Brazilian ethical regulations. Volunteers, aged 18 to 30 years, were selected based on good general and oral health, as well as normal unstimulated (>0.1 mL/min) and stimulated (>0.7 mL/min) salivary flow rate. Included volunteers did not use antibiotics 1 month prior to the study and were not under orthodontic treatment.

Experimental design

In a double-blind, crossover, in situ design, 10 volunteers wore, in 4 phases of 14 days, a palatal appliance containing four sound bovine enamel slabs covered by a plastic mesh to allow the accumulation of biofilm. The slabs were treated with 20% sucrose solution eight times per day to simulate a high cariogenic challenge. In order to assess the enhancement of F effect by a Ca pre-rinse, and the dose-response of the model to the high levels of F anticipated with the pre-rinse, the following set of two-rinse treatments were tested: 1. a 150 mM sodium lactate pre-rinse (placebo solution for the Ca pre-rinse) followed by a purified water (0 ppm F) rinse (negative control); 2. a 150 mM sodium lactate pre-rinse followed by a 250 ppm F (as NaF) rinse (250 ppm F group); 3. a 150 mM sodium lactate pre-rinse followed by a 1000 ppm F rinse (1000 ppm F group); and 4. a 150 mM calcium lactate pre-rinse followed by a 250 ppm F rinse (Ca+ 250 ppm F group). The rinses were performed twice per day (in the morning and at night), after the oral hygiene with non-F toothpaste, for 1 min each. After 14 days, 10 h after the last exposure to sucrose and treatment solutions, the biofilm formed and the slabs were collected for analyses. Response variables were biofilm fluid F concentration, total concentration of F in the biofilm

solids, percent surface hardness loss (%SHL), area of surface hardness loss (ΔS), and F concentration in enamel as loosely or firmly-bound F. Wash-out periods of at least 7 days were allowed between each phase. ANOVA followed by tukey test was used to compare all groups.

Preparation of enamel slabs and appliance

Bovine teeth, stored in formaldehyde solution 2% (pH 7.0) for at least 30 days [Cury et al., 2000], were used to prepare 4 x 4 x 2 mm enamel slabs. These were polished flat and selected for the study based on their baseline Knoop hardness values [Cury et al., 1997] (average 338.5 ± 17.8), determined using a Future Tech FM-7 microhardness tester, as previously described [Cury et al., 2003]. The slabs were accommodated on cavities in custom-made, acrylic resin appliances, and covered by a plastic mesh to promote biofilm accumulation [Hara et al., 2003].

Treatments

In order to simulate a high cariogenic challenge, volunteers dripped a 20% sucrose solution extra orally on the slabs eight times per day (8:00, 9:30, 11:00, 13:30, 15:00, 16:30, 18:00, 19:30 h) and replaced the appliance in the mouth, after 5 min [Ccahuana-Vásquez et al., 2007]. The Ca pre-rinse was prepared from calcium lactate (Sigma® - C8356) at concentration of 150 mM. Its placebo was a sodium lactate solution (Sigma® L7022) at concentration of 150 mM. The F rinse was prepared from sodium F (Merck® - 106449) at concentrations of 250 and 1000 ppm F (13.16 and 52.63 mM F, respectively). These set of rinses (15 mL each) were used in the morning, before the first sucrose exposure, and at night, after the last sucrose exposure, after oral hygiene with non-F toothpaste. A 30-min period was allowed to pass between morning and evening toothbrushing and the rinses to avoid the influence of the detergent present in toothpaste in the treatments (rinses). Each rinsing solution was sequentially swished in the mouth for 1 minute and then expectorated, with the second rinse being used immediately after the first.

Biofilm analyses

On the 15th day of each experimental phase, in the morning, 10 h after the last rinse, the biofilm and slabs were collected for analysis. The plastic meshes were removed and the biofilm samples covering the enamel slabs were collected, and

immediately immersed in mineral oil to avoid evaporation. The biofilm fluid and solids were separated by centrifugation and the fluid collected using a micropipette, as previously described [Vogel et al., 1992, 2000, 2008a]. A F ion-specific electrode adapted for microanalysis was used to determine F concentration in the biofilm fluid [Vogel et al., 1983, 1990, 2000; Tenuta et al., 2006]. After the fluid separation, the biofilm solids were extracted twice with 0.5 M HCl [Cury et al., 2000; Tenuta et al., 2006] for 60 min. The separate extract was neutralized with NaOH, diluted with TISAB and analyzed using the same methodology used for the fluid [Vogel et al., 1990, 2000; Tenuta et al., 2006]. The concentration in the 2 extracts was summed.

Enamel analyses

The slabs were collected for determination of enamel demineralization and F concentration in enamel as loosely or firmly-bound F. Of the four slabs present in each appliance: (1) all were analyzed for the surface hardness; (2) two were also analyzed for cross-sectional microhardnes; (3) the other two slabs were used to determine F concentration in enamel.

Enamel demineralization determination

Surface hardness of all enamel slabs was re-assessed at 100-μm distance from the initial indentations to determine enamel demineralization. The percentage of surface hardness loss (%SHL) was calculated by: (baseline hardness – final hardness) / baseline hardness x 100.

Two of these slabs were further sectioned to assess cross-sectional hardness, by measuring hardness at 14 distances (from 10 to 300 μm) from the enamel surface. The hardness at each depth was plotted in a graph and the area of hardness loss (ΔS) was calculated using the trapezoidal rule [Cury et al., 2010].

Enamel loosely and firmly-bound F determination

Two slabs were isolated with wax, leaving only the surface area free. They were then immersed in 1M KOH and agitated for 24 h [Caslavska et al., 1975] for extraction of enamel loosely-bound F. After that, slabs were treated with 0.5 M HCl and agitated for 30 s for extraction of enamel firmly-bound F [Koo and Cury, 1998]. F concentrations in both extracts were determined using an ion-specific electrode, after appropriate neutralization and buffering with TISAB II, using standards prepared in

the same conditions of the samples. The results for both loosely and firmly-bound F were calculated as µg F/cm^2.

Statistical analysis

The normality of errors and homocedasticity of data was checked for each response variable, and when necessary the data were transformed: the area of hardness loss (ΔS), F in the biofilm fluid, F in the biofilm solids and F in enamel (loosely- and firmly-bound) were transformed using $\log_{10}$. Groups were compared using ANOVA, considering the volunteers as statistical blocks, followed by post hoc Tukey test. For the transformed data, the geometric mean and 95% confidence intervals (calculated from the transformed data and then converted to the non-log scale) are presented. One volunteer did not complete one experimental phase while in the control group, so for all variables n=9 for this group. The SAS system 9.4 (SAS Institute, Cary, USA) was used in these calculations and a significance level of 5% was adopted.

Results

Significant differences in the %SHL were found among groups 0, 250 and 1000 ppm F (fig. 1a). The %SHL in the Ca + 250 ppm F group was not different from that in the 1000 ppm F group, but was significantly higher than in the 250 ppm F rinse (fig. 1a). In relation to area of hardness loss (ΔS) no difference was observed between any of the F rinse groups (p>0.05), but all F rinse groups resulted in significantly lower ΔS values when compared to the negative control group (p<0.05) (fig. 1b).
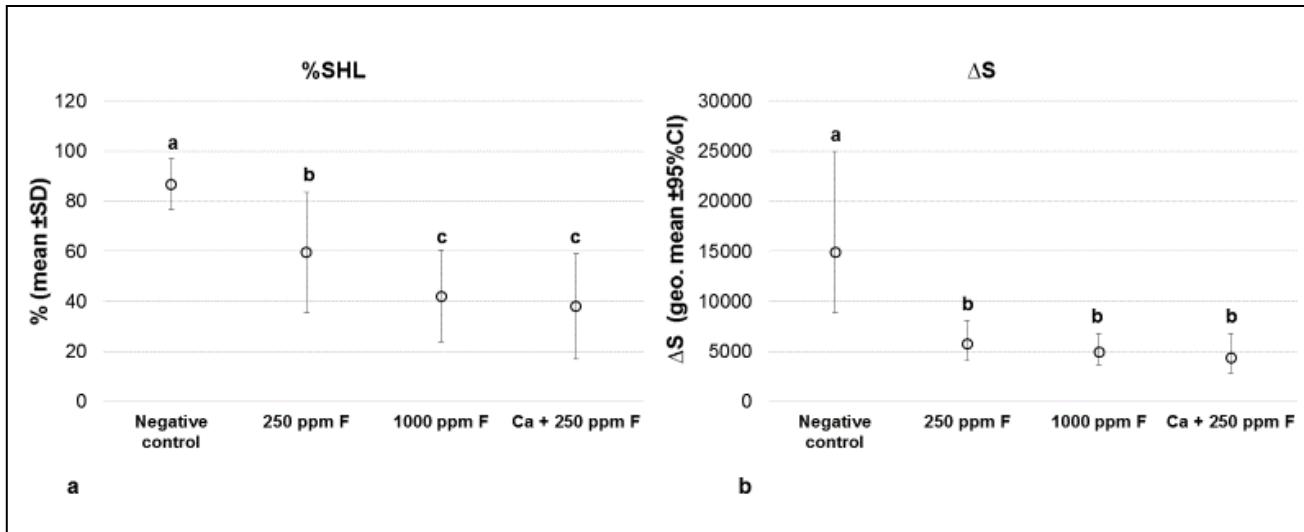
Figure 1 – Analysis of enamel demineralization, according to the treatment groups. (a) %SHL (mean ± SD); (b) ΔS (geometric mean ± 95% CI). Different letters represent statistical differences (p<0.05).

All F containing rinses increased F concentrations in the fluid relative to the placebo, non-F rinse. However, although the Ca pre-rinse increased biofilm F (~2x), only the 1000 ppm F rinse produced a significant (4x) increase from above the 250 ppm F rinse. The Ca pre-rinse increased F in the biofilm solids (22x above the 250 ppm F rinse), being significantly higher than the other groups, including the 1000 ppm F rinse (fig. 2).
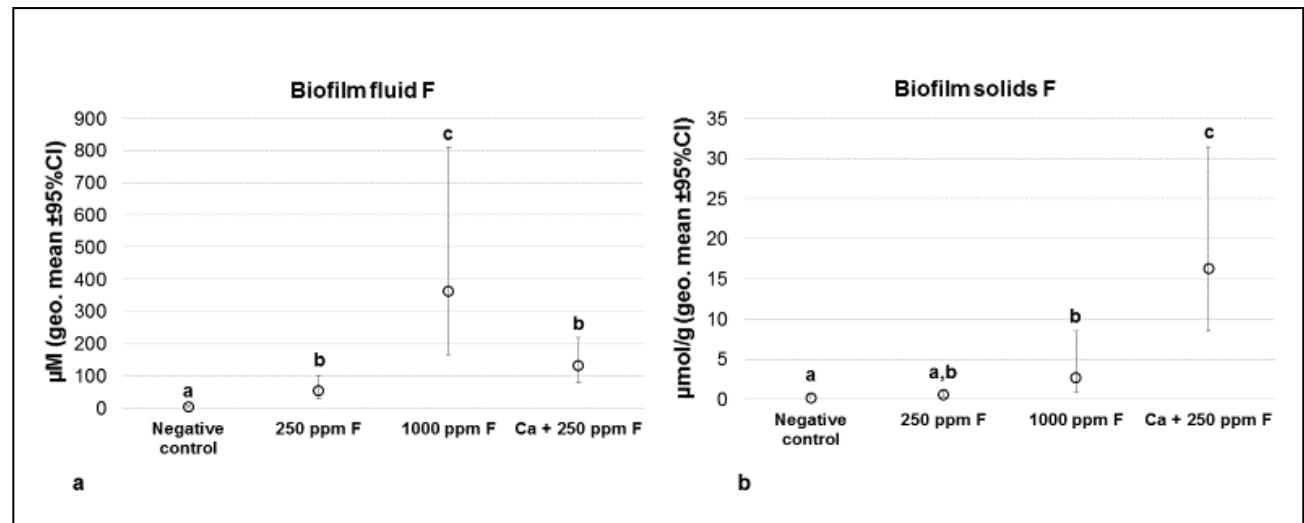
Figure 2 – Analysis of the biofilm fluid and solids (geometric mean ± 95% CI), according to the treatment groups. (a) F in the biofilm fluid; (b) total F in the biofilm

solids. Different letters represent statistical differences (p<0.05). For F in the biofilm fluid, one outlier (value = 7.70 µM) was removed from the 1000 ppm F group (n=9).

The loosely-bound F concentration in enamel increased significantly with the increasing F rinse concentration, however only the 1000 ppm F rinse was significantly different from the other F-containing rinses. The firmly-bound F concentrations were all significantly higher than the negative control (fig. 3), with the 250 ppm F and 1000 ppm F groups having the highest values. The Ca pre-rinse group did not differ significantly from the 1000 ppm F group in this concentration (fig. 3).
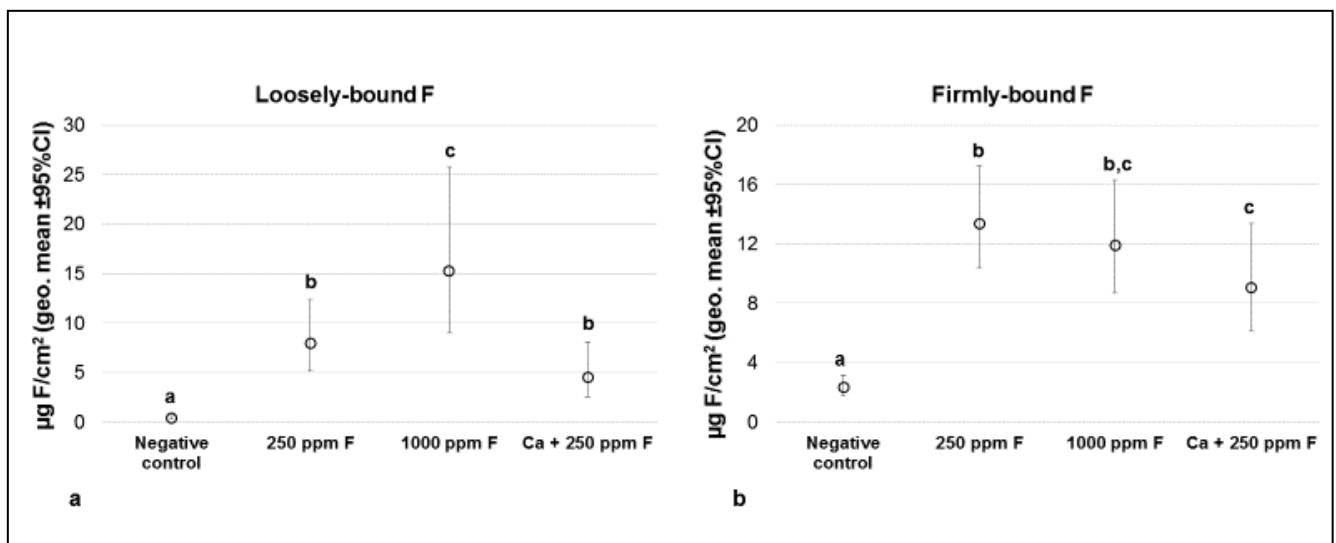
Figure 3 – Analysis of loosely-bound (a) and firmly-bound (b) fluoride in enamel (geometric mean ± 95% CI), according to the treatment groups. Different letters represent statistical differences (p<0.05).

Discussion

As shown in Fig. 1a, with respect to surface hardness, the in situ model was sensitive to increases in F concentration to at least 1000 ppm F. This provides confidence in the data of this figure demonstrating that the Ca + 250 ppm F group significantly reduce the %SHL, when compared with the 250 ppm F rinse alone: the inhibition of mineral loss was similar to that found with the 1000 ppm F rinse. Surface hardness data is taken as the only indicator of mineral loss [Cury et al., 2003; 2010; Kusano et al., 2011; Vale et al., 2011] in this study since, as shown in Fig. 1b, the area of hardness loss was not sensitive to levels above 250 ppm F.

Surprising, given its central role in the mechanism of F in reducing caries [Vogel et al., 1990; Margolis and Moreno, 1990; Vogel 2011], overnight biofilm fluid F levels only increased about twice with the use of the Ca pre-rinse, and this increase was not significantly higher (p=0.068) then the 250 ppm F rinse alone. Only the 1000 ppm F rinse produced a 4 times, significant increase in this parameter. The fact that nearly 10 h elapsed since the rinses suggest that biofilm fluid F should be highly elevated by the Ca pre-rinse procedure at shorter time periods: as noted above, 1 h biofilm samples collected from human teeth [Vogel et al., 2008a] showed nearly 5 x increase.

The ~22x increase in the biofilm solids F (Fig. 2b) with use of the Ca pre-rinse is greater than then the 6x increase observed in samples also collected from an in situ model when the Ca pre-rinse was used with a dentifrice [Magalhães et al., 2007]. Differences in protocol, such as the use of extra oral or intra oral rinses, and the presence of calcium binding moieties in dentifrices [Vogel et al., 2006], appear to explain these differences. In any case, the very large increase in biofilm F in both these studies, as well as the 12x increase in this parameter found in samples of biofilm collected from human teeth 1 h after use of this same Ca pre-rinse/250 ppm F rinse combination [Vogel et al., 2008a], appear to explain the high level of protection against demineralization observed here. Such biofilm F trapping is consistent with the fixing of fluoride by Ca mechanism by which the rinse has been postulated to increase oral levels of fluoride [Rose et al., 1996; Vogel et al., 2008a, 2008b, 2011, 2014]. Although the nature of such biofilm reservoirs requires further investigation, recent studies have suggested that that unlike a conventional F rinse [Vogel et al., 2010], the Ca pre-rinse has been shown to deposit nearly 30% of its F as calcium fluoride-like precipitates [Vogel et al., 2014]. With the 1000 ppm F rinse, the higher levels of biofilm fluid F coupled with a lower level of F in the biofilm solids suggest that the biofilm F deposits induced by this rinse are more labile that those produce by the Ca pre-rinse. Specifically, they may be in the form of calcium mediated biological/bacterial bound F reservoirs [Rose et al., 1996] that have previously been found to be the form that F is held in plaque 1 h after use of a 250 ppm F rinse [Vogel et al., 2010].

Loosely-bound F may be an important source of F on the enamel surface which can slowly release F to oral fluids to induce remineralization and reduce demineralization during periods of caries attack, but it is mostly formed after the use

of professional methods of F use [Tenuta et al., 2008]. Firmly-bound F appears to represent the F incorporated into the tooth mineral, mainly as a result of re-precipitation of fluoridated apatites from enamel mineral dissolved during the periods of low pH in the presence of F [Margolis and Moreno, 1990]. Both these reservoirs were increased by the F regimen used here (fig. 3). However, in spite of the fact that the Ca + 250 ppm F group greatly reduced demineralization, this rinse combination did not significantly enhance either of these F reservoirs compared with the 250 ppm F alone. In fact, the Ca pre-rinse appears to decrease these parameters (significantly in the case of firmly-bound F). This suggests that, in accordance with the very high levels of biofilm F observed with the Ca + 250 ppm F group, the F in this combination of rinses appears to have been trapped in the plaque and hence did not reach the surface of the enamel to form these F reservoirs. Thus, when a conventional F rinse is being used, at least some of the fluid F at tooth surface may be released from loosely-bound enamel F, but when a Ca pre-rinse/F rinse combination is used, the very high levels of F found in the biofilm appears to be the primary source of biofilm fluid F.

Some inhibition of bacterial metabolism by the high F concentrations found in the biofilm fluid could also explain the lower demineralization in the Ca pre-rinse/F rinse group, with no increase in firmly-bound F by re-precipitation. However, the F concentration found in the biofilm fluid in this group, although high, is not enough to significantly inhibit bacterial metabolism [Bradshaw et al., 2002]. It may be, however, that immediately after the F rinses and for some time after that, the F concentration reached inhibitory concentration of at least 10 ppm F (526.3 µM); this would be important for the morning rinse, which was performed before the first sugar exposure of the day. However, such effect would be even higher in the 1000 ppm F group than in the Ca pre-rinse + F group, considering that higher resting fluid F values were observed for the first.

In conclusion, the results showed that a Ca pre-rinse greatly increases the deposition of F in the biofilm, and that this increased biofilm F deposition appears to be responsible for the large decrease in tooth enamel demineralization observed in this study after use of this pre-rinse.

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Author Contributions

Conceived and designed the experiment: L.M.A.T., A.A.D.B.C., G.L.V., J.A.C.; performed the experiment: J.G.S.S., L.M.A.T., A.A.D.B.C., D.F.N., R.R.B., M.X.Q.; analyzed the data: J.G.S.S., L.M.A.T.; wrote the draft manuscript: J.G.S.S., L.M.A.T.; reviewed and approved the final manuscript: all authors

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3 CONCLUSÃO

O presente estudo demonstrou que a utilização de um pré-bochecho com cálcio, antes do uso de bochecho fluoretado, é capaz de reduzir significativamente a perda mineral do esmalte submetido a um alto desafio cariogênico em relação ao uso isolado do bochecho fluoretado na mesma concentração, o que está ligado a sua capacidade de aumentar a concentração de fluoreto no biofilme dental.

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ANEXOS

<u>Anexo 1</u>

Comprovante de submissão

● Computer-Generated E-Mail: Caries Research - Account Created in Manuscript Central

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Anexo 2 –Dose-resposta modelo

Análise de regressão linear considerando apenas os grupos 0, 250 e 1000 ppm F para verificar o efeito dose-resposta do "modelo in situ de Piracicaba" para diferentes concentrações de bochecho fluoretado.
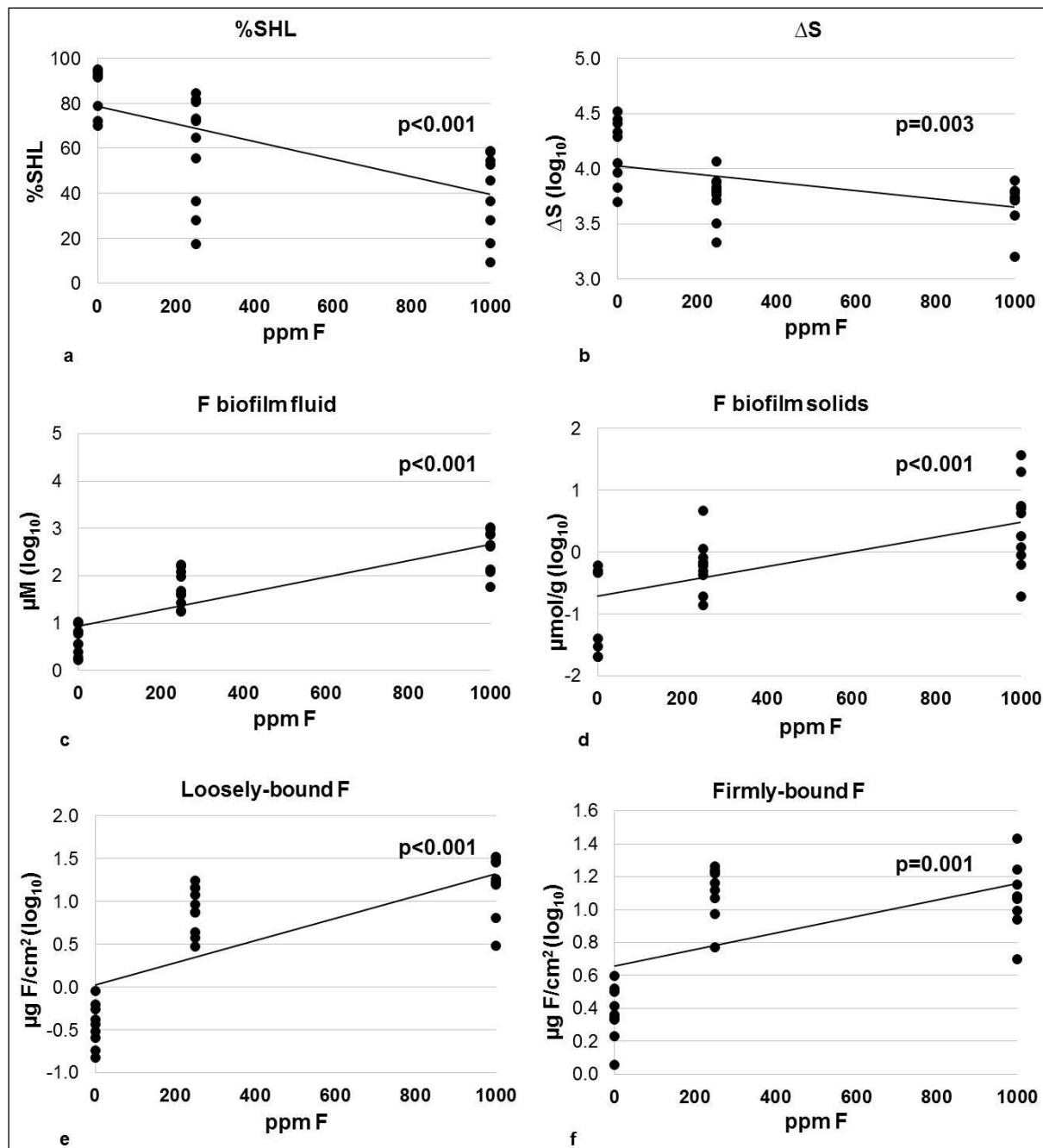
Anexo 3 – Análise biofilme

Análise da porção fluida e sólida do biofilme, considerando as concentrações de F (descrita no artigo), Ca iônico livre e pH do fluido do biofilme, determinado de acordo com metodologia previamente padronizada (Vogel et al., 2000), utilizando eletrodos de íon especifico. Determinou-se também as concentrações de F (descrita no artigo), Ca e fósforo (Pi) na porção sólida do biofilme. As concentrações de Ca e Pi foram realizadas utilizando reagentes colorimétricos (Arsenazo III e verde malaquita, respetivamente) (Vogel et al., 1983; Tenuta et al., 2006).