UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

Diego Figueiredo Nóbrega

Avaliação do potencial anticárie dos reservatórios de fluoreto do biofilme dental

Evaluation of the anticaries effect of dental biofilm fluoride reservoirs

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Evaluation of the anticaries effect of dental biofilm fluoride reservoirs

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

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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

Embora o efeito anticárie do fluoreto esteja claramente estabelecido na literatura, ainda não se sabe qual o papel dos reservatórios de fluoreto do biofilme dental nesse efeito. Tem sido sugerido que o fluoreto retido no biofilme dental, quer seja ligado a superfície de bactérias ou precipitado na forma de fluoreto de cálcio (CaF_2), poderia ser liberado para a porção fluida do biofilme, funcionando assim como reservatórios do íon. Ambos os tipos de reservatórios (bacteriano ou mineral) dependem da presença de cálcio, que no primeiro caso funciona como uma ponte para a ligação dos íons fluoreto, e no segundo caso determina a saturação com relação ao CaF_2, necessária para que ocorra sua formação. No entanto, a dinâmica de formação e a importância relativa de cada um desses reservatórios na redução da desmineralização dental ainda são desconhecidas. Assim, o objetivo desse estudo foi avaliar a formação desses reservatórios em pellets bacterianos e seu efeito anticárie. Para tal, foram realizados dois estudos. No primeiro, foi avaliada *in vitro*, a retenção de fluoreto a pellets de *S. mutans* tratados com concentrações crescentes de cálcio e fluoreto, abaixo (forma apenas reservatórios bacterianos) ou acima do produto de solubilidade do mineral fluoreto de cálcio (Ksp_{CaF_2}) (forma reservatórios bacterianos e de CaF_2). Os resultados mostraram que abaixo do Ksp_{CaF_2}, a adição de cálcio à solução de tratamento não resultou em maior retenção de fluoreto nos pellets bacterianos ($p > 0,05$). Por outro lado, quando as concentrações de cálcio e fluoreto superaram o Ksp_{CaF_2}, a retenção de fluoreto aumentou significativamente em função da concentração de cálcio utilizada no tratamento ($p < 0,05$). No segundo estudo, testamos *in situ* o efeito anticárie dos dois tipos de reservatórios de fluoreto no biofilme dental. Doze voluntários utilizaram dispositivos palatinos contendo blocos de esmalte dental bovino, montados em dois holders em contato com pellets de *S. mutans*, simulando placas-teste, previamente tratadas segundo 4 grupos: G1. Placa-teste sem reservatórios de F (controle negativo); G2. Placa-teste contendo apenas reservatórios bacterianos de F; G3. Placa-teste contendo apenas CaF_2 (controle ativo); e G4. Placa-teste contendo reservatórios bacterianos e CaF_2. Os voluntários utilizaram os aparelhos por 30 min, quando metade das placas-teste foram coletadas para análise de fluoreto. Quarenta e cinco minutos após a realização de um bochecho com solução de sacarose a 20%, a outra metade das placas-teste e blocos de esmalte foram coletadas para análises de fluoreto no fluido do biofilme e da % de perda de dureza de superfície (%PDS). Os resultados mostraram que apenas os grupos contendo CaF_2 (G3 e G4) foram capazes de manter elevadas concentrações de fluoreto no

fluido do biofilme durante todo o experimento ($p<0,05$; ANOVA). Consequentemente, nestes grupos a %PDS foi significativamente menor ($p<0,05$; ANOVA). Em resumo, nossos resultados sugerem que o aumento da concentração de fluoreto em biofilmes expostos a altas concentrações de cálcio e fluoreto se deve principalmente a precipitação de CaF_2. Estes reservatórios são capazes de manter concentrações elevadas de fluoreto no fluido do biofilme, reduzindo a desmineralização do esmalte.

Palavras chave: Cárie dentária. Fluoretos. Placa dentária. Desmineralização do dente. Fluoreto de cálcio.

ABSTRACT

Despite the recognized anticaries effect of fluoride, the role of dental biofilm fluoride reservoirs in this effect is unknown. It has been suggested that fluoride retained in dental biofilm, whether bound to the bacterial surface or precipitated in the form of calcium fluoride (CaF_2), could be released to the biofilm fluid phase acting as an ion reservoir. Both reservoirs (bacterially-bound or precipitated CaF_2) depend on the presence of calcium, which in the former works as a bridge for the binding of fluoride ions, and in the second case determines the saturation with respect to CaF_2, necessary for its formation. However, the formation of these reservoirs and their relative importance in reducing dental demineralization is unknown. Thus, the aim of this study was to evaluate the formation of fluoride reservoirs in bacterial pellets and their anticaries effect. For this, two studies were carried out. In the first, we assessed *in vitro* the fluoride retention to *S. mutans* pellets treated with increasing concentrations of calcium and fluoride, either below (forms only bacterially-bound reservoirs) or above the solubility product of calcium fluoride ($KspCaF_2$) (forms both bacterially-bound and CaF_2 reservoirs). The results showed that below the $KspCaF_2$, the addition of calcium to the treatment solution did not result in higher fluoride retention in the bacterial pellets ($p>0.05$). On the other hand, when calcium and fluoride concentrations exceeded the $KspCaF_2$, fluoride retention increased significantly as a function of the calcium concentration used in the treatment solution ($p<0.05$). In the second study, we tested *in situ* the anticaries effect of the two types of biofilm fluoride reservoirs. Twelve volunteers used palatal appliances containing bovine enamel blocks, mounted on two holders in contact with *S. mutans* pellets, simulating test-plaques, previously treated according to 4 treatment groups: G1. test-plaque containing no fluroeto reservoirs (negative control); G2. test-plaque containing only bacterially-bound fluoride; G3. test-plaque containing only CaF_2 (active control); and G4. test-plaque containing both bacterially-bound and CaF_2 reservoirs. The volunteers used the devices for 30 min, when half of the test-plaques were collected for fluoride analysis. Forty-five minutes after a rinse with 20% sucrose solution, the other half of the test-plaques and enamel blocks were collected for analysis of biofilm fluid fluoride and % of surface hardness loss (% SHL). The results showed that only those groups containing CaF_2 (G3 and G4) were able to maintain high fluoride concentrations in the biofilm fluid throughout the experiment ($p<0.05$, ANOVA). Consequently, the %SHL was significantly lower in these groups ($p<0.05$, ANOVA). In summary, our results suggest that the increased

fluoride retention in biofilms exposed to high calcium and fluoride concentrations should be mainly attributed to CaF_2 precipitation. These reservoirs are able to maintain increased fluoride concentrations in the biofilm fluid, reducing enamel demineralization.

Key words: Dental caries. Fluorides. Dental plaque. Tooth demineralization. Calcium fluoride.

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1 INTRODUÇÃO

O efeito do fluoreto no controle de cárie dental é amplamente descrito na literatura mundial (ten Cate, 2004; Tenuta & Cury, 2010). Sua utilização em meios de abrangência coletiva, como água fluoretada (Iheozor-Ejiofor et al., 2015), ou individual, como dentifrícios fluoretados (Marinho et al., 2003), tem sido relacionada com o declínio da prevalência de cárie no Brasil (Cury et al., 2004) e ao redor do mundo (Pitts et al., 2017)). Revisões sistemáticas da literatura mundial tem mostrado evidências de que a utilização de fluoreto a partir de diferentes meios é capaz de reduzir a prevalência de cárie, quando comparado a um grupo controle ou placebo (água fluoretada 25-36%, dentifrício fluoretado 24%, bochecho fluoretado 27%, gel fluoretado 28% e verniz fluoretado 45%) (Iheozor-Ejiofor et al., 2015; Marinho et al., 2003, 2013, 2015, 2016). O fluoreto age reduzindo a perda mineral dental quando presente de forma constante no meio bucal, para interferir com os processos de des e remineralização ao qual as superfícies dentárias estão expostas diariamente, pelo acúmulo de biofilme e sua exposição a açúcares fermentáveis da dieta (Tenuta & Cury, 2010). O efeito físico-químico do fluoreto na inibição da desmineralização dental acontece quando, no biofilme dental exposto a açúcar fermentável, a presença de fluoreto no fluido do biofilme é capaz de reduzir a perda mineral, uma vez que parte dos minerais dissolvidos da estrutura dental durante a queda de pH retorna ao dente como um mineral fluoretado. Por outro lado, sua ação na ativação da remineralização acontece quando o pH do biofilme volta ao normal, por potencializar a capacidade remineralizadora da saliva, repondo minerais contendo fluoreto na estrutura dental (Cury & Tenuta 2009).

De fato, tendo em vista o papel fundamental do biofilme dental no processo de cárie, sua remoção é ideal para o controle de cárie. Neste sentido, a escovação diária com dentifrício fluoretado é considerada o meio mais racional de uso de fluoretos, pois além do

enriquecimento do meio bucal com fluoreto, ocorre a desorganização do biofilme pelo o ato mecânico da escovação (Tenuta & Cury, 2013). No entanto, devido à deficiência que grande parte dos indivíduos possui no controle de placa, o acúmulo de biofilme inevitavelmente ocorrerá, principalmente em áreas de difícil acesso (Nyvad, 2015). Nestes locais, o enriquecimento de residuais de biofilme com fluoreto será fundamental para seu efeito anticárie, pois no biofilme dental não removido pela escovação, a manutenção de fluoreto será capaz de reduzir a perda mineral (Tenuta et al., 2009).

Logo após a escovação com dentifrício fluoretado, a porção fluida do biofilme dental fica enriquecida com fluoreto (Cury et al., 2010). Esta concentração, no entanto, cai nas horas subsequentes, por difusão do íon desde o fluido do biofilme até a saliva (Cury et al., 2010). No entanto, o biofilme continuamente exposto a dentifrício fluoretado possui uma concentração total de fluoreto mais elevada (Cenci et al., 2008; Cury et al., 2010). Assim, o biofilme é capaz de reter fluoreto, e poderia funcionar como um reservatório deste, sendo liberado para o fluido do biofilme nos momentos em que a concentração de fluoreto nesse compartimento é reduzida. Entretanto, pouco se sabe sobre o efeito da liberação de fluoreto a partir destes reservatórios para o fluido do biofilme, bem como a importância desse mecanismo para o controle de cárie.

Existem basicamente duas formas de retenção de fluoreto no biofilme, ambas dependem de cálcio: 1) Reservatórios biológicos: ligação de fluoreto a íons cálcio adsorvidos a cargas negativas presentes na superfície bacteriana, ou em proteínas da matriz do biofilme (Rose et al., 1996); ou 2) Reservatórios minerais: no qual o fluoreto está ligado ao cálcio na forma de minerais precipitados, tais como fluoreto de cálcio (CaF_2) (Vogel, 2011). A capacidade desses reservatórios de fluoreto se formarem e dissolverem parece ser distinta (Vogel, 2011): 1. Os reservatórios de fluoreto na superfície bacteriana parecem ser função da

concentração de fluoreto e do pH no fluido do biofilme. Se a concentração de fluoreto aumenta, mais fluoreto se ligaria aos íons cálcio na superfície bacteriana e vice versa; se o pH do fluido baixa, íons cálcio seriam deslocados de seu sítio de ligação pelos íons H^+, culminando com a liberação também dos íons fluoreto que a eles estavam ligados. Essa dinâmica de formação e liberação de fluoreto de reservatórios da superfície bacteriana, no entanto, só foi estudada *in vitro*, e em condições de exposição a altas concentrações de cálcio e fluoreto, (Rose et al., 1996), sendo necessários mais estudos para confirmar essa hipótese e a importância desses reservatórios quando utilizadas menores concentrações destes íons, ou seja, aquelas normalmente encontradas em produtos fluoretados. 2. Os reservatórios minerais de fluoreto são formados em função da concentração dos íons que os compõem no fluido do biofilme. Por exemplo, o mineral fluoreto de cálcio poderá se formar no biofilme dental se as concentrações de cálcio e fluoreto no fluido excederem o produto de solubilidade deste mineral. No entanto, para a formação desses minerais parece ser necessário um alto grau de supersaturação em relação aos íons que os compõem, e não apenas concentrações que excedem seu produto de solubilidade. Assim, embora logo após um bochecho com solução fluoretada concentrações de cálcio e fluoreto que excedem o produto de solubilidade do fluoreto de cálcio sejam observadas no fluido do biofilme, este mineral não parece se formar (Vogel et al., 2010). Para sua formação, estratégias como o aumento da disponibilidade de cálcio (por exemplo, por um bochecho com cálcio) antes do uso do fluoreto parecem ser necessárias (Vogel et al., 2014).

Por outro lado, a importância de aumentar esses reservatórios de fluoreto no biofilme, para que de fato liberem o íon para o fluido do biofilme ainda precisa ser confirmada por estudos que induzam a liberação desses reservatórios. Embora o efeito do fluoreto no controle de cárie seja suportado por revisões sistemáticas da literatura (Iheozor-Ejiofor et al., 2015;

Marinho et al., 2015, 2016), não está claro o quanto desse efeito se dá a partir do fluoreto que fica retido nos reservatórios do biofilme para ser posteriormente liberado, ou se apenas o efeito momentâneo do fluoreto que penetra através do fluido do biofilme é suficientemente importante. Estudo recente confirmou que o uso de cálcio previamente ao uso de fluoreto aumenta o efeito anticárie do fluoreto utilizado isoladamente (Souza et al., 2016), porém a importância relativa do aumento da disponibilidade de fluoreto nos fluidos bucais promovido por esse tratamento logo após sua realização, em relação a retenção aumentada de fluoreto em reservatórios do biofilme, é desconhecida.

Considerando a importância do fluoreto para o controle de cárie e a possibilidade de desenvolver estratégias para potenciar seu efeito pelo aumento de sua retenção no biofilme dental, este trabalho objetivou estudar a formação dos reservatórios de fluoreto no biofilme dental e seu efeito anticárie.

2 ARTIGOS

2.1 ARTIGO: *Artigo submetido ao periódico Caries Research.

Fluoride binding to dental biofilm bacteria: synergistic effect with calcium questioned

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Abstract

It has been suggested that fluoride binding to dental biofilm is enhanced when more bacterial calcium binding sites are available. However, this was only observed at high calcium and fluoride concentrations (i.e. above Ksp_{CaF_2}). We assessed fluoride binding to *S. mutans* pellets treated with calcium and fluoride at concentrations below and above Ksp_{CaF_2}. Increasing calcium concentration resulted in a linear increase ($p<0.01$) in fluoride concentration in the pellets only in experiments above Ksp_{CaF_2}. The results suggest that CaF_2 precipitation, rather than bacterially-bound fluoride, is responsible for the increased in fluoride binding to dental biofilm with the increase in calcium availability.

Introduction

Dental biofilm has a considerable fluoride binding capacity and could act as a reservoir, releasing fluoride to biofilm fluid phase to interfere with the caries process when the fluoride concentration in that compartment is low [Margolis and Moreno, 1992; Pearce, 1999; Vogel, 2011]. Although this may have important consequences for the development of products to control caries based on fluoride retention in dental biofilm, as recently shown in situ by the use of a calcium prerinse followed by a fluoride rinse [Souza et al., 2016], the nature of this binding is poorly understood.

Basically, there are two recognized forms of fluoride retention in dental biofilm, both of them related to calcium: 1) precipitated minerals, mainly CaF_2 - formed when the calcium and fluoride concentrations in biofilm fluid exceed the solubility product (KSP) of the minerals [Vogel, 2011]; 2) bacterially-bound fluoride – fluoride binding to calcium ions which are adsorbed to anionic sites present on the surface of bacteria, or biofilm matrix proteins [Rose et al., 1996].

It has been suggested that the presence of calcium increases the bacterial fluoride binding in dental biofilm, and also that the amount of bacterial-bound calcium doubles in the presence of fluoride [Rose et al., 1996; Domon-Tawaraya, 2013]. However, this supposed synergism between calcium and fluoride in bacterial binding was only studied in conditions of high concentrations of these ions (above the KSP_{CaF_2}) and the results may have been overestimated by the precipitation of CaF_2.

Therefore, the aim of this *in vitro* study was to evaluate the fluoride binding to biofilm bacteria using solutions containing calcium and fluoride concentrations either below the KSP_{CaF_2} (able to form only bacterially-bound reservoirs) or above the KSP_{CaF_2} (able to form both bacterially-bound reservoirs and precipitated CaF_2), in order to check the synergism between calcium and fluoride in the retention of the latter in dental biofilms.

Materials and Methods

Bacterial preparation

Streptococcus mutans was used since it is a major caries-related species [Bowen and Koo, 2011], and there are not marked differences in calcium binding to different species of streptococci [Rose et al., 1993], one of the major genus of dental biofilm bacteria [Richards

et al., 2017]. Pellets of *S. mutans* Ingbritt-1600 were obtained from cultures grown in TYB medium (tryptone + yeast extract) supplemented with 0.25 % glucose for 18 hours at 37 °C, 10% CO_2. Bacterial pellets were separated from culture media by centrifugation (10.000 *g*, 10 min, 4 ºC). In order to remove remnants of the culture media, neutralize the pH and quelate calcium, the pellets were sequentially washed in 0.05 M PIPES buffer, pH 7.0, followed by 0.01 M EDTA solution, and again in PIPES buffer [Rose et al., 1993], using vortex followed by sonication at 7 W for 1 min (Vibra Cell sonicator, Sonics and Materials, Danbury, USA) to disrupt bacterial masses at each wash. Between each wash, the pellets were recovered by centrifugation (10,000 *g*, 10 min, 4 °C). After this procedure, the pellets were re-suspended in 20 mL of 0.05 M PIPES buffer and aliquots of 400 μL (to contain about 10 mg of bacteria) were transferred to pre-weighted microcentrifuge tubes. These tubes were centrifuged (21,000 *g*, 5 min, 4 °C) and the supernatant carefully discarded under microscope. Lastly, the bacterial pellets were weighed (± 0.01 mg) for calculation of the amount of calcium and fluoride treatment solution to be added.

Treatments

The treatments consisted of 0.05 M PIPES buffer, pH 7, containing combinations of increasing fluoride and calcium concentrations (0, 1 or 10 mM), divided into 9 groups: G1 – negative control group (0 mM F and 0 mM Ca); G2 (0 mM F and 1 mM Ca); G3 (0 mM F and 10 mM Ca); G4 (1 mM F and 0 mM Ca); G5 (1 mM F and 1 mM Ca); G6 (1 mM F and 10 mM Ca); G7 (10 mM F and 0 mM Ca); G8 (10 mM F and 1 mM Ca); G9 (10 mM F and 10 mM Ca). The pellets were treated with the respective solutions for 30 min, 10 % CO_2 and 37 °C, and immediately vortexed. To facilitate comparisons, we used equimolar concentrations of fluoride and calcium (1 and 10 mM). For fluoride treatments, we used 1.5 mL of PIPES buffer containing 0, 1 or 10 mM F (from sodium fluoride) for each 10 mg of pellet. For calcium treatments, we added 0.015 mL of 0.1 M and 1M calcium standards (from $CaCl_2$) to the fluoride treatment solutions for each 10 mg of pellet, to form groups containing 1 and 10 mM Ca, respectively. In groups 8 and 9, the Ca and F concentrations exceed the solubility of CaF_2 (KSP_{CaF_2}= 3,0 x $10^{-10.4}$ M [McCann, 1968]). Therefore, in such conditions, additionally to bacterial binding, the precipitation of CaF_2 is also expected [Leitão et al., 2017].

Determination of total fluoride and calcium concentrations in the bacterial pellets

After treatment, the microtubes containing the samples were centrifuged, the supernatant carefully discarded under microscope and the pellets weight was determined. Calcium and fluoride extracted from these pellets using strong acid were considered to be bound to the bacteria or matrix proteins. The bacterial pellets were sequentially extracted with 0.5 M HCl (1 h per extraction), as follows: G1 to 6 (lower fluoride concentration): one extraction with 0.1 mL HCl/10 mg of pellet followed by one extraction with 0.05 mL HCl/10 mg of pellet; G7 to 9 (higher fluoride concentration): one extraction with 1.0 mL HCl/10 mg of pellet, followed by another extraction with 0.5 mL/10 mg of pellet. These extraction conditions were previously determined in a pilot study [Salvaterra et al., 2014].

The acid extracts were neutralized with 2.5 M NaOH (1:5) and TISAB III (1:10) (Thermo Electron, Waltham, MA, USA) and the total fluoride concentration in the bacterial pellets was determined by an inverted ion-specific electrode [Vogel et al., 1997]. The calcium binding to *S. mutans* pellets as a function of the fluoride treatment was also determined to support the results of fluoride binding. The total calcium concentration was measured using the Arsenazo III colorimetric reagent, after neutralization of the acid extracts with 0.5 M NaOH (1:1). The absorbance of the mixtures was read in 96-well microplates, using a Multiskan Spectrum (Thermo Scientific) microplate reader at 650 nm [Vogel et al., 1983]. The total fluoride and calcium concentrations were expressed in nmol / mg.

Statistical Analysis

The experiments were repeated 3 times, with triplicate samples. Total fluoride and calcium concentrations on the bacterial pellets exposed to the different treatments were compared by one-way ANOVA, followed by Tukey test. Also, the effect of calcium at increasing concentrations on the total bacterial F concentration was estimated by linear regression analysis. The assumptions of equality of variances and normal distribution of errors were checked and data that did not fit these assumptions were transformed [Box et al., 2005]. All analyses were performed using the software SPSS® Statistics (version 18.0) and the significance level was set at 5%.

Results

In groups treated with calcium and fluoride below the KSP_{CaF_2} (G 1-7), the increasing calcium concentrations did not affect fluoride binding to bacteria ($p>0.05$). Indeed, the

fluoride binding in these groups seems to reflect only the fluoride concentration used in the treatment solution (0 < 1 < 10 mM, $p<0.05$) (figure 1). In contrast, in groups treated above the KSP_{CaF_2} (G 8-9), the total fluoride concentration on the bacterial pellets increased significantly ($p<0.05$) according to the calcium concentration used in the treatment solution (figure 1). Similarly, in groups treated with zero (G 1, 4 and 7), 1 (G 2 and 5), or 10 mM Ca (G 3 and 6) below the KSP_{CaF_2}, the increasing fluoride concentrations did not affect bacterial calcium binding ($p>0.05$). Again, the total calcium concentration found on the bacterial pellets was a function of the calcium concentration used during treatment (0 < 1 < 10 mM, $p<0.05$) (figure 2). Conversely, in groups treated above the KSP_{CaF_2} (G 8 and 9), the total calcium concentration on the bacterial pellets increased considerably ($p<0.05$) according to the fluoride concentration contained in the treatment solution (figure 2).

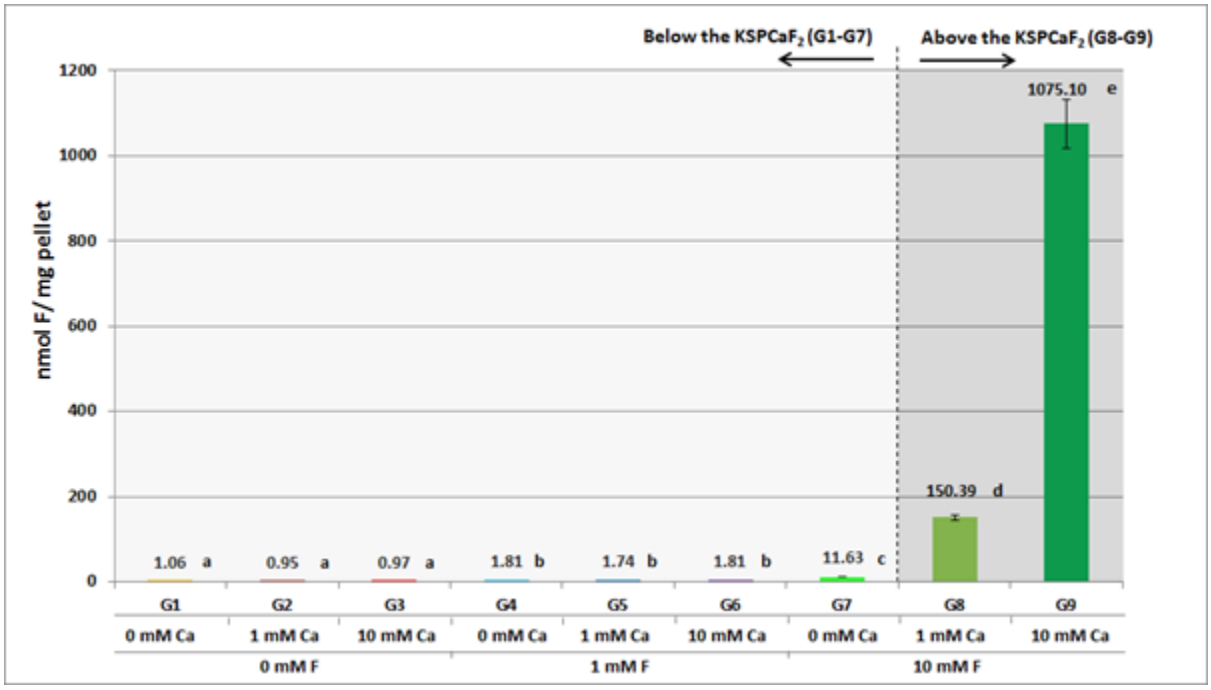

Fig.1. Whole fluoride concentration on the bacterial pellets according to the treatment group (nmol F/mg pellet, mean ± SD; n = 9 for each treatment group). Groups 1-7 were treated below the $KSPCaF_2$, and groups 8 and 9 above. Different letters represent statistical differences ($p<0.05$).Values were transformed by log 10.

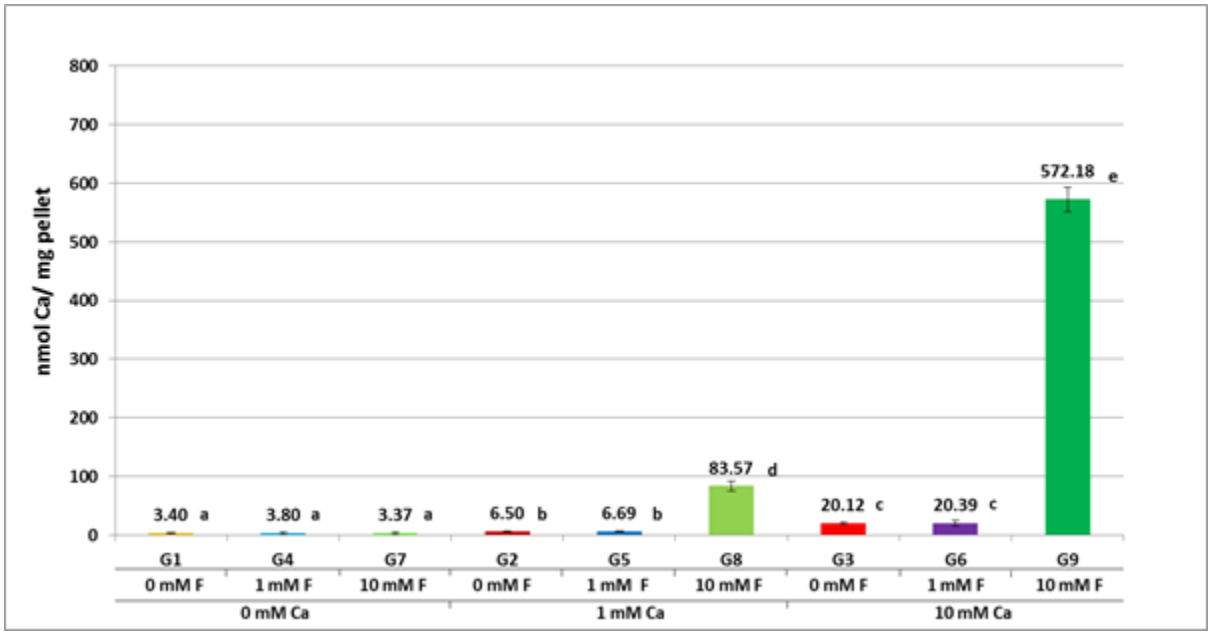

Fig.2. Whole calcium concentration on the bacterial pellets according to the treatment group (nmol F/mg pellet, mean ± SD; n = 9 for each treatment group). Groups 1-7 were treated below the $KSPCaF_2$, and groups 8 and 9 above. Different letters represent statistical differences ($p < 0.05$). Values were transformed by square root. An outlier was removed from group 9 (data 56: whole Ca concentration = 662.68 nmol F/mg).

Regression analyses showed a significant linear fit between the calcium concentration in the treatment solution (0, 1 or 10 mM) and the total fluoride concentration retained on the bacterial pellets in groups treated with high fluoride concentration ($p<0.05$), but not for groups treated with low fluoride concentrations ($p>0.05$) (figure 3).

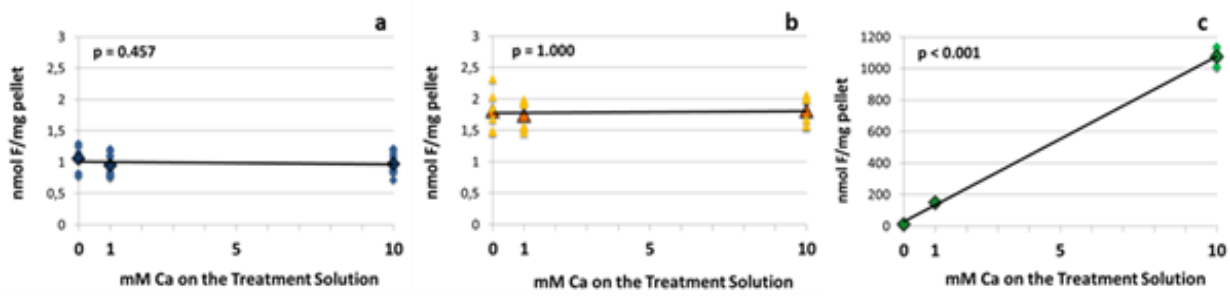

Fig.3. Linear regression fits of the whole F concentration found on the bacterial pellets (nmol F / mg pellet) as a function of the Ca concentration contained on the treatment solution (0, 1 or 10 mM). Graph "a", "b" and "c" represents groups treated with 0, 1 or 10 mM of fluoride, respectively. The fits were significant only for groups treated with high fluoride concentration ($p<0.05$). In these groups, a strong correlation ($r = 0.997$) between the F retained on the bacterial pellets and the Ca concentration used in treatments was found. For groups treated with 10 mM F (3c) the values were transformed to the $\log_{10}$.

Discussion

To exert its anticaries effect, fluoride must be maintained constantly in the oral fluids, particularly in the biofilm fluid phase, considering its role on the caries process [Cury and Tenuta, 2008]. In addition, fluoride bound to dental biofilm, which could be released to the fluid, have been subject of several investigations [Rose et al., 1996; Pearce et al., 1999; Tenuta et al., 2006; Vogel et al., 2011]. Here we assessed the fluoride binding to *S. mutans* pellets treated with different calcium and fluoride concentrations. Our findings shown that in the presence of low calcium and fluoride concentrations, fluoride binding to S. *mutans* is not influenced by calcium, and vice versa. On the other hand, in the presence of high concentrations of these ions, there seem to be a synergism between calcium and fluoride on their binding to the bacterial pellets, but this might be attributed mainly to the precipitation of CaF_2, rather than to bacterial F binding.

The mechanisms by which calcium improves fluoride binding to *S. mutans* were first proposed by Rose et al. [1996]. According to these authors, calcium as a divalent ion could bind to two anionic sites on the bacterial surface (in the same bacterium or between adjacent bacteria). The addition of fluoride would be able to break such bidentate calcium bonds, exposing new anionic sites, and hence more calcium and fluoride could bind to the exposed bacterial sites. However, this supposed synergism between calcium and fluoride in bacterial binding was tested in the presence of high calcium and fluoride concentrations (5 mM Ca and 5 mM F), which also favors the precipitation of calcium fluoride [Rose et al., 1996]. In the present study, we could observe similar results when high calcium and fluoride concentrations were used (G 8 and 9, above the KSP_{CaF_2}). Increasing calcium concentrations resulted in increased fluoride binding to the bacterial pellets (12x and 89x higher for G8 and 9, respectively) when compared to the group exposed to fluoride alone (G7) (figures 1 and 3c). The same was observed for the calcium binding, since only those groups treated with calcium and fluoride above the KSP_{CaF_2} had higher total calcium concentration (13x and 28x higher for G 8 and 9, respectively), when compared to groups treated with the same calcium concentration (figure 2). Furthermore, only when the KSP_{CaF_2} was exceeded, the mean total fluoride concentration found in the pellets was higher than the mean total calcium concentration (almost twice for G8 and 9, figures 1 and 2).

On the other hand, in the pellets treated below the KSP_{CaF_2} (G 1 to 7), the addition of calcium (0, 1, or 10 mM) did not alter the concentration of fluoride bound to the bacterial pellets (figure 1). Similarly, the addition of fluoride (0, 1 or 10 mM) had no effect on the concentration of calcium bound to the bacterial pellets (figure 2). This result suggests that the bacterial Ca-F binding model proposed by Rose et al. [1996] is only valid for high calcium and fluoride concentrations and in such conditions, the fluoride binding to bacteria may be overestimated by the precipitation of CaF_2.

The model proposed by Rose et al. [1996] may also be questioned based on the higher binding affinity for calcium than fluoride found by them: the estimated dissociation constants were 0.94 mM for calcium and 8.4 mM for fluoride. Here, we also found higher calcium than fluoride binding when equimolar concentrations of both were used: calcium bound to the bacterial pellets after treatment with 1 and 10 mM Ca (G2 and 3) is higher than fluoride bound after treatment with 1 and 10 mM F (G4 and 7) (figures 1 and 2). This higher affinity of calcium to bacterial binding sites compared with fluoride may not allow for the latter to easily break the calcium-bacterium complex; also, there are enough calcium bridges for fluoride binding when the concentration of both in the surrounding fluid is the same. Calcium and fluoride binding to dental biofilm bacteria seem to be governed by the concentration of both in the surrounding fluids. The decrease in the binding affinity for calcium in the presence of fluoride (dissociation constants increasing from 0.94 mM to 7.5 mM), estimated by Rose et al. [1996], may have been overestimated by the concomitant precipitation of CaF_2.

Although a technique to differentiate the nature of the biofilm fluoride reservoirs (bacterially bound or CaF_2 reservoirs) at saturating conditions (G8 and 9) is not available, it seems clear that the increase of total fluoride concentration in the bacterial pellets is conditioned to the precipitation of CaF_2. Nevertheless, part of the increase in calcium and fluoride bound in groups exceeding the KSP_{CaF_2} (G8 and 9) may be associated with bacterial fluoride binding. The extent of the contribution of both reservoirs to the increase, however, is yet to be determined. Nevertheless, it is unlikely that the lack of synergism observed at low concentrations changes when high concentrations of both ions are used and the KSP_{CaF_2} is exceeded. It should be noted in this regard that the precipitation of CaF_2, happening in G8 and 9, reduces drastically the free calcium and fluoride concentrations. On the other hand, fluoride concentration in the pellets of these groups was approximately twice higher than calcium concentration (figures 1 and 2), as would be expected by the precipitation of CaF_2.

Also, considering the use of monospecific non-matrix bacterial pellets, rather than naturally-formed biofilms, any extrapolation of the results to *in vivo* conditions must be done carefully.

In summary, the findings of this in vitro study suggest that unless high calcium and fluoride concentrations had been used, there is no synergistic effect between calcium and fluoride affecting fluoride binding to dental biofilm bacteria. Thus, the increased fluoride binding observed when dental biofilm is exposed to high concentrations of these ions should be mainly attributed to CaF_2 precipitation.

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References

Bowen WH, Koo H. Biology of Streptococcus mutans-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. Caries Res. 2011;45:69-86.

Box GEP, Hunter JS, Hunter WG: Statistics for Experimenters: Design, Innovation, and Discovery, ed 2. New York, John Wiley and Sons, 2005.

Cury JA, Tenuta LM. How to maintain a cariostatic fluoride concentration in the oral environment. Adv Dent Res. 2008;20:13-6.

Domon-Tawaraya H, Nakajo K, Washio J, Ashizawa T, Ichino T, Sugawara H, Fukumoto S, Takahashi N. Divalent cations enhance fluoride binding to Streptococcus mutans and Streptococcus sanguinis cells and subsequently inhibit bacterial acid production. Caries Res. 2013;47:141-9.

Leitão TJ, Tenuta LM, Cury JA. Kinetics of calcium binding to dental biofilm bacteria. Plos One, 2017. Submitted.

McCann HG. The solubility of fluorapatite and its relationship to that of calcium fluoride. Arch Oral Biol. 1968;13:987-1001.

Margolis HC, Moreno EC. Composition of pooled plaque fluid from caries-free and caries-positive individuals following sucrose exposure. J Dent Res. 1992;71: 1776-84.

Pearce EI, Margolis HC, Kent RL Jr. Effect of in situ plaque mineral supplementation on the state of saturation of plaque fluid during sugar-induced acidogenesis. Eur J Oral Sci. 1999;107:251-9.

Richards VP, Alvarez AJ, Luce AR, Bedenbaugh M, Mitchell ML, Burne RA, Nascimento MM. Microbiomes of Site-Specific Dental Plaques from Children with Different Caries Status. Infect Immun. 2017 Jul 19;85(8).

Rose RK, Dibdin GH, Shellis RP. A quantitative study of calcium binding and aggregation in selected oral bacteria. J Dent Res. 1993;72:78-84.

Rose RK, Shellis RP, Lee AR. The role of cation bridging in microbial fluoride binding. Caries Res. 1996;30:458-64.

Salvaterra C, Tenuta LMA, Oliveira TJL, Cury JA. Padronização da extração de cálcio e flúor em biofilmes contendo altas concentrações de minerais. Brazilian Oral Research. 2014;24:191-191.

Souza JG, Tenuta LM, Del Bel Cury AA, Nóbrega DF, Budin RR, de Queiroz MX, Vogel GL, Cury JA. Calcium Prerinse before Fluoride Rinse Reduces Enamel Demineralization: An in situ Caries Study. Caries Res. 2016;50:372-7.

Tenuta LM, Del Bel Cury AA, Bortolin MC, Vogel GL, Cury JA. Ca, Pi, and F in the fluid of biofilm formed under sucrose. J Dent Res. 2006;85:834-8.

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

Vogel GL, Mao Y, Carey CM, Chow LC. Increased overnight fluoride concentrations in saliva, plaque, and plaque fluid after a novel two-solution rinse. J Dent Res. 1997;76:761-7.

Vogel GL. Oral fluoride reservoirs and the prevention of dental caries. Monogr Oral Sci. 2011;22:146-57.

2.2 ARTIGO: *Artigo a ser submetido ao periódico Caries Research.

CaF_2 acts as a fluoride reservoir in test plaques and reduces mineral loss

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Declaration of Interests

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Abstract

The relevance of fluoride reservoirs in dental biofilm, either bound to bacteria or in the form of precipitated calcium fluoride (CaF_2), on enamel demineralization is unknown. In a crossover, double-blind, split-mouth, short-term *in situ* study we evaluated the fluoride release from these two reservoirs to biofilm fluid and the effect on enamel demineralization. Twelve volunteers wore palatal appliances containing bovine enamel blocks with known surface hardness (SH), mounted in two holders in contact with *Streptococcus mutans* test-plaques, performing four treatment groups: G1) negative control group: no calcium or fluoride reservoirs were formed; G2) F-Bio: the test plaque contained only biological, bacterially-bound reservoirs; G3) CaF_2: powdered CaF_2 was added to the pellets to simulate mineral CaF_2 reservoirs in test plaque (active control group); and G4) F-Bio / CaF_2: the test plaque contained both biological and mineral reservoirs . The volunteers wore the intraoral appliance for 30 min when half of the samples of test plaque were collected for fluoride determination. The appliances were re-inserted in the mouth and a cariogenic challenge was made by rinsing with 20% sucrose solution. After 45 min, the rest of samples of test plaque and enamel blocks were collected for the determination of plaque fluid fluoride concentration and enamel %SH Loss (%SHL), respectively. After 30 min of intraoral appliance use the test-plaque fluid fluoride concentration (μM) was highest in G3 and G4 ($p>0.05$), followed by G2 and G1 ($p<0.05$). After the cariogenic challenge, the test-plaque F concentration decreased in all groups, but only groups 3 and 4 maintained significantly higher fluoride concentrations than G1 ($p < 0.05$). Accordingly, G1 had the highest %SHL, followed by G2, with no significant difference between G3 and G4 ($p>0.05$). These results suggest that CaF_2 is able to maintain increased fluoride concentrations in the biofilm fluid phase, reducing enamel demineralization.

Introduction:

Dental caries is considered the major chronic oral disease, representing a public health problem that affects millions of people all over the world [Marcenes et al., 2015]. The manifestation of the disease is dependent on bacterial accumulation on dental surfaces (necessary factor) and its frequent exposure to dietary sugars (determinant factor) [Fejerskov and Manji, 1990]. Although fluoride does not have a direct effect on the etiological factors responsible for the disease (biofilm accumulation and sugar exposure), it is recognized as the main anticaries agent (positive determinant factor), acting on the dynamics of the caries process and retarding the progression of caries lesions by its physicochemical effect [Tenuta and Cury, 2010].

It has been suggested that dental biofilms can retain fluoride [Rose et al., 1996; Vogel et al., 2008, 2010, 2014], and could act as an ion reservoir, releasing fluoride to the biofilm fluid phase when the fluoride concentration in that compartment is low [Margolis and Moreno, 1992, Pearce et al., 1999]. The biofilm fluid phase is the dynamic interface between the tooth and the oral environment, and the maintenance of fluoride in this compartment is relevant for the balance between de- and remineralization of teeth [Vogel et al., 1990, Vogel, 2011].

There are two major forms of fluoride retention in dental biofilm, both of them involving calcium: 1) bacterially-bound (or biological) reservoirs and 2) mineral reservoirs. In the former, fluoride is held in dental biofilm bound to calcium ions, which are adsorbed to anionic sites on the surface of bacteria or matrix proteins [Rose et al., 1996]. In the latter, fluoride is found in the form of precipitated salts, mainly calcium fluoride (CaF_2), considering the low solubility of fluorapatite (FAp) in the oral fluids [Vogel, 2011]. Although the formation of biological reservoirs can occur in the presence of low calcium and fluoride concentrations [Vogel et al., 2010], the formation of CaF_2 reservoirs depends on the saturation degree reached in biofilm fluid (above the $KSPCaF_2$) when concentrated fluoridated products are used, mainly in association with a calcium pretreatment [Vogel et al., 2014].

Data from clinical studies have shown that in the absence of a previous calcium treatment, the use of a 228 ppm (12 mM) fluoride rinse is able to form only biological reservoirs [Vogel et al., 2010], while when its use is preceded by a calcium pre-rinse (150

mM), CaF_2 reservoirs are also formed [Vogel et al., 2014]. However, the relative importance of both fluoride reservoirs to maintain increased fluoride levels in the biofilm is not known. Also, the different solubility of these two biofilm fluoride sources may interfere with their anticaries effect, but this has not been experimentally assessed. Therefore, the aim of our study was to evaluate the fluoride releasing from test plaques containing only biological or mineral fluoride reservoirs, or a combination of both to the biofilm fluid phase and their effect on enamel demineralization.

Methods

Experimental design

This short-term *in situ* study involved a crossover, double-blind, split-mouth design, conducted in 2 experimental phases. Ethical approval was obtained from the Research and Ethics Committee of Piracicaba Dental School, and volunteers signed a written, informed consent. During each phase, twelve healthy adult volunteers (absence of active caries lesions and normal unstimulated (0.6 ± 0.4 mL/min) and stimulated (1.7± 0.4 mL/min) salivary flow rate), aged 25 – 32 years, wore palatal appliances containing two holders, each one with 4 bovine enamel blocks with known surface hardness (SH). The blocks were mounted in contact with a layer of bacteria ('test-plaque'), obtained from a culture of *Streptococcus mutans* IB 1600, and fixed on the palatal appliances through acrylic holders [Zero et al., 1992; Cury et al., 2003]. These test-plaques had been previously treated or not with calcium and fluoride, in order to form different types of F reservoirs: G1) negative control group, no calcium or fluoride reservoirs; G2) only bacterially-bound reservoirs (formed); G3) CaF_2 control group (added) – simulating precipitated CaF_2 reservoirs (powdered CaF_2 added directly to the pellets) and G4) both bacterially-bound and CaF_2 reservoirs (formed). After 30 minutes of intraoral appliance use, half of the enamel blocks and test plaques were collected, two from each side of the appliance, for initial SH and test plaque F analyses. Then, the appliance was reinserted into the mouth and the volunteers rinsed for 1 minute with a 20% sucrose solution. Forty-five minutes after the cariogenic challenge, the other half of the enamel blocks were collected for determination of the percentage of SH loss (%SHL) and the test plaque was collected and analyzed for fluoride concentration in the fluid and solid phases. All volunteers lived in an optimally fluoridated area (0.6 – 0.8 µg F/ml). The experiments were performed after a two hour fasting period, and a placebo non-fluoridated toothpaste was used during the lead-in and washout periods for at least 3 days [Fernández et

al., 2015]. The placebo toothpaste was formulated by Colgate-Palmolive and differed from the commercially available fluoridated toothpaste only in relation to the presence of sodium fluoride (NaF).

Preparation of enamel blocks and baseline SH determination

Enamel blocks (5 × 5 × 2 mm) obtained from bovine incisor crowns were polished flat and had their baseline SH determined by a Future-Tech FM microhardness tester with a Knoop indenter using a 50-gram load for 5 s. In each enamel block, 10 indentations were made at 50, 100, 200, 300, 400, 500, 1,000, 1,500, 2,000 and 2,500 µm from one block edge [Tenuta et al., 2009], to simulate enamel demineralization at different plaque thickness. The upper corners of this side of the enamel block was marked to serve as a reference for proper positioning of the blocks in the holders (the side were the baseline indentations were made) (for further details, please see Tenuta et al., 2008). Then, the mean SH of these 10 indentations was calculated and a total of 192 blocks (336.6 ± 5.75 kg/mm^2) were selected based on the intra-block (±10% of the block's mean) and inter-block variability (±10% of all blocks' mean). The selected blocks were randomly assigned to the treatment groups.

Test plaque preparation and treatment

S. mutans Ingbritt 1600 was grown in Todd-Hewitt Broth (THB) (Difco Labs., Detroit, USA) supplemented with 1% sucrose for 18 h at 37°C and 10% CO_2. Bacterial pellets were separated by centrifugation. In order to remove remnants of culture media and unbound Ca, the bacterial pellets were sequentially washed in 0.05 M PIPES buffer, pH 7.0, followed by 0.01 M EDTA solution, and again in PIPES buffer [Rose et al., 1993] using vortex followed by sonication at 7 W for 1 min (Vibra Cell sonicator, Sonics and Materials, Danbury, USA) to disrupt the pellets at each wash. Between each wash, the pellets were recovered by centrifugation (10,000 *g*, 10 min, 4 °C). The washed pellets were treated with PIPES buffer (10 min at 37°C and 10% CO_2), containing or not calcium and fluoride according to the treatment groups: In group 1, the negative control group, the pellets were treated with PIPES buffer containing no calcium or fluoride. In group 2 the pellets were treated with PIPES buffer containing 0.5 mM fluoride (from sodium fluoride, 1.5 mL / 10 mg) and a 4 mM calcium solution (from a concentrated 1 M calcium solution, made from $CaCl_2$ and used in the proportion of 6 µL /10 mg) to form only bacterially-bound deposits (at the same level of group 4). These concentrations were defined by determination of calcium

and fluoride in supernatants of group 4 in a pilot study (4.02 ± 0.41 mM calcium; 0.55 ± 0.01 mM fluoride). In group 3, the calcium fluoride control group, the pellets were treated with Pipes buffer containing no calcium or fluoride. Then, the suspension was centrifuged and the supernatant discarded. The bacterial pellets were weighted (± 0.01 mg) and powdered CaF_2 (JT Baker®) was added directly to the pellets (55.6 mg / g) and gently spread, to simulate only precipitated CaF_2 reservoirs (at the same level of group 4). The amount of CaF_2 added in this group (corresponding to 0.71 mmol CaF_2/g) was determined from the amount of fluoride precipitated in group 4 in a previous pilot study (1.42 mmol F). A CaF_2 control group was necessary because in natural conditions, when calcium and fluoride concentrations in the oral fluids are high enough to form CaF_2, the biological fluoride reservoir is also formed. Lastly, in group 4 the pellets were treated with PIPES buffer containing 10 mM fluoride (from sodium fluoride, 1.5 mL / 10 mg) and with a 10 mM calcium solution (from concentrated 1M calcium solution, 15 µL /10 mg), to form both bacterially-bound and CaF_2 reservoirs (at the same levels of groups 2 and 3). After the treatments, the pellets were recovered by centrifugation and spread on filter paper to remove excess treatment solution (except for group 3, in which this procedure was done before adding powdered CaF_2). Duplicate samples of each freshly prepared test-plaque were collected for determination of the baseline test-plaque fluoride concentration.

Palatal appliance mounting

Acrylic palatal appliances carrying two plastic holders were constructed for each volunteer. Four bovine enamel blocks with known SH were mounted in each holder in contact with the *S. mutans* test-plaques. The plastic holders were mounted with the marked edge of the enamel blocks, where the baseline hardness measurements were made, facing the center of the palatal appliance. This is relevant to assure the access of saliva and sugar to the test-plaque (further details can be found in Cury et al., 2003). Also, given the split mouth design of the study, test plaques containing treatments 1 and 4 were used in one phase, and treatments 2 and 3 in the other. These combinations were chosen to check any cross-contamination effect. Different colors were used to identify the holders containing different test-plaques.

Intra oral demineralization test

Immediately after mounting, the palatal appliance holding the enamel blocks and test-plaques was kept inside the volunteer's mouth for 30 min. After this period, half of the enamel blocks were removed and the test-plaques were collected for fluoride analysis. Then, the appliance was reinserted into the mouth and the volunteers gently rinsed for 1 minute with a 20% sucrose solution, to simulate a cariogenic challenge. The appliances were used for a subsequent 45-min period, when the other half of samples were collected for determination of the percentage of SH Loss in the blocks (%SHL) and for determination of the test-plaque fluoride concentration in the fluid and solids. Throughout the intraoral test, subjects were instructed to avoid talking, drinking or eating.

Collection and fluoride analysis of the test-plaque fluid phase

The test-plaque samples were collected with a plastic spatula, and immediately placed inside an oil-filled centrifuge tube [Vogel et al., 1990]. After weighing, the tube was centrifuged (5 min, at 21,000 g and 4 °C) to separate the fluid from the plaque solids. Then the test-plaque fluid phase was recovered using oil-filled capillary micropipettes under microscope and the fluoride concentration was immediately determined, using an inverted fluoride electrode, as described previously [Vogel et al., 1990, Tenuta et al., 2009].

Enamel demineralization assessment

Enamel blocks removed from the holders were washed with deionized water and had their SH measured again. A new set of ten indentations was made 150 μm distant from the baseline indentations. From this block edge, sucrose solution and saliva had access to the enamel surface covered by test plaque, simulating the diffusion through dental plaque thickness of up to 2.5 mm [Zero, 1995]. The percentage of Surface Hardness Loss (%SHL) was calculated at each distance from the block edge according to the formula: [%SHL = 100 (SH after in situ test – baseline SH) / baseline SH].

Statistical analysis

As all the volunteers used all combinations of treatments, they were considered as statistical blocks in the statistical analysis, to reduce unknown variability in the experimental error. Baseline fluoride concentration in the fluid phase of test plaques (before the intra-oral test) was analyzed by one-way ANOVA. The results of the two test plaques and enamel blocks of each side of the appliance (same treatment) from the same collection time (pre or post cariogenic challenge) were averaged. Factors under study were treatments, at 4 levels,

and collection time, at 2 levels. The fluoride concentration in the fluid phase of the test plaques was analyzed by two-way ANOVA. For %SHL, a split-plot ANOVA was used and pairwise differences were tested using the Tukey test (comparisons between treatments at each distance and between distances (simulating plaque thickness) within each treatment). The assumptions of equality of variances and normal distribution of errors were checked and data that did not fit these assumptions were transformed [Box et al., 2005]. SAS software/LAB (version 9.2; SAS Institute Inc., Cary, N.C., USA) was used for all analyses and the significance limit was set at 5%.

Results

The F concentration in the fluid phase of the test plaques at baseline (before the intraoral test, n = 6-8) was higher in group 4, followed by groups 3, 2 and 1 (Fig. 1, time zero). In groups containing CaF_2 reservoirs (groups 4 and 3), the high F concentrations found in the test plaques at baseline were maintained during the first 30 min of intraoral appliance use, in contrast with the group containing only biological reservoirs, group 2 (Fig 1, time 30 minutes), in which the F concentration decreased sharply ($p < 0.05$). After the cariogenic challenge, the test-plaque F concentration decreased in all groups ($p < 0.05$), but only those groups containing CaF_2 maintained significantly higher fluoride concentrations than the negative control group throughout the experiment (Fig 1, time 75 minutes, $p < 0.05$).

Accordingly, at the first 500 μm distance from the block edge, groups 1 and 2 had the highest percentage of surface hardness loss ($p < 0.05$); while in groups containing CaF_2 the enamel demineralization was negligible (figure 2). These differences gradually decreased with deeper plaque thickness ($p > 0.05$). In addition, the effect of distance from the block edge was different according to treatment group. For groups containing CaF_2, no significant effect of distance from the block edge was observed (groups 4 and 3; $p > 0.05$). On the other hand, for the group containing only bacterially-bound reservoirs hardness loss was significantly smaller at 2,000 μm (group 2; $p < 0.05$), while for the negative control group the %SHL was higher at 400 and 500 μm ($p < 0.05$).

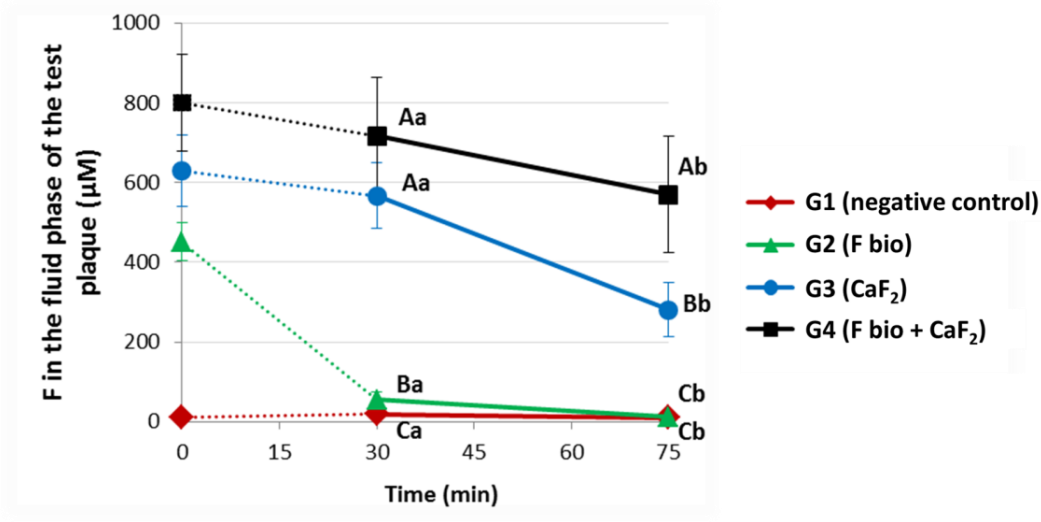

Figure 1: Fluoride concentration in the fluid phase of the test plaques according to the treatment group. Before the intra-oral test (time zero, n = 6-8) the test-plaque F concentration differed among all groups ($p<0.05$). Different capital letters indicates differences between groups at the same collection time (mean ± SD, n = 12). Different lowercase letters indicates differences between collections made before (30 min) and after (75 min) the cariogenic challenge (two-way ANOVA; $p<0.05$).

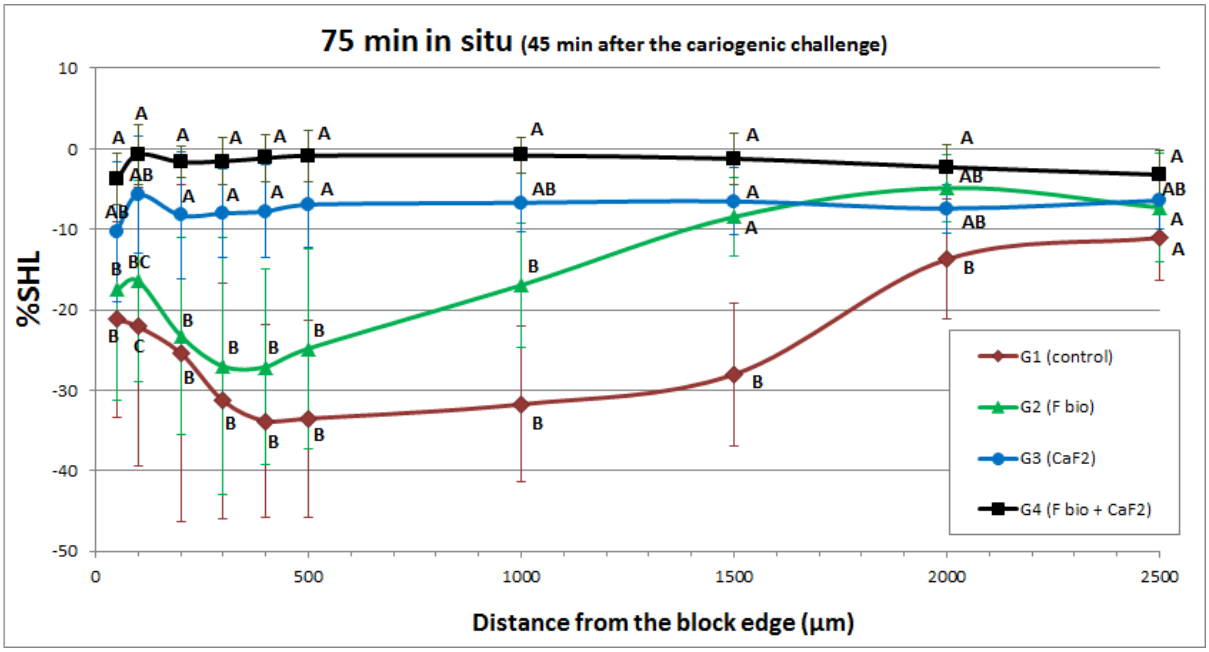

Figure 2: %SHL according to the treatments and distance from the block edge after 75 minutes of intraoral appliance use (mean ± SD, n=12). At each distance, means with different capital letters indicates significant differences between the treatment groups ($p<0.05$). No significant effect of distance from the block edge was observed for G4 and G3, but for G2 hardness loss at 2,000 µm was significantly lower than at the other distances, as well as for G1 at 400 and 500 µm (split-plot ANOVA; $p<0.05$).

Discussion

Fluoride is able to reduce the progression of caries lesions ("preventive effect") and also to reverse the pre-existing ones ("therapeutic effect") [Nóbrega et al., 2016]. Nevertheless, these effects depends on the constant maintenance of fluoride in the oral fluids, especially in the biofilm fluid phase, considering its central role in the caries process [Vogel, 1990]. In this study, the fluoride release from different test plaque fluoride reservoirs, i.e., biological and mineral reservoirs, or a combination of both, to its fluid phase, was studied. Our results showed that at baseline (before the intraoral test), higher test-plaque fluid fluoride concentrations were found in the group containing the combination of biological and mineral reservoirs (group 4), followed by the groups that contained only one of these two pools (groups 3 and 2) and by the negative control group (figure 1, time zero). We expected that the combination of groups 2 and 3 would reflect the F concentration found in the fluid phase of group 4. This differences may be attributed to the different sources used to produce the CaF_2 reservoirs in groups 3 (added from powdered CaF_2 salt) and 4 (naturally formed by treatment with high concentrated Ca and F solutions). However, irrespective of the F concentration found at baseline, only those groups containing CaF_2 mineral reservoirs were able to maintain significantly increased fluoride concentrations in the test plaque fluid phase during the first 30 minutes of exposure to saliva (figure 1, time 30 min). This result suggests that CaF_2 mineral reservoirs are more persistent, acting as a long-term fluoride reservoir, in contrast with the biological reservoirs, from which fluoride is rapidly lost.

Previous studies have reported increased fluoride concentrations in the biofilm fluid phase immediately after the isolated use of fluoridated agents such as rinses and toothpastes (able to form only bacterially-bound reservoirs) [Cenci et al., 2008; Cury et al., 2010; Tenuta et al., 2010]. However, this elevated fluoride concentrations falls in the subsequent hours by diffusion of the fluoride ion from the biofilm fluid to saliva [Cury et al., 2010; Tenuta et al., 2010]. Vogel et. al [2010] found a 4-fold reduction in the biofilm fluid F concentration (from 85 ± 0.55 μmol/l to 22 ± 0.38 μmol/l) in samples collected *in vivo*, under undisturbed sugar conditions, 30 and 60 minutes after a NaF rinse. Conversely, when the use of a fluoride rinse is preceded by a calcium prerinse (able to form both bacterially-bound reservoirs and precipitated CaF_2), more persistent increases have been reported in biofilm fluid F levels (5-7 x higher), when compared to the use of NaF rinse alone [Vogel et al., 2008; 2014]. These results support the hypothesis that CaF_2 reservoirs contributes to the long-term maintenance

of elevated fluoride levels in biofilm fluid, overcoming the poor fluoride retention following topical fluoride administration.

After the cariogenic challenge, a reduction in the test-plaque fluid fluoride concentration was observed in all groups (figure 1, time 75 min). Our results are similar to those observed in a previous study using the same short-term *in situ* model and should be mainly attributed to the diffusion of fluoride to saliva [Tenuta et al., 2009]. Nevertheless, even considering the large interval between the cariogenic challenge and the final biofilm collection (45 min), those groups containing CaF_2 reservoirs were able to maintain significantly higher test plaque fluoride concentrations than G1 and G2.

This is the first study to evaluate the anticaries effect of fluoride released from different types of biofilm fluoride reservoirs to the biofilm fluid phase. As a consequence of the higher fluoride availability in the test-plaque fluid phase, no demineralization was observed in groups containing CaF_2 reservoirs. Moreover, in the first 500 μm distance from the block edge, the enamel demineralization was significantly higher in the group containing only biological reservoirs, that did not differ from the negative control group (figure 2). Also, no significant effect of distance from the block edge was observed in the CaF_2 groups. These results are consistent with the current understanding that fluoride available in the biofilm fluid is able to interfere with the caries process, reducing demineralization and enhancing remineralization of enamel and root dentine [Tenuta and Cury, 2010].

Several studies have shown a clear relationship between the increased biofilm fluid fluoride concentrations and caries control, after usage of fluoridated agents [Cenci et al., 2008; Tenuta et al., 2009; Cury et al., 2010; Fernandéz et al., 2017]. In such conditions, only bacterially bound fluoride reservoirs are expected to be formed. On the other hand, Vogel et al. [2014] found that the use of a calcium treatment prior to a fluoride rinse was able to produce CaF_2 reservoirs in dental biofilms, in addition to biological reservoirs. However, up to date, only one study evaluated the effect of this combination on enamel demineralization [Souza et al., 2016], but the relative importance of the increased fluoride availability in oral fluids promoted by this treatment, in relation to the increased retention of fluoride in biofilm reservoirs, is unknown. Therefore, our experimental results extend this knowledge by showing that precipitated CaF_2 is the main responsible for the anticaries effect of biofilm fluoride reservoirs.

The experimental short-term in situ demineralization model used in the present study [Brudevold et al., 1984; modified by Zero et al., 1992] has been successfully used to test the mechanism of action of several fluoridated agents, such as toothpastes [Tenuta et al., 2009 and 2010], gels [Tenuta et al., 2008] and F-releasing dental materials [Tenuta et al., 2005]. In our study, this model allowed the distinction of different levels of inhibition of enamel hardness loss by different fluoride biofilm reservoirs, i.e. bacterially-bound fluoride and/or precipitated CaF_2. This would simulate two distinct clinical conditions: (1) use of topical over the counter fluoridated products alone; or (2) in combination with a calcium pretreatment. However, limitations of the model mean that the results cannot be extrapolated directly to *in vivo* conditions. One limitation is the use of a monospecific matrix rich artificial test-plaque, rather than naturally-formed biofilms. Also, considering the high fluoride concentration found in "plaque fluid" of groups containing CaF_2 at the moment of the cariogenic challenge, it is possible that some inhibition of acid production by test-plaque bacteria may have contributed to the lower enamel demineralization found in these groups (since the inhibitory concentration is about 10 ppm ≈ 526.3 μM F [Marsh and Bradshaw, 1990]). Nevertheless, the model was able to identify the differences in fluoride released from different test-plaque pools to the biofilm fluid, as well as its anticaries effect. Moreover, given the short-term effect observed for bacterially bound fluoride reservoirs on biofilm fluid fluoride (drops quickly during the first 30 minutes), the use of a long-term in situ model would not be appropriate.

In summary, the findings of this in situ study suggest that CaF_2, rather than bacterially-bound reservoir, is able to maintain increased fluoride concentrations in the biofilm fluid phase, reducing enamel demineralization.

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J.A.C.; performed the experiments: D.F.N, L.M.A.T., A.A.D.B.C; analyzed the data: D.F.N, L.M.A.T.; J.A.C. wrote the draft manuscript: D.F.N., L.M.A.T.; reviewed the paper: D.F.N, L.M.A.T, A.A.D.B.C., J.A.C.

References:

Box GEP, Hunter JS, Hunter WG: Statistics for Experimenters: Design, Innovation, and Discovery, ed 2. New York, John Wiley and Sons, 2005.

Brudevold F, Attarzadeh F, Tehrani A, van Houte J, Russo J. Development of a new intraoral demineralization test. Caries Res 1984;18:421-9.

Cenci MS, Tenuta LM, Pereira-Cenci T, Del Bel Cury AA, ten Cate JM, Cury JA. Effect of microleakage and fluoride on enamel-dentine demineralization around restorations. Caries Res 2008;42:369-379.

Cury JA, Francisco SB, Simões GS, Del Bel Cury AA, Tabchoury CP. Effect of a calcium carbonate-based dentifrice on enamel demineralization in situ. Caries Res. 2003;37:194-9.

Cury JA, Amaral RC, Tenuta LMA, Del Bel Cury AA, Tabchoury CPM. Low-fluoride toothpaste and deciduous enamel demineralization under biofilm accumulation and sucrose exposure. Eur J Oral Sci 2010;118:370-375.

Fejerskov O, Manji F. Risk assessment in dental caries: In: Bader J, editor. Risk assessment in dentistry. Chapel Hill: University of North Carolina Dental Ecology, 1990. p 215-17.

Fernández CE, Tenuta LMA, Cury JA: Wash-out period for crossover design experiments using high fluoride concentration dentifrice (in Spanish). Rev Clin Periodoncia Implantol Rehabil Oral 2015;8:1–6.

Marcenes W, Kassebaum NJ, Bernabé E, Flaxman A, Naghavi M, Lopez A, Murray CJ. Global burden of oral conditions in 1990-2010: a systematic analysis. J Dent Res. 2013;92:592-7.

Margolis HC, Moreno EC. Composition of pooled plaque fluid from caries-free and caries-positive individuals following sucrose exposure. J Dent Res. 1992;71:1776-84.

Marsh PD, Bradshaw DJ. The effect of fluoride on the stability of oral bacterial communities in vitro. J Dent Res 1990;69 Spec Iss: 668-71.

Nóbrega DF, Fernández CE, Del Bel Cury AA, Tenuta LM, Cury JA. Frequency of Fluoride Dentifrice Use and Caries Lesions Inhibition and Repair. Caries Res. 2016;50:133-40.

Pearce EI, Margolis HC, Kent RL Jr. Effect of in situ plaque mineral supplementation on the state of saturation of plaque fluid during sugar-induced acidogenesis. Eur J Oral Sci. 1999;107:251-9.

Rose RK, Dibdin GH, Shellis RP. A quantitative study of calcium binding and aggregation in selected oral bacteria. J Dent Res. 1993;72:78-84.

Rose RK, Shellis RP, Lee AR. The role of cation bridging in microbial fluoride binding.Caries Res 1996;30:458-64.

Souza JG, Tenuta LM, Del Bel Cury AA, Nóbrega DF, Budin RR, de Queiroz MX, Vogel GL, Cury JA. Calcium Prerinse before Fluoride Rinse Reduces Enamel Demineralization: An in situ Caries Study. Caries Res. 2016;50(4):372-377.

Tenuta LM, Ribeiro CC, Gonçalves NC, Del Bel Cury AA, Aires CP, Tengan C, Tagliaferro EP, Pecharki GD, Napimoga MH, Tabchoury CP, Cury JA. The short-term in situ model to evaluate the anticariogenic potential of ionomeric materials. J Dent. 2005;33:491-7.

Tenuta LM, Cerezetti RV, Del Bel Cury AA, Tabchoury CP, Cury JA. Fluoride release from CaF2 and enamel demineralization. J Dent Res. 2008;87:1032-6.

Tenuta LM, Zamataro CB, Del Bel Cury AA, Tabchoury CP, Cury JA. Mechanism of fluoride dentifrice effect on enamel demineralization. Caries Res. 2009;43:278-285.

Tenuta LM, Cury JA. Fluoride: its role in dentistry. Braz Oral Res. 2010;24 Suppl 1:9-17.

Tenuta LM, Del Bel Cury AA, Tabchoury CP, Moi GP, Silva WJ, Cury JA. Kinetics of monofluorophosphate hydrolysis in a bacterial test plaque in situ. Caries Res. 2010;44:55-9.

Vogel GL, Carey CM, Chow LC, Tatevossian A. Micro-analysis of plaque fluid from single-site fasted plaque. J Dent Res. 1990; 69:1316-23.

Vogel GL, Schumacher GE, Chow LC, Takagi S, Carey CM. Ca pre-rinse greatly increases plaque and plaque fluid F. J Dent Res. 2008;87:466-9.

Vogel GL, Tenuta LM, Schumacher GE, Chow LC. No calcium-fluoride-like deposits detected in plaque shortly after a sodium fluoride mouthrinse. Caries Res. 2010;44:108-15.

Vogel GL. Oral fluoride reservoirs and the prevention of dental caries. Monogr Oral Sci. 2011;22:146-57.

Vogel GL, Tenuta LM, Schumacher GE, Chow LC. A calcium prerinse required to form calcium fluoride in plaque from a sodium fluoride rinse. Caries Res. 2014;48:174-8.

Zero DT, Fu J, Anne KM, Cassata S, McCormack SM, Gwinner LM. An improved intra-oral enamel demineralization test model for the study of dental caries. J Dent Res. 1992;71 Spec No:871-8.

Zero DT: In situ caries models. Adv Dent Res. 1995; 9: 214–230.

3. DISCUSSÃO

Tendo em vista o papel do biofilme no desenvolvimento da cárie dental e a importância da manutenção de fluoreto neste local para o seu efeito anticárie (Tenuta et al., 2009), esta tese teve como objetivo principal avaliar a dinâmica de formação dos reservatórios de fluoreto presentes no biofilme dental e a contribuição de cada um deles para o controle da cárie dental. Espera-se que o conhecimento gerado por esta pesquisa possibilite o melhor entendimento do mecanismo de ação destes reservatórios de fluoreto, pelo estudo de sua capacidade de suprir o fluido do biofilme com fluoreto, assim como o seu efeito na redução da desmineralização dental.

Em geral, foi observado que o sinergismo entre cálcio e fluoreto na retenção de flúor a *S. mutans* só ocorre na presença de altas concentrações destes íons (artigo 1). Nestas condições, o aumento da retenção de fluoreto nos pellets bacterianos foi atribuído principalmente à precipitação do mineral fluoreto de cálcio (CaF_2), e não apenas a ligação do fluoreto à bactérias do biofilme dental, como havia sido proposto anteriormente (Rose et al., 1996) . Além disto, os resultados do nosso estudo in situ (artigo 2) demonstram que apenas os reservatórios de CaF_2 são capazes de manter concentrações elevadas de fluoreto no fluido do biofilme, mantendo um prolongado efeito anticárie.

No que diz respeito à retenção fluoreto na superfície de bactérias do biofilme dental, os dados do estudo *in vitro* apresentados no artigo 1 desta tese mostraram que na presença de baixas concentrações de Ca e F, ou seja, abaixo do limite de solubilidade do CaF_2, a ligação de fluoreto à *S. mutans* não é influenciada pela disponibilidade de cálcio e vice-versa. Este resultado já havia sido relatado anteriormente (Leitão et al., 2013) e contradiz o modelo proposto por Rose et al. (1996), no qual o Ca, por ser um íon divalente, se ligaria a dois grupamentos aniônicos na mesma bactéria ou entre bactérias adjacentes. A adição de F seria capaz de quebrar estas ligações, expondo novos sítios aniônicos para que mais Ca e F pudessem se ligar. No entanto, nos experimentos de Rose et al. (1996) o sinergismo de efeito entre Ca e F na ligação a bactérias do biofilme dental só foi estudado na presença de altas concentrações destes íons, o que também favorece a precipitação de fluoreto de cálcio. Nossos resultados demonstram que abaixo do limite de solubilidade do CaF_2, a retenção de fluoreto não se alterou com o aumento da concentração de cálcio nos tratamentos. Este efeito parece ser justificado não pela ausência de Ca ligado na superfície bacteriana, mas devido a

limitação de fluoreto livre para se ligar ao Ca, uma vez que a concentração total de fluoreto retido nos pellets bacterianos tratados com 0 (0.98 ± 0.19 nmol/mg) e 1 mM de flúor (1.79 ± 0.26 nmol/mg) foi sempre menor que a concentração de cálcio retida nos pellets tratados com estas mesmas concentrações de cálcio (3.53 ± 1.51 nmol/mg e 6.57 ± 0.60 nmol/mg, respectivamente).

Por outro lado, quando os pellets de *S. mutans* foram expostos a altas concentrações destes íons (acima do limite de solubilidade do CaF_2), a retenção de fluoreto nos pellets bacterianos aumentou significativamente (12-89 vezes) e de maneira proporcional a concentração de Ca utilizada no tratamento. O mesmo foi observado em relação a retenção de cálcio, quando os pellets foram expostos a concentrações crescentes de fluoreto (13-28 vezes maior). Estes resultados inéditos sugerem que o "sinergismo" de efeito observado por Rose et al. (1996) quando da exposição do biofilme a altas concentrações de Ca e F, se deve principalmente à precipitação de CaF_2 e não à ligação de fluoreto à bactérias, via pontes de Ca. Neste sentido, o aumento exponencial das concentrações de F na porção sólida do biofilme (12-22 vezes maior) relatado em estudos *in situ* e *in vitro*, após o uso combinado de bochechos contendo concentrações elevadas de cálcio e flúor (Vogel et al., 2008; Vogel et al., 2014; Souza et al., 2016), também deve estar relacionado à formação de reservatórios de CaF_2 no biofilme dental.

No artigo 1 foi estudada apenas a dinâmica de formação de reservatórios bacterianos/biológicos e minerais de fluoreto em diferentes condições de exposição a cálcio e flúor. Diante da constatação de que a formação desses dois diferentes tipos de reservatórios de fluoreto no biofilme dental poderia ser controlada *in vitro*, pelo emprego de soluções sub ou supersaturadas em relação ao limite de solubilidade do CaF_2, o próximo passo do estudo foi avaliar a contribuição relativa de cada um destes reservatórios para o controle da cárie dental. Assim, com base nos nossos resultados *in vitro*, foi desenvolvido um estudo *in situ* (artigo 2), com o objetivo de avaliar a liberação de fluoreto a partir de reservatórios biológicos, minerais, ou uma combinação deles, para o fluido do biofilme e seu efeito na desmineralização do esmalte dental.

Para isto foi utilizado o modelo *in situ* de curta duração proposto por Brudevold (1984) e adaptado por Zero (1992), para a mensuração do potencial anticárie dos reservatórios de fluoreto no biofilme. Neste modelo, uma placa-teste de rica em matriz extracelular é

preparada a partir de *S. mutans* e utilizada para causar uma desmineralização no esmalte dental em diferentes profundidades, simulando assim a difusão de açúcar pelo biofilme dental. Este modelo tem sido utilizado com sucesso no estudo do mecanismo de ação de diferentes produtos fluoretados, tais como dentifrícios (Cury et al., 2003 and 2005; Tenuta et al., 2009 and 2010), géis (Tenuta et al., 2008) e materiais dentários liberadores de flúor (Tenuta et al., 2005). A grande vantagem deste modelo *in situ* é a possibilidade de avaliar o desenvolvimento da cárie dental em condições controladas, mimetizando na medida do possível o que ocorreria naturalmente na cavidade bucal (pH, temperatura, concentração de O_2, uso de microorganismo cariogênico, acesso à saliva, etc). Além disto, o modelo in situ de curta duração permite a utilização de técnicas análiticas laboratóriais de alta sensibilidade e validade científica, fornecendo informações clinicamente relevantes em um curto período de tempo, a um custo relativamente baixo e sem causar danos irreversíveis a dentição natural (Zero et al., 1995).

Para avaliar o efeito anticárie dos diferentes tipos de reservatórios de fluoreto no biofilme dental, foram utilizados 4 grupos de tratamento: G1. Placa-teste sem reservatórios de F (controle negativo); G2. Placa-teste contendo apenas reservatórios bacterianos de F (formado); G3. Placa teste contendo apenas CaF_2 (adicionado) (controle ativo); e G4. Placa teste contendo reservatórios bacterianos e CaF_2 (formado). Os resultados mostraram que no baseline (antes do teste intra-oral) foram encontradas diferentes concentrações de fluoreto na fase fluida da placa teste: G4 > G3 > G2 > G1 (figura 1 do artigo 2, tempo zero). A princípio era esperado que a concentração de fluoreto encontrada no fluido do biofilme do grupo 4 (reservatórios biológicos + CaF_2) fosse semelhante àquela resultante da soma dos grupos 2 (reservatórios biológicos) e 3 (reservatórios de CaF_2), uma vez que as concentrações de Ca (4,02 ± 0,41 mM) e F (0,55 ± 0,01 mM) adicionadas no grupo 2, assim como a concentração de pó de CaF_2 adicionado no grupo 3 (0,7125 mmol CaF_2 /g), foram determinadas em um estudo piloto, com base no grupo 4. Estas diferenças na concentração de flúor encontradas no baseline parecem ser explicadas pelas diferentes origens dos reservatórios de CaF_2 utilizados para formar os grupos 3 e 4. No grupo 3, foi adicionado CaF_2 em pó, diretamente ao pellet preparado in vitro; enquanto no grupo 4 o CaF_2 foi formado naturalmente, pela exposição a soluções contendo altas concentrações de Ca e F. No entanto, a utilização de um controle ativo de CaF_2 faz-se necessária, pela impossibilidade deste reservatório ser formado separadamente em condições naturais, uma vez que quando o nível de saturação de Ca e F no

fluido do biofilme é alto o suficiente para que haja a precipitação de CaF_2, invariavelmente o reservatório biológico também é formado.

A análise realizada na placa-teste coletada após 30 minutos de utilização intra-oral (para equilíbrio com a saliva) mostrou que apenas os grupos que continham CaF_2 (grupos 3 e 4) foram capazes de manter as elevadas concentrações de fluoreto encontradas no fluido da placa-teste no baseline, em comparação aos demais grupos ($p < 0.05$) (figura 1 do artigo 2, tempo 30 min). Por outro lado, no grupo que continha apenas reservatórios biológicos (grupo 2) a concentração de fluoreto caiu bruscamente durante este intervalo (de 451,5 ± 47,3 µM para 55,2 ± 18,6 µM). Estes resultados sugerem que os reservatórios de CaF_2 seriam mais persistentes e poderiam ser liberados por mais tempo (até 75 minutos neste estudo), ao contrário dos reservatórios biológicos que parecem ter uma natureza muito mais lábil e se difundem rapidamente para a saliva (nos primeiros 30 minutos).

A redução na concentração de fluoreto no fluido do biofilme durante os primeiros 30 minutos de experimento já era esperada, tendo em vista o efeito da difusão do fluoreto para a saliva observado em estudos prévios (Cury et al., 2010, Tenuta et al., 2010). Um estudo *in vivo* chegou a reportar uma redução de 75 % na concentração de fluoreto no fluido de biofilmes tratados com bochecho fluoretado (capaz de formar apenas reservatórios biológicos de F), após um intervalo de 30 minutos de exposição à saliva (Vogel et al., 2010). No entanto, quando o uso deste mesmo bochecho fluoretado foi precedido de um bochecho com cálcio (capaz de formar tanto reservatórios biológicos e de CaF_2), a concentração de fluoreto encontrada no fluido do biofilme foi 7 vezes maior que aquela do grupo que havia utilizado apenas o bochecho fluoretado (Vogel et al. 2014), mostrando que a utilização de Ca e F em altas concentrações é capaz de potencializar a disponibilidade de fluoreto no biofilme dental.

A coleta realizada 45 minutos após a realização do desafio cariogênico mostrou uma redução na concentração de fluoreto no fluido da placa-teste em todos os grupos, como esperado em virtude da exposição à saliva. Apesar disto, aqueles grupos que continham reservatórios de CaF_2 (grupos 3 e 4) foram capazes de manter concentrações elevadas de fluoreto no fluido do biofilme em comparação aos demais, durante todo o experimento (figura 1 do artigo 2, tempo 75 minutos). Estes achados reafirmam que os reservatórios de CaF_2 estão relacionados a manutenção de fluoreto no fluido do biofilme a longo prazo.

No que diz respeito aos resultados da análise da porcentagem de perda de dureza de superfície, como consequência da maior disponibilidade de flúor no fluido do biofilme, a desmineralização do esmalte nos grupos contendo reservatórios de CaF_2 foi desprezível. Por outro lado, a desmineralização no grupo contendo apenas reservatórios biológicos de flúor na placa teste foi significativamente maior, não diferindo do grupo controle nos primeiros 500 mM do bloco (simulando a "profundidade da placa") (figura 2 do artigo 2). Os resultados de %PDS refletem a disponibilidade de fluoreto encontrado no fluido do biofilme no momento do desafio cariogênico (G1 < G2 < G3 = G4), e estão de acordo com o atual conhecimento de que a presença de fluoreto no fluido do biofilme é capaz de interferir físico-quimicamente no desenvolvimento da cárie, pela redução da desmineralização e ativação da remineralização dental (Tenuta et al., 2017).

Sabe-se que o uso de dentifrícios e géis fluoretados aumenta a disponibilidade de fluoreto no fluido do biofilme imediatamente após o uso (Tenuta et al., 2009; Cury et al. 2010), mantendo este efeito por até 10 horas (Cenci et al., 2008; Cury et al., 2010; Fernandéz et al., 2017), o que resulta na redução da desmineralização do esmalte e da dentina. Além disto, a associação de bochecho fluoretado com um pré-bochecho de cálcio já mostrou ser capaz de potencializar a retenção de fluoreto no biofilme, assim como seu o efeito anticárie (Souza et al., 2016). No entanto, a importância relativa de cada tipo de reservatório para este efeito era desconhecida. e foram demonstradas pela primeira vez no presente estudo.

Além disto, se considerarmos a alta concentração de fluoreto encontrada no fluido da placa teste dos grupos que continham CaF_2 no momento da realização do desafio cariogênico (G4 = 717,0 ± 147,8 μM e G3 = 567,1 ± 83,1 μM), é possível que tenha havido algum efeito antibacteriano, uma vez que a manutenção constante de fluoreto em concentrações superiores a 10 ppm F (526.3 μM F) é capaz de afetar a capacidade dos organismos de produzir ácidos (Bradshaw et al., 2002). Portanto, a inibição da acidogenicidade bacteriana também pode ter contribuído para o melhor efeito anticárie observado nos grupos 3 e 4.

4. CONCLUSÃO

Diante do exposto, os resultados observados in vitro e in situ permitem concluir que:

1) A menos que altas concentrações de cálcio e fluoreto sejam utilizadas, não existe efeito sinérgico entre estes dois íons na ligação à superfície bacteriana. Assim, o aumento da retenção de fluoreto observada quando o biofilme dental é exposto a altas concentrações de cálcio e flúor deve ser atribuído principalmente à precipitação de fluoreto de cálcio, e não a ligação de fluoreto a bactérias do biofilme.

2) O efeito anticárie do fluoreto retido no biofilme está diretamente relacionado a presença de reservatórios de fluoreto de cálcio, uma vez que estes são capazes de manter concentrações elevadas de fluoreto no fluido do biofilme, reduzindo a perda mineral.

REFERÊNCIAS

Bradshaw DJ, Marsh PD, Hodgson RJ, Visser JM: Effects of glucose and fluoride on competition and metabolism within in vitro dental bacterial communities and biofilms. Caries Res. 2002; 36: 81–86.

Bratthall D, Hänsel-Petersson G, Sundberg H: Reasons for the caries decline: what do the experts believe? Eur J Oral Sci 1996; 104: 416-422.

Brudevold F, Attarzadeh F, Tehrani A, van Houte J, Russo J. Development of a new intraoral demineralization test. Caries Res 1984;18(5):421-9.

Cenci MS, Tenuta LM, Pereira-Cenci T, Del Bel Cury AA, ten Cate JM, Cury JA. Effect of microleakage and fluoride on enamel-dentine demineralization around restorations. Caries Res 2008;42:369-379.

Cury JA, Francisco SB, Simões GS, Del Bel Cury AA, Tabchoury CP. Effect of a calcium carbonate-based dentifrice on enamel demineralization in situ. Caries Res. 2003;37(3):194-9.

Cury JA, Tenuta LM, Ribeiro CC, Paes Leme AF. The importance of fluoride dentifrices to the current dental caries prevalence in Brazil. Braz Dent J. 2004;15(3):167-174.

Cury JA, Simões GS, Del Bel Cury AA, Gonçalves NC, Tabchoury CP. Effect of a calcium carbonate-based dentifrice on in situ enamel remineralization. Caries Res. 2005;39(3):255-7.

Cury JA, Tenuta LM. Enamel remineralization: controlling the caries disease or treating early caries lesions? Braz Oral Res. 2009;23 Suppl 1:23-30.

Cury JA, Amaral RC, Tenuta LMA, Del Bel Cury AA, Tabchoury CPM. Low-fluoride toothpaste and deciduous enamel demineralization under biofilm accumulation and sucrose exposure. Eur J Oral Sci 2010;118:370-375.

Fernández CE, Tenuta LMA, Del Bel Cury AA, Nóbrega DF, Cury JA. Effect of 5,000 ppm Fluoride Dentifrice or 1,100 ppm Fluoride Dentifrice Combined with Acidulated Phosphate Fluoride on Caries Lesion Inhibition and Repair. Caries Res. 2017;51(3):179-187.

Iheozor-Ejiofor Z, Worthington HV, Walsh T, O'Malley L, Clarkson JE, Macey R, et al. Water fluoridation for the prevention of dental caries. Cochrane Database Syst Rev. 2015. 18;(6):CD010856.

Leitão TJ , Tenuta LM, Borges PA, Salvaterra C, Cury JA. Calcium and Fluoride Binding to Streptococcus mutans at Below-KSP CaF_2 Concentrations [ORCA abstract 203]. Caries Res 2013;47(Spec Issue):516-516.

Marinho VC, Higgins JP, Sheiham A, Logan S. Fluoride toothpastes for preventing dental caries in children and adolescents. Cochrane Database Syst Rev. 2003;(1):CD002278.

Marinho VC, Worthington HV, Walsh T, Clarkson JE. Fluoride varnishes for preventing dental caries in children and adolescents. Cochrane Database Syst Rev. 2013 Jul 11;(7):CD002279.

Marinho VC, Worthington HV, Walsh T, Chong LY. Fluoride gels for preventing dental caries in children and adolescents. Cochrane Database Syst Rev. 2015. 15;(6):CD002280.

Marinho VC, Chong LY, Worthington HV, Walsh T. Fluoride mouthrinses for preventing dental caries in children and adolescents. Cochrane Database Syst Rev. 2016. 29;7:CD002284.

Nyvad B: The role of oral hygiene; in Fejerskov O, Nyvad B, Kidd E (eds): Dental Caries. The Disease and Its Clinical Management, ed 3. Oxford, Wiley-Blackwell, 2015, pp 277–285.

Pitts NB, Zero DT, Marsh PD, Ekstrand K, Weintraub JA, Ramos-Gomez F, Tagami J, Twetman S, Tsakos G, Ismail A. Dental caries. Nat Rev Dis Primers. 2017. 25;3:17030.

Rose RK, Shellis RP, Lee AR. The role of cation bridging in microbial fluoride binding.Caries Res 1996;30:458-64.

Souza JG, Tenuta LM, Del Bel Cury AA, Nóbrega DF, Budin RR, de Queiroz MX, et al. Calcium Prerinse before Fluoride Rinse Reduces Enamel Demineralization: An in situ Caries Study. Caries Res. 2016 Jun 30;50(4):372-377.

Ten Cate JM. Fluorides in caries prevention and control: empiricism or science. Caries Res. 2004 May-Jun;38(3):254-7.

Tenuta LM, Ribeiro CC, Gonçalves NC, Del Bel Cury AA, Aires CP, Tengan C, et al. The short-term in situ model to evaluate the anticariogenic potential of ionomeric materials. J Dent. 2005 Jul;33(6):491-7.

Tenuta LM, Cerezetti RV, Del Bel Cury AA, Tabchoury CP, Cury JA. Fluoride release from CaF2 and enamel demineralization. J Dent Res. 2008;87(11):1032-6.

Tenuta LM, Zamataro CB, Del Bel Cury AA, Tabchoury CP, Cury JA. Mechanism of fluoride dentifrice effect on enamel demineralization. Caries Res. 2009;43(4):278-285.

Tenuta LM, Cury JA. Fluoride: its role in dentistry. Braz Oral Res. 2010;24 Suppl 1:9-17.

Tenuta LM, Cury JA. Laboratory and human studies to estimate anticaries efficacy of fluoride toothpastes. Monogr Oral Sci. 2013;23:108-24.

Tenuta LM, Marín LM, Cury JA. Mecanismo de ação do fluoreto. In: Cury JA, Tenuta LMA, Tabchoury CPM. Bioquímica Oral. 1a. ed. São Paulo: Artes Médicas; 2017. p. 110-123.

Vogel GL, Schumacher GE, Chow LC, Takagi S, Carey CM. Ca pre-rinse greatly increases plaque and plaque fluid F. J Dent Res. 2008;87(5):466-9.

Vogel GL, Tenuta LM, Schumacher GE, Chow LC. No calcium-fluoride-like deposits detected in plaque shortly after a sodium fluoride mouthrinse. Caries Res. 2010;44(2):108-15.

Vogel GL. Oral fluoride reservoirs and the prevention of dental caries. Monogr Oral Sci. 2011;22:146-57.

Vogel GL, Tenuta LM, Schumacher GE, Chow LC. A calcium prerinse required to form calcium fluoride in plaque from a sodium fluoride rinse. Caries Res. 2014;48(2):174-8.

Zero DT, Fu J, Anne KM, Cassata S, McCormack SM, Gwinner LM. An improved intraoral enamel demineralization test model for the study of dental caries. J Dent Res. 1992;71(Spec Iss):871-8.

Zero DT. In situ caries models. Adv Dent Res. 1995 Nov;9(3):214-30; discussion 231-4.

APÊNDICE I

Artigos publicados durante o período do doutorado:

1. Fernández CE, Tenuta LMA, Del Bel Cury AA, **Nóbrega DF**, Cury JA. Effect of 5.000 ppm Fluoride Dentifrice or 1.100 ppm Fluoride Dentifrice Combined with Acidulated Phosphate Fluoride on Caries Lesion Inhibition and Repair. Caries Res.2017;51(3):179-187.

2. Figuero E, **Nóbrega DF**, García-Gargallo M, Tenuta LM, Herrera D, Carvalho JC. Mechanical and chemical plaque control in the simultaneous management of gingivitis and caries: a systematic review. J Clin Periodontol. 2017 Mar;44 Suppl 18:S116-S134.

3. **Nóbrega DF**, Fernández CE, Del Bel Cury AA, Tenuta LM, Cury JA. Frequency of Fluoride Dentifrice Use and Caries Lesions Inhibition and Repair. Caries Res. 2016;50(2):133-40.

4. Souza JG, Tenuta LM, Del Bel Cury AA, **Nóbrega DF**, Budin RR, de Queiroz MX, Vogel GL, Cury JA. Calcium Prerinse before Fluoride Rinse Reduces Enamel Demineralization: An in situ Caries Study. Caries Res. 2016;50(4):372-7.

5. Cury JA, Vieira-Dantas ED, Tenuta LMA, Romão DA, Tabchoury CPM, **Nóbrega DF**, Velo MMAC; Pereira CM. Concentração de fluoreto nos dentifrícios a base de MFP/CaCO3 mais vendidos no Brasil, ao final dos seus prazos de validade. Rev. Assoc. Paul. Cir. Dent. 2015; 69(3):248-51.

Artigos aceitos para publicação:

1. Nóbrega DF, Assis ACBM, Souza JG, Martins AMEBL, Bulgareli JV. Association of normative and subjective oral health conditions and the dissatisfaction with dental services among brazilian adults. Ciência e Saúde Coletiva. 2017.

ANEXO I

Aprovação do Comitê de Ética em Pesquisa da FOP-UNICAMP

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "Avaliação in situ do potencial anticárie de diferentes tipos de reservatórios de fluoreto no biofilme dental", protocolo CAAE nº 60264316.0.0000.5418, dos pesquisadores Diego Figueiredo Nóbrega, Lívia Maria Andaló Tenuta, Jaime Aparecido Cury e Antoninha Del Bel Cury, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 21 de Outubro de 2016.

The Ethics Committee in Research of the Piracicaba Dental School, University of Campinas, certify that the project "The anticaries effect of different types of F reservoirs on dental biofilm: An in situ study", CAAE 60264316.0.0000.5418, of Diego Figueiredo Nóbrega, Lívia Maria Andaló Tenuta, Jaime Aparecido Cury e Antoninha Del Bel Cury, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee on October 21, 2016.

Profa. Fernanda Miori Pascon
Vice Coordenador
CEP/FOP/UNICAMP

Prof. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo e a lista de autores aparece como fornecidos pelos pesquisadores, sem qualquer edição.
Notice: The title and the list of researchers of the project appears as provided by the authors, without editing.