MARÍLIA FERREIRA CORREIA

POTENCIAL ANTICÁRIE DOS RESERVATÓRIOS DE CÁLCIO, FOSFATO E FLUORETO DO BIOFILME DENTAL

Dissertação de mestrado apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, como requisito para obtenção do título de Mestre em Odontologia, Área de Cariologia.

Orientador: Prof. Dr. Jaime Aparecido Cury

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RESUMO

O biofilme dental apresenta reservatórios orgânicos e inorgânicos de cálcio (Ca), fosfato (P_i) e fluoreto (F) que se liberados para o fluido do biofilme mediante quedas de pH, interfeririam com o grau de saturação do fluido, reduzindo a desmineralização dental. Entretanto, ainda se desconhece a origem responsável pelas mudanças observadas na concentração de Ca, P_i e F no fluido após queda de pH: se derivadas dos reservatórios ou da desmineralização do próprio substrato dental. Em acréscimo, ainda não se determinou a solubilidade desses reservatórios em pHs considerados importantes para interferir com o processo de desmineralização dental. Assim, este trabalho de dissertação teve como objetivos avaliar a influência que a solubilidade dos substratos e dos reservatórios do biofilme exercem na concentração inorgânica do fluido do biofilme, avaliando a cinética dos íons Ca, P_i e F para o fluido após desafio acidogênico. Para isto foram realizados dois estudos in situ, ambos cegos e cruzados. No primeiro foi contemplada a hipótese de que a concentração inorgânica do fluido do biofilme seria influenciada pela desmineralização do substrato, refletindo o grau de solubilidade do mineral que o compõe. Para isto, blocos de esmalte (menos solúvel), dentina (mais solúvel) e acrílico (não solúvel) foram expostos, durante 4 dias, a desafios cariogênicos utilizando glicose a 20%, 8x/ao dia; as variáveis analisadas ao final de cada fase foram composição microbiológica, pH no fluido, Ca, P_i e F no fluido e no estroma (parte sólida) do biofilme antes e 5 min após desafio acidogênico. A influência da quantidade dos reservatórios na concentração inorgânica do fluido foi avaliada no segundo in situ, onde em acréscimo, determinou-se a solubilidade dos mesmos em função de pHs decrescentes. Assim, biofilme dental foi formado, durante 14 dias, sobre blocos de esmalte e acrílico, utilizando diferentes freqüências de exposição a glicose a 20% (0, 2 e 8x/dia), para obtenção de biofilmes com diferentes quantidades de reservatórios. O pH, Ca, P_i e F foram determinados no fluido antes e 5 min após desafio acidogênico, enquanto a solubilidade dos reservatórios foi determinada através da extração de Ca, P_i e F no estroma do biofilme, utilizando tampões com pHs decrescentes (6,5; 5,5; 4,5 e ácido forte). Os resultados desses estudos permitiram observar que houve um aumento significativo de Ca no fluido após queda de pH, ocorrendo de modo semelhante independentemente do substrato onde o

biofilme foi formado e da quantidade de reservatórios presentes no seu estroma. Além disso, a solubilidade dos reservatórios demonstrou ser função inversa do pH e direta da concentração de Ca e PI presente. Assim, sugere-se que os reservatórios representam uma importante fonte de mobilização iônica para o fluido do biofilme, porém o fluido não refletiu, na condição analisada, a diferença de solubilidade dos substratos utilizados e dos reservatórios presentes.

Palavras-chave: biofilme dental, fluido do biofilme, reservatórios do biofilme, pH, cálcio, fosfato, fluoreto, esmalte, dentina, acrílico.

ABSTRACT

Dental biofilm presents organic and inorganic reservoirs of calcium (Ca), phosphate (P_I) and fluoride (F) that could act as a source of these ions to biofilm fluid during pH drops, reducing dental demineralization. It's still unknown the source responsible for the increase of such ions in biofilm fluid after pH drop, since they could be also derived from mineral dissolution of dental structure. In addiction, there's no clear evidence about the solubility of these reservoirs according to the pHs considered important to interfere with dental demineralization. Thus, this study aimed to evaluate the influence of mineral dissolution of dental structure and also of biofilm reservoirs in the inorganic composition of biofilm fluid, analyzing the kinetics of Ca, P_I and F to fluid just after pH drop. Two crossover and blind in situ studies were performed, which the aim of the first one was to evaluate the hypothesis of the inorganic composition of biofilm fluid after pH drop would reflect the mineral solubility of substrate where biofilm was formed. Thus, dental biofilm was formed during 4 days on enamel (less soluble), dentine (more soluble) and acrylic (not soluble) slabs, which were exposed to 8 times/day to 20% glucose solution. In the end of each phase, the acidogenicity, microbiological composition and inorganic concentration of these biofilms were analyzed. The second in situ study evaluated the relation between the amount of Ca, P_i and F reservoirs with the inorganic composition of biofilm fluid and moreover, the potential of these reservoirs to release Ca, P_i and F was estimated according to decreasing pHs (6.5; 5.5; 4.5 and acid). Therefore during 14 days, dental biofilm was formed on enamel and acrylic slabs, which were exposed to different frequencies of 20% glucose (0, 2 and 8 times/day) to form biofilms with different amounts of Ca, P_i and F reservoirs. The biofilm fluid analyses were the same performed in the first study, however the concentration of Ca, P_i and F in biofilm solids were determined after extraction with buffers of 6.5, 5.5 and 4.5 and with acid. A significant increase in Ca concentration was observed after pH fell; nevertheless this increase was the same regardless of the substrate where biofilm was formed and regardless of the amount of Ca, P_i and F reservoirs. Furthermore, biofilm reservoirs showed potential to release Ca, P_i and F at those pHs analyzed, although their solubility were inversely related with the frequency of glucose exposure that biofilm was formed.

These findings suggest that biofilm reservoirs are important source of Ca, P_i and F to biofilm fluid after pH drop, however the inorganic concentration of fluid does not reflect the mineral solubility of substrates, the amount and solubility potential of biofilm reservoirs.

Key words: dental biofilm, biofilm fluid, biofilm reservoirs, pH, calcium, phosphate, fluoride, enamel, dentine, acrylic

SUMÁRIO

INTRODUÇÃO

A cárie dental é uma doença biofilme-açúcar dependente, sendo o processo de desmineralização governado pelo grau de saturação do fluido do biofilme em relação aos minerais da estrutura dental. Sabe-se que o processo de desmineralização dental está condicionado a existência de um estado de subsaturação do meio e a determinação do grau de saturação sofre influência do pH e da concentração dos íons cálcio (Ca), fosfato (P_i) e fluoreto (F) (Vogel et al., 1990; Margolis and Moreno, 1994; Pearce, 1998).

Como o biofilme dental pode apresentar reservatórios de Ca, P_i e F sob a forma de depósitos minerais (Kaufman e Kleinberg, 1976) e/ou ligados a grupamentos aniônicos das paredes das bactérias (Rose et al., 1993; 1996) e proteínas (Gao et al., 2001), a mobilização iônica provenientes de tais reservatórios para o fluido poderia ser capaz de interferir no grau de saturação do mesmo, reduzindo a perda mineral da estrutura dental quando quedas de pH estivessem presentes. Assim, tem-se sugerido que as mudanças inorgânicas observadas no fluido do biofilme, após queda de pH, sejam provenientes desses reservatórios. De fato, tem sido observado um aumento significativo na concentração de Ca no fluido do biofilme após desafio acidogênico (Margolis e Moreno, 1992; Rankine et al., 1996; Tanaka e Margolis, 1999; Tenuta et al., 2006), porém com ausência de resultados consistentes tanto para P_i, quanto para o F. A falta de aumento na concentração de P_i e F no fluido após desafio acidogênico, pode ser decorrente da existência de um mecanismo antagônico: ao mesmo tempo que estão sendo liberados, sugere-se que P_i pode ser utilizado pelas bactérias no processo de fermentação de carboidratos (Pearce, 1998) e o F pode ser tanto incorporado a estrutura dental sob a forma de fluorapatita (Tanaka e Margolis, 1999) ou acumulado no interior das bactérias sob a forma de ácido fluorídrico (Hamilton et al., 1990).

Adicionalmente, observou-se que a quantidade de Ca, P_i e F encontrada na parte sólida do biofilme demonstrava ter relação inversamente proporcional com a freqüência de exposição do biofilme a carboidrato fermentável, como também é influenciada pelo grau de acidogenicidade do carboidrato utilizado (Cury et al., 1997, 2000, 2003;; Ccahuana-Vasquez et al., 2007; Ribeiro et al., 2005; Tenuta et al., 2006; Vale et al.,

2007), o que poderia representar um fator adicional sobre a cariogenicidade do biofilme, já que o potencial de liberação desses reservatórios poderia estar vinculado com a sua quantidade. Diante dessa constatação, seria esperado que a composição inorgânica do fluido do biofilme refletisse relação semelhante a observada nos reservatórios, entretanto foi demonstrado que essa menor concentração encontrada na parte sólida do biofilme (estroma) não se refletia no fluido (Tenuta et al., 2006).

A estimação da relevância desses reservatórios é dificultada pelo fato do biofilme dental ser formado sobre uma estrutura mineral, que por conter Ca, P_i e F representa uma fonte passível de disponibilização de íons para o fluido do biofilme, durante o processo de desmineralização (Rankine et al., 1996). Assim, a utilização de um substrato inerte (isento de Ca, P_i e F em sua composição), propiciaria avaliar a influência da desmineralização do substrato na liberação dos íons para o fluido do biofilme. Em acréscimo, considerando que a dentina apresenta uma maior solubilidade, ou seja, o pH considerado critico para a desmineralização do mineral presente na dentina é maior do que para o do esmalte (Hoppenbrouwers et al., 1986), o grau de solubilidade do substrato poderia ser refletido na concentração inorgânica do fluido do biofilme.

Em contra-partida, diante da constatação da importância dos reservatórios na cinética dos íons para o fluido, tornar-se-ia necessário avaliar se a solubilidade desses reservatórios em pHs considerados importantes para interferir com o processo de desmineralização dental teria relação com quantidade de Ca, P_i e F encontrada na parte sólida do biofilme.

Dessa forma, o entendimento da cinética dos íons Ca, P_i e F no biofilme dental é fundamental para avaliar a importância dos reservatórios em interferir nos processos de desmineralização dental, assim como determinar o papel que a desmineralização do substrato exerce na concentração inorgânica do fluido do biofilme.

PROPOSIÇÃO

O presente estudo teve como objetivo estudar a influência da solubilidade do substrato dental e dos reservatórios de Ca, P_i e F do biofilme dental na concentração inorgânica do fluido do biofilme, avaliando a cinética dos íons Ca, P_i e F para o fluido do biofilme após desafio acidogênico. Adicionalmente, a relação entre quantidade de reservatórios e sua solubilidade foram determinadas em função de pHs considerados importantes para interferir no processo de desmineralização dos diferentes substratos dentais.

Este trabalho foi realizado no formato alternativo, conforme deliberação número 002/06 da Comissão Central de Pós-Graduação (CCPG) da Universidade Estadual de Campinas (UNICAMP) (Anexo 1).

CAPÍTULO 1

Kinetics of Ca, P_i and F in enamel and dentine biofilms during sugar challenge[1]

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ABSTRACT

We hypothesized that the change in inorganic composition of biofilm fluid during a pH drop would reflect the solubility of substrate on which the biofilm is formed. Thus, in a crossover, blind study, dental biofilm was formed in situ on slabs of enamel (less soluble), dentine (more soluble) or acrylic (not a source of ions to the fluid), under exposure to 20% glucose 8 times/day for 4 days. Compared to the baseline values, the pH decreased and Ca concentration increased significantly in biofilm fluid 5 min after glucose exposure for all substrates, but the differences between them was not significant. The findings suggest that biofilm reservoirs are important source of ions to the fluid when the pH drops and that the composition of the biofilm fluid shortly after sugar exposure does not explain the difference of solubility between enamel and dentine.

INTRODUCTION

Dental demineralization is governed by the degree of saturation of the biofilm fluid with respect to tooth mineral, which is function of the pH and the concentrations of calcium (Ca), inorganic phosphate (P_i) and fluoride (F) in the fluid (Vogel *et al.*, 1990; Margolis and Moreno, 1994; Pearce, 1998).

Dental biofilm presents inorganic and organic reservoirs of Ca, P_i and F (Kaufman and Kleinberg, 1973; Rose *et al.*, 1993, 1996; Gao *et al.,* 2001) that could act as sources of these ions to biofilm fluid during sugar exposure, reducing the driving force for dental demineralization. Furthermore, the surface on which the biofilm is formed could also be a source of Ca, P_i and F and the changes observed in biofilm fluid after sugar exposure could be promoted by the mineral dissolution of dental structure during pH drop (Rankine *et al.,* 1996). Therefore, considering that dentine is more soluble than enamel (Hoppenbrouwers *et al.,* 1987) it would be expected a greater mobilization of ions from this substrate during sugar fermentation. However, the kinetics of mineral dissolution from enamel or dentine to the biofilm fluid has not been studied previously. On the other hand, if biofilm were formed on a non-mineral surface such as acrylic, the only source of ions would be from the biofilm reservoirs (Rankine *et al.,* 1996), which could help elucidating their relative contribution to the kinetics of mineral ions in the fluid during a pH drop.

We thus hypothesized that the concentration of Ca, P_i and F in biofilm fluid after sugar fermentation and pH drop would be highest in biofilm formed on a more soluble mineral such as dentine and lowest in biofilm formed on acrylic. For this purpose, biofims were formed on enamel, dentine and acrylic slabs with the same surface roughness, and their microbiological composition and acidogenicity were checked.

MATERIALS AND METHODS

Experimental design

This was a crossover and blind in situ study, approved by the Ethics Committee of Piracicaba Dental School. During 3 phases of 4 days each, 10 volunteers who fulfilled

the inclusion criteria (described in Ccahuana-Vásquez *et al.,* 2007) wore an acrylic palatal appliance containing 10 slabs of human enamel or root dentine or acrylic resin. The sequence of substrate to be tested in each phase per volunteer was created by a computer-generated randomization list (Excel, Microsoft Corporation). Since surface roughness could interfere on microorganism adherence (Teughels *et al.,* 2006), the surface roughness of the three substrates was standardized (Ra mean of 0.30 μm and SD of 0.08 μm; Surfcorder SE 1700, Kosaka Laboratory) by grinding with 400, 500 and 600-grade Al_2O_3 papers for enamel, acrylic and dentine, respectively. Five slabs were fixed on each side of the appliance, 1 mm below the acrylic level and covered with a plastic mesh to allow dental biofilm accumulation (Cury et al., 1997). The volunteers subjected extra-orally the slabs to 20% glucose solution 8 times/day, and after 5 minutes the appliances were re-inserted in the mouth. Also, throughout the entire study they used a silica-based dentifrice (1100 μg F/g, as NaF) 3 times/day and consumed optimally fluoridated water (0.6 – 0.8 mg F/L).

On the 5^{th} day, the plastic mesh covering the slabs was removed and an aliquot of biofilm was collected in resting condition for microbiological analysis. The remaining not disturbed biofilm was exposed, at one side of the appliance, to distilled water (for baseline evaluation) and at the other side to 20% glucose solution. Each slab was exposed to one single drop of the respective solution. Water was used to counteract for the possible dilution effect of the fluid due to the volume of glucose solution used (Vogel *et al.,* 2001). After 1 minute the appliance was replaced in the volunteers' mouth and 4 min later the appliance was removed and biofilms were separately collected for analyses of biofilm fluid pH and inorganic composition, and also for analysis of the biofilm solids.

Microbiological Analysis

The biofilm collected was analyzed according to Tenuta *et al.* (2006a) and the results were expressed in colony forming unit (CFU) per mg of biofilm wet weight and percentage of CFU in relation to total microbiota.

Concentrations of Ca, P_i and F in the biofilm fluid and solids

The procedures of biofilm collection, separation of fluid from solids and the analyses of concentration of Ca, P_i and F in the two biofilm compartments are described in Tenuta *et al.* (2006b).

Analysis of biofilm fluid pH

The analysis was done mainly as described in Vogel *et al.* (2000). Micro samples and pH standards were deposited on the surface of a F electrode (only as a stand), covered with mineral oil which was bubbled with water-saturated 4% CO_2/96% N_2 gas, to prevent CO_2 loss. pH was determined using constructed micro pH and reference electrodes, connected to an electrometer (FD223, WPI Instruments).

Statistical analyses

The data were analyzed with SAS software (version 8.01, SAS Institute Inc., Cary, NC, USA), with p level fixed at 5%. The volunteers were considered as statistical blocks and ANOVA followed by Tukey test were used for comparisons between substrates. The normality of error distribution and the homogeneity of variances were checked for each response variable using the SAS/Lab package (SAS software) and data were transformed as suggested by the software, according to Box et al. (1978). When no transformation was possible, the Friedman test was used. Paired tests (*t* test for normally distributed variables or the sign test for not normally distributed ones) were used to compare the baseline values and those after glucose exposure for each substrate.

RESULTS

The microbiological composition of biofilms formed on enamel, dentine and acrylic (Table 1) was similar, except for the lactobacilli counts, which were higher in biofilm formed on acrylic when compared to dentine.

The concentration of mineral ions in the biofilm solids was not significantly different among the substrates at baseline (Table 2). In the fluid, the baseline values of pH and Ca and P_i concentrations also did not differ among substrates; nevertheless F

concentration in the fluid was higher in the biofilm formed on acrylic when compared to dentine.

When the biofilms were exposed to glucose (Table 2), their pHs decreased and Ca concentrations in the fluid increased significantly, irrespective of the substrate considered. P_i concentration in the fluid did not change, and F concentration increased only for the biofilm formed on dentine. In the biofilm solids, the comparison of baseline and after sugar exposure concentrations showed a significant increase in Ca in the biofilm formed on dentine and a decrease in P_i in biofilms formed on dentine and enamel. The resulting concentrations of ions in the biofilm fluid and solids after sugar exposure was not significantly different among the substrates, except for F concentration in the solids, which was lower in biofilm formed on enamel when compared to dentine.

DISCUSSION

In the present study, similar biofilms with respect to microbiological composition, acidogenic potential and amount of biofilm inorganic reservoirs were formed on different substrates to evaluate the movement of mineral ions to the biofilm fluid during a pH drop. Considering that carbohydrates and frequency of exposure significantly affect the mineral ions accumulation in biofilms (Paes Leme *et al.*, 2006 for a review), and that the study focused on dissolution of minerals during a pH drop, the biofilm formation was standardized under exposure to glucose 8 times/day.

The main results showed a significant increase in Ca concentration in the biofilm fluid as the pH decreases, irrespective of the substrate on which the biofilm was formed. This increase agrees with previous studies (Margolis and Moreno, 1992; Tanaka and Margolis, 1999; Tenuta *et al.*, 2006b), and is expected considering that all Ca biofilm reservoirs, *i.e.* calcium phosphate minerals (Kaufman and Kleinberg, 1973) or Ca bound to bacterial cell wall or to proteins by phosphate and/or carboxyl groups (Rose *et al.*, 1993; 1996; Gao *et al.*, 2001), are sensitive to pH changes. Given the condition of biofilm formation (frequent exposure to sugar), limiting the presence of precipitated calcium phosphates in the biofilms, and the pH reached after the sugar challenge (around 5.0), not low enough to reach the pK_a of acid radicals present in bacteria cells,

the main source of Ca to the fluid would probably be Ca bound to organic phosphate reservoirs.

Interestingly, P_i concentration in the biofilm solids was significantly lower in samples collected after sugar exposure than in samples collected at baseline, while its concentration in the fluid did not change significantly. Such result may be due to an antagonist effect: while P_i in biofilm reservoirs is released to fluid, fermenting bacteria simultaneously take it up to use in phosphorylation processes. In previous studies, a significant decrease in P_i in the fluid was observed 5 min after a sugar challenge (Tenuta *et al.,* 2006b). Differences in the experimental protocols may have caused the different results, since in the present study baseline samples were exposed to water, while in the previous one this step was omitted and a dilution effect could be present in samples collected after sugar exposure (Vogel *et al.,* 2001; Tenuta *et al.,* 2006b). Also, the length of the experiments was different. In fact, higher values of P_i in the solids were found in the present study when compared to that of Tenuta *et al.* (2006b), suggesting a greater availability and mobility of phosphate in biofilm reservoirs in younger biofilms as the one evaluated in the present study.

In the present study, F was used by volunteers through water and dentifrice. Given that F is retained in the biofilm mainly bound to Ca, an increase of its concentration in the fluid would be expected during the pH drop. However, as with P_i, the lack of a consistent change in F concentration post-glucose challenge may be caused by the simultaneous diffusion of F into bacterial cytoplasm as HF (Hamilton, 1990) or its uptake by dental structure as fluorapatite (Tanaka and Margolis, 1999). The last mechanism is supported by the degree of saturation of the biofilm fluid with respect to FAp after sugar challenge ($pKIAP_{FAp} \approx 109$, Chemist, version 1.0.1, Salt Lake City, UT, USA).

Although our hypothesis that after sugar exposure and pH drop different concentration of ions would be present in the fluid of biofilms formed on substrates of different solubilities, this does not exclude that, in addition to biofilm reservoirs as discussed above, Ca is also derived from dental surface. In fact, considering the high cariogenic condition of the present study, caries lesions would be forming in enamel (Cury et al., 2000) and dentine (Aires et al., 2008). Also, 5 min after sugar challenge, the biofilm fluid was slightly undersaturated with respect to hydroxyapatite ($pKIAP_{HAp} \approx$

116, Chemist). Thus, considering that solubility products for dental minerals are higher than that for hydroxyapatite (Patel and Brown, 1975), it seems that both enamel and dentine dissolved and after 5 min the saturation of the fluid is being reached.

On the other hand, the increase in Ca concentration found in the fluid of biofilm formed on acrylic 5 min after sugar exposure would exclusively be the result of biofilm reservoirs dissolution. This increase was not observed by Rankine et al. (1996), who analyzed the biofilm fluid 15 min after sugar exposure, suggesting that this increase lasts for a short time. The apparent no decrease of Ca concentration in the solids after sugar exposure may be explained by the fact that the increase observed in the fluid would occur at expenses of less than 10% of the acrylic biofilm reservoirs, and the variability on the Ca concentrations in samples collected at baseline or after sugar challenge would make difficult the observation of a significant change.

Moreover, the lack of difference between enamel and dentine in Ca concentration in bioflm fluid 5 min after sugar challenge does not explain why dentine is more caries susceptible than enamel. Thus, the explanation for the greater dissolution of dentine, in comparison with enamel, would not lie on the difference of concentration of mineral ions in the fluid at the time minimum pH is reached, but rather on the time needed for the pH to rise above the minimum for the solubility of both, but this should be tested further. Although the role of biofilm reservoirs as mineral buffers during a pH drop could not be determined in the present study, the results of the acrylic biofilm suggest they could be important sources of Ca to the fluid. Moreover, the present results are in accordance with our previous publication (Tenuta *et al.,* 2006b) showing that the baseline concentration and the increase of mineral ions to the fluid soon after a pH drop are not influenced by the amount of reservoirs available in the biofilm. Thus, the study of potential solubility of these reservoirs at decreasing pHs would help ascertain their role during pH drops, and is under evaluation in our lab.

Considering that the driving forces for dental demineralization are based on the concentration of ions in the biofilm fluid and its pH, the results suggest that the increase in Ca concentration in the fluid would be a self-limiting step for dental demineralization soon after exposure of the biofilm to carbohydrates, as discussed previously (Kashket and Yaskell, 1992). However, the kinetics of ions presently studied would be relevant for

surface phenomena involved in caries development, but the sub-superficial progression of mineral dissolution would be much more influenced by the conditions of the fluid within enamel or dentine.

In summary, the increase of Ca concentration in fluid of biofilm formed over acrylic suggests that organic reservoirs present in dental biofilms may be important source of ions to buffer the effect of pH drop after sugar challenge. Also, the difference on caries susceptibility between dentine and enamel is not simply explained by the difference in mineral dissolved shortly after sugar exposure, suggesting that the time that the pH remains under critical values for enamel or dentine dissolution would be more relevant.

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Table 1: Microbiological analysis of dental biofilm according to substrates [Mean (SD), n = 10]

Substrate	Total microbiota (CFU / mg biofilm wet weight x 10^7)	Total Streptococci (CFU / mg biofilm wet weight x 10^6)	Mutans Streptococci (CFU/ mg biofilm wet weight x 10^3)	% Mutans Stretococci in relation to total microbiota	Lactobacilli (CFU / mg biofilm wet weight x 10^3)	% Lactobacilli in relation to total microbiota
Acrylic	1.4 (1.1)	8.1 (6.3)	1.8 (3.0)	0.01 (0.01)	74.8[a] (214.7)	1.8 (3.8)
Enamel	1.8 (1.2)	7.3 (5.2)	7.4 (12.9)	0.06 (0.12)	4.3 (9.3)	0.04 (0.09)
Dentine	2.4 (2.1)	11.1 (7.9)	1.8 (2.5)	0.01 (0.02)	0.5 (1.1)	0.00 (0.01)

[a] Significantly different from dentine ($p<0.05$).

For ANOVA, data for total microbiota, total streptococci, mutans streptococci and lactobacilli were transformed to the power of 0.3,0.5, 0.1 and -0.1, respectively; data of % mutans streptococci and lactobacilli in relation to total microbiota could not be transformed and the non-parametric Friedman test was used.

Table 2: Analysis of acidogenicity (pH) and inorganic composition of biofilm solids and fluid at baseline and after glucose exposure, according to the substrates [Mean (SD), n]

Condition	Substrates	Biofilm solids (wet weight)			Biofilm fluid			
		Ca (µmol/g)	P_i (µmol/g)	F (µmol/g)	pH	Ca (mM)	P_i (mM)	F (µM)
Baseline	Acrylic	12.7 (7.7), n=8[a]	31.1 (17.4), n=8[a]	0.26 (0.49), n=8[a]	6.6 (0.5), n=8[a]	1.1 (0.4), n=8[a]	8.2 (4.1), n=8[a]	8.9[b] (10.1), n=8[a]
	Enamel	8.9 (7.1), n=10	28.9 (19.7), n=10	0.17 (0.39), n=10	6.4 (0.6), n=9[a]	1.2 (0.8), n=9[a]	12.1(13.2), n=9[a]	7.4 (7.3), n=10
	Dentine	9.8 (5.9), n=10	29.0 (14.7), n=10	0.24 (0.42), n=10	6.7 (0.4), n=9[a]	1.5 (1.4), n=9[a]	7.4 (2.5), n=9[a]	4.0 (1.1), n=10
After glucose exposure	Acrylic	12.6 (8.7), n=9[a]	21.6 (12.5), n=9[a]	0.28 (0.60), n=9[a]	5.0[c] (0.2), n=9[a]	4.6[c] (2.9), n=9[a]	9.3 (4.8), n=9[a]	7.7 (4.6), n=9[a]
	Enamel	9.5 (7.9), n=10	20.8[c] (17.6), n=10	0.19[b] (0.45), n=10	4.9[c] (0.2), n=9[a]	4.5[c] (2.4), n=9[a]	8.8 (5.7), n=10	10.0 (9.1), n=10
	Dentine	11.7[c] (6.5), n=9[a]	21.3[c] (10.7), n=9	0.27 (0.46), n=9[a]	5.0[c] (0.3), n=9[a]	4.7[c] (1.3), n=9[a]	7.6 (3.2), n=9[a]	9.0[c] (3.9), n=10

[a] n values lower than 10 refer to missing data due to insufficient amount of sample for analysis.

[b] Significantly different from dentine, under the same condition ($p<0.05$).

[c] Significantly different from baseline values for comparisons made under the same substrate (paired test; $p<0.05$).

For ANOVA data were transformed as follows: Biofilm solids: Ca and F concentrations were $\log_{10}$-transformed in both conditions; P_i concentrations after glucose exposure v transformed to the square root. Biofilm fluid: in both conditions, Ca and F concentrations were transformed, to the $\log_{10}$ and to the inverse, respectively; P_i concentration baseline and after glucose exposure were transformed to the inverse and $\log_{10}$, respectively.

CAPÍTULO 2

Anticaries potential of Ca, P_i and F presents in biofilm reservoirs

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ABSTRACT

Ca, P_i and F stored in biofilm reservoirs may be released to biofilm fluid after pH drop by sugar exposure and interfere with the caries process of de-remineralization, but how much is released according to the pH is unknown; also the relative importance of these ions from biofilm or enamel surface is uncertain. Biofilm was formed in situ over enamel and acrylic under frequencies of glucose exposure (0, 2 and 8x/day) to form biofilms with different amounts of Ca, P_i and F reservoirs. Ca, P_i and F and pH were determined in the biofilm fluid before (baseline) and after one glucose exposure. Ca, Pi and F were also extracted from the solids of biofilms not exposed to glucose, using buffers simulating different pHs drop. The concentration of Ca in fluid increased and the pH decreased after glucose exposure, irrespective of where and how the biofilms were formed. The amount of Ca, P_i and F extracted from the biofilm solids increased according to decreasing pHs, irrespective of enamel or acrylic biofilms. These results suggest that biofilms reservoirs represent an important source of Ca to the biofilm fluid when the pH drop after sugar exposure and that the solids reservoirs could be source of P_i and F only when the pH reached was very low.

INTRODUTION

Dental biofilm contains organic and inorganic reservoirs of ions Calcium (Ca), Inorganic Phosphate (P_i) and Fluoride (F) that are sensitive to pH fluctuations (Kaufman and Kleinberg, 1976; Rose et al., 1993, 1996; Gao et al., 2001). Thus, these reservoirs have been associated with the changes observed in the inorganic composition of biofilm fluid after pH drops due to carbohydrate fermentation. Such changes serve to determine the state of fluid saturation and consequentially become important to regulate the dental de-remineralization process. However, it has been shown that biofilm fluid is not affected by the low concentration of Ca, P_i and F observed in whole biofilm (Margolis and Moreno, 1992; Cury et al. 1997, 2000; Tenuta et al., 2006). In fact, biofilm was formed over dental substrate and this evidence could be due to mineral dissolution of dental structure during pH drop (Rankine et al., 1996). Therefore, the relevance of the amount of Ca, P_i and F stored in biofilms reservoirs as source of ions mobilization to fluid during pH drops remains unclear. In order to clarify this mechanism, dental biofilm should also be formed on a non-mineral substrate. Additionally, the relation between the amount and the respective solubility of Ca, P_i and F stored in biofilm reservoirs under pHs considered important to interfere with dental demineralization has not been determined previously.

Thus, the purposes of this in situ study were primarily evaluate if biofilm reservoirs contribute for the inorganic modification observed in fluid thereupon pH drop; and secondly evaluate the solubility of Ca, P_i and F stored at different quantities in dental biofilm reservoirs. In order to clarify these mechanisms dental biofilm should also be formed on a non-mineral substrate and the solubility of reservoirs could be estimated by submitting these biofilms (with different amounts of Ca P_i an F) to extraction with buffers solutions that simulate the same pH fluctuations related with dental demineralization (pH in the range of 6.5 to 4.5).

MATERIALS AND METHODS

Experimental Design

This in situ study, approved by the Ethics Committee of Faculty of Dentistry of Piracicaba, had a crossover and blind design and was conducted in 3 phases of 14 days each. Twelve volunteers wore intraoral palatal appliances containing 7 slabs of human enamel in one side and 7 slabs of acrylic resin in the other side of the appliance, with dimensions of 4x4x2 mm, renewed after each phase but the side of each substrate was fixed per volunteer. All slabs were placed 1 mm below acrylic appliance level and covered with plastic mesh to allow dental biofilm accumulation (Hara et al., 2003; for details). The enamel and acrylic surface roughness were standardized around Ra of 0.3 μm to normalize bacteria adherence (Teughels et al., 2006). The volunteers were randomly assigned to the three groups of treatments, which were based in frequency of glucose exposure (0, 2 or 8 times/day). The dripping procedure was the same described in others in situ studies (Tenuta et al., 2006; Ccahuana-Vasquez et al., 2007, among others). In all experimental phase, the volunteers used a NaF silica-based dentifrice (1100 ug F/g) 3 times/day and consumed optimally fluoridated water (0,7 mg F/l). In the end of each phase, biofilms were collected five minutes after the exposure to distilled water (baseline condition) or to 20% glucose. Each slab was exposed to one single drop of the respective solution. Water was used to counteract for the possible dilution effect of the fluid due to the volume of glucose solution used it (Vogel et al., 2001). The procedure of collection was done as described: distilled water was extra-orally dripped on 5 of the 7 slabs of enamel and acrylic and after 1 minute the appliance was replaced in volunteers' mouth. After 4 minutes, the appliance was removed and the biofilms were collected, homogenized and split in 4 parts, which were separately placed in oil-filled centrifuge tubes. The solid part of these 4 samples exposed to water was destined to extraction with three different pH buffers and acid. Then, the other 2 slabs of enamel and acrylic were exposed to glucose solution in the same way as described for water and the biofilms of each substrate were collected and inserted in an oil-filled centrifuge tube.

Biofilm fluid analyses

After determination of sample weight, the oil-filled centrifuge tube containing each sample were centrifuged for 5 min (21,000 g) at 4ºC to separate fluid from biofilm solids. The fluid was recovered with a capillary micropipette, and only the fluid of the 4 samples exposed to distilled water was pooled. The analyses of Ca, P_i and F in the biofilm fluid, are described in Tenuta et al., 2006.

The pH analysis was done mainly as described by Vogel et al., 2000. Samples of biofilm fluid and pH standards were deposited on the surface of a F electrode (only as a stand), covered with mineral oil. The oil was bubbled with water-saturated 4% CO_2 / 96% N_2, to prevent CO_2 loss. Micro pH and reference electrodes were constructed and mounted in micro-manipulators, which helped to touch each sample or standard drop under microscope view. The electrodes were mounted into a FD223 electrometer (WPI Instruments) and the mV was recorded using the Plot Program (American Dental Association Health Foundation).

Biofilm solid analyses

The extraction buffers with their respective pH and the acid were: 0.1 M cacodylate buffer (pH 6,5); 0.1 M acetate buffer (pH 5.5 and 4.5) and 0.5 M HCL. After the fluid of these samples was recovered and pooled with capillary micropippete, the tip of each centrifuge tube was cut and the biofilm solid was centrifuged into a microcentrifuge tube containing one of the buffers solution or acid (0.1 ml/10 mg of biofilm wet weight) for extraction of Ca, P_i and F soluble into respective pH buffers and acid. The extraction procedure was the same described in Cury et al. (1997). After the obtainment of supernatants, the samples were neutralized with NaOH and Ca and P_i were spectrophotometrically determined with sensitive colorimetric reagents (Vogel et al., 1983, Tenuta et al., 2006) and the absorbance of the mixture (200 μl) was read in spectrophotometer microplate reader (Thermo Multiskan Spectrum), using 96-well culture plate. The F in supernatants was determined after neutralization with NaOH, using the same dilution and technique of F fluid samples.

Statistical analyses

All statistical tests were done using SAS software (SAS Institute Inc. version 8.01, Cary, NC, USA), considering the volunteer as statistical block and fixing the significance level at 5%. The assumption of equality of variance and normal distribution were checked for each variable, and those that did not satisfy these assumptions were transformed (Box et al., 1978). Two-way ANOVA followed by Tukey's test were used to analyze the effect of substrate and frequency, as well as substrate/frequency interaction. Paired *t* test was used to compare the baseline values with those after glucose exposure for each frequency of glucose challenge fixed. When the normality requirement for the t test was not satisfied non-parametric signed test (Wilcoxon signed-rank or sign test) were used.

RESULTS

There was no statistical difference between enamel and acrylic for all dependent variables of biofilm fluid and solid.

With regard to biofilm fluid results (Table 1), the baseline pH was statistically higher in the biofilm formed with the absence of glucose challenge when compared to other frequencies. However, after glucose exposure, the pH significantly decreased in all frequencies but with no longer differences among them. Ca concentration showed no difference among frequencies at baseline, but after glucose exposure Ca significantly increased in all frequencies; nevertheless without difference among them. The baseline concentrations of P_i and F in fluid were the same among frequencies of glucose challenge. Only after glucose exposure, the highest values of P_i were found in biofilm formed with the absence of glucose challenge, which statistically differed from that exposed to glucose 2 times/day. When the values of baseline and after glucose exposure are compared, no significant change was observed for P_i concentration in any frequency analyzed, while F significantly increased just after glucose exposure in the biofilm exposed 8 times/day to glucose solution.

The biofilm solid results (Table 2) demonstrated that regardless of the extraction pH, Ca and P_i concentration significantly decreased with increasing frequency of glucose exposure. Moreover, the F extracted from biofilm exposed 8 times/day to glucose showed significantly lower values than biofilm formed with absence of glucose (pH 4.5 and acid) and exposed 2 times/day to glucose (pH 5.5; pH 4.5 and acid).

DISCUSSION

The fluid of the biofilms formed on enamel and acrylic showed the same baseline inorganic composition and also post-glucose exposure (Table 1), regardless of the frequency of glucose challenge. Since acrylic substrate is not a mineral source of Ca, P_i and F, this finding may demonstrate the important function of biofilm reservoirs to mobilize ions to fluid, contributing to its inorganic composition.

With regard do pH values, the clear observation that the biofilm formed in the absence of glucose challenge presents higher baseline pH than the one formed in the presence of high and even low frequency of glucose challenge may be associated with the bacterial consumption of intracellular polysaccharide, which represents an endogenous source of carbohydrate, able to be metabolized and produced to acids during the fasting periods (Tanzer et al., 1976).

Although Ca concentration in biofilm fluid is related to be pH-mediated, the difference in baseline pH was not reflected in Ca concentration and this data is unlike the result observed by Tenuta et al. (2006). However, when these biofilms were submitted to glucose exposure, the pH fell with no longer difference among frequencies and also exhibited a significant increase in Ca concentration with no difference among them. The increase in the concentration of Ca after pH fall is the most consistent result cited by innumerous studies (Margolis and Moreno, 1992; Tenuta et al., 2006, among others), whose attribute this finding to the release of Ca present in biofilm reservoirs. The lack of an increase trend in P_i and F concentrations in biofilm fluid, as also observed in this current study, has been postulated with the existence of two opposing mechanisms operating simultaneously: their release to fluid followed by the Pi-uptake by fermenting bacteria to be used in phosphorylation processes (Pearce, 1998) and F-

uptake as HF to bacteria cytoplasm (Hamilton, 1990) or as FA to dental structure (Tanaka and Margolis, 1999). Consequentially, the mechanisms behind P_i and F mobilization to fluid are difficult to discern.

Tenuta et al. (2006) have already pointed out the presence of homeostatic mechanism responsible for the maintenance of ion concentration in fluid regardless of inorganic concentration of Ca, P_i and F in whole biofilm. However, the importance of Ca, P_i and F accumulated in different quantities in dental biofilm still required investigation about their solubility and potential to interfere with dental demineralization. Previous studies extracted the Ca, P_i and F present in biofilm solids using only acid (HCl) that couldn't determine the avaliability of biofilm reservoirs to be released with the same pH fluctuations related with the demineralization process. Thus, the extraction procedure of biofilm solid using buffers with decreasing pHs could estimate the solubility of different biofilm reservoirs to fluid are difficult to discern. The models of Ca, P_i and F stored in dental biofilm involve the presence of organic binding sites, i.e. by anionic groups present in bacteria cell wall (Rose et al., 1993; 1996) and proteins (Gao et al., 2001), and the presence of solid mineral phase (Kaufman and Kleinberg, 1973) but these reservoirs would be important only if they become soluble in pHs higher than those considered critical for dental demineralization.

Our results showed that despite the different avaliability of the biofilm solids to mobilized Ca, P_i and F to milieu according to the frequency of glucose exposure, the inorganic concentration of the biofilm fluid was the same for all frequencies. However, these results represent a shortly time after pH fall, and it's still not clear if these reservoirs could maintain their ionic mobilization as long as pH remains critical to dental demineralization. The kinetics of Ca, P_i and F in dental biofilms reservoirs were not fully explored by this in situ study; therefore, studies evaluating the effect of multiple pH drops or extended period after sugar exposure could propitiate a better understanding of the maintenance of biofilm reservoirs as a source of ionic mobilization to fluid.

Thus, our findings suggest that biofilm reservoirs represent a source for inorganic composition of biofilm fluid either in resting condition or shortly after pH drop. Although the solubility of Ca, P_i and F were directly related with their accumulation in dental biofilm, the fluid just after pH drop did not reflect this finding. It's seems that how long

these reservoirs can serve as a source of ions to the biofilm fluid may be more relevant to determine their anticaries potential.

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Table 1: Analysis of biofilm fluid before (baseline) and after one glucose exposure (after GE) according to where (substrates) and how the biofilm were formed (glucose/day) [Mean (SD), n].

Substrate	Frequency of glucose/ day	Biofilm fluid [a]							
		pH		Calcium (mM)		Phosphate (mM)		Fluoride (uM)	
		Baseline	After GE	Baseline	After GE	Baseline	After GE	Baseline	After GE
Enamel	0x	7.4 (0.3), n= 12	5.0[c] (0.4), n=9[d]	1.2 (1.1), n=10[d]	4.1[c] (1.8), n=9[d]	7.9 (2.4), n=11[d]	8.0 (2.9), n=10[d]	4.0 (0.9), n=11[d]	5.5 (3.3), n=10[d]
	2x	6.7[b] (0,8), n=11[d]	5.1[c] (0.6), n=11[c]	1.9 (2.1), n=10[d]	3.8[c] (3.0), n=9[d]	8.8 (3.1), n=11[d]	7.2[b] (3.2), n=10[d]	11.7 (13.1), n=12	8.9[b] (6.6), n=11[d]
	8x	6.5[b] (0,8), n=10[d]	5.1[c] (0.6), n=11[d]	1.5 (1.0), n=11[d]	3.6[c] (1.3), n=10[d]	8.1 (1.9), n=11[d]	7.5 (2.2), n=12	6.1 (10.1), n=11[d]	6.9[c] (2.8), n=11[d]
Acrylic	0x	7.5 (0.4), n=12	5.0[c] (0.5), n=12	1.0 (0.9), n=11[d]	3.8[c] (2.7), n=11[d]	7.7 (1.6), n=12	7.8 (2.5), n=11[d]	8.4 (10.6), n=12	5.9 (3.2), n=12
	2x	6.8[b] (1.0), n=12	5.0[c] (0.6), n=12	1.4 (1.5), n=10[d]	3.0[c] (1.2), n=11[d]	7.8 (3.3), n=11[d]	7.0[b] (3.3), n=11[d]	6.1 (2.8), n=12	11.1[b] (10.9), n=12
	8x	6.5[b] (0.8), n=12	5.1[c] (0.5), n=12	1.3 (0.5), n=12	2.8[c] (1.2), n=12	8.7 (2.1), n=12	7.8 (1.8), n=11[d]	5.6 (3.4), n=12	7.2[c] (3.1), n=12

[a] No significant difference between substrates for all variables.
[b] Significantly different from the frequency of 0x, under the same condition: baseline or after GE ($p<0,05$).
[c] Significantly different from baseline values for comparisons made under the same frequency (paired test; $p<0,05$).
[d] n values lower than 12 refer to missing data due to insufficient amount of sample for analysis Baseline data were transformed for statistical analysis as follows: pH was elevated to 3; Ca was elevated to -0,1; P_i was transformed to square root and F to inverse of square root; and data for Ca, P_i after GE were log-transformed.

Table 2: Concentrations of Ca, Pi and F in extracts of biofilm solids, according to extraction conditions and how the biofilm were formed (glucose/day) [Mean (SD), n].

Extraction condition (buffer pH)	Biofilm formation glucose/day	Biofilm solid [a]					
		Calcium (μmol/g)		Phosphate (μmol/g)		Fluoride (μmol/g)	
		Enamel	Acrylic	Enamel	Acrylic	Enamel	Acrylic
6.5	0x	13.8 (6.2), n=9[d]	13.5 (5.9), n=9[d]	24.5 (11.4), n=9[d]	20.6 (6.3), n=9[d]	0.07 (0.11), n=9[d]	0.06 (0,06), n=8
	2x	6.8[b] (3.1), n=8[d]	6.3[b] (3.9), n=9[d]	16.1 (3.9), n=8[d]	17.0 (5.5), n=9[d]	0.02 (0.02), n=7[d]	0.03 (0.04), n=9
	8x	4.7[bc] (1.9), n=9[d]	4.2[bc] (1.4), n=8[d]	14.1[b] (4.2), n=9[d]	14.0[b] (2.7), n=8[d]	0.02 (0.01), n=9[d]	0.01 (0.01), n=8
5.5	0x	31.5 (12.4), n=9[d]	36.2 (15.3), n=9[d]	42.2 (14.1), n=9[d]	47.0 (16.0), n=9[d]	0.4 (0.4), n=9[d]	0.5 (1.0), n=9[d]
	2x	22.5[b] (21.2), n=9[d]	18.1[b] (14.2), n=9[d]	31.0[b] (21.4), n=9[d]	26.9[b] (16.0), n=9[d]	0.4 (0.5), n=9[d]	0.2 (0.2), n=9[d]
	8x	7.1[bc] (3.4), n=11[d]	6.9[bc] (2.9), n=9[d]	13.4[bc] (5.8), n=11[d]	11.7[bc] (4.4), n=9[d]	0.1[c] (0.1), n=11[d]	0.07[c] (0.07), n=9[d]
4.5	0x	76.7 (51.3), n=9[d]	56.2 (21.3), n=9[d]	69.2 (31,24), n=9[d]	73.1 (39.8), n=9[d]	1.3 (1.2), n=9[d]	1.4 (1.1), n=9[d]
	2x	33.2[b] (16.6), n=9[d]	38.1[b] (41.6), n=9[d]	39.0[b] (20.3), n=9[d]	42.4[b] (42.9), n=9[d]	1.2 (0.9), n=8[d]	1.4 (1.3), n=8[d]
	8x	12.3[bc] (9.3), n=12	13.0[bc] (11.0), n=10[d]	17.2[bc] (6.4), n=11[d]	16.5[bc] (7.2), n=10[d]	0.4[bc] (0.5) n=11[d]	0.4[bc] (0.6), n=10[d]
Acid (HCl)	0x	94.1 (52.0), n=11[d]	102.7 (41.7), n=11[d]	90.2 (40.9), n=11[d]	89.7 (44.8), n=11[d]	2.5 (1.9), n=11[d]	2.6 (2.4), n=11[d]
	2x	45.7[b] (27.5), n=12	42.0[b] (39.2), n=12	45.1[b] (18.6), n=12	50.3[b] (37.1), n=12	1.8 (2.0), n=12	1.7 (1.9), n=12
	8x	14.0[bc] (9.0), n=12	16.1[bc] (8.7), n=12	18.5[bc] (11.0), n=12	19.2[bc] (9.0), n=12	0.6[bc] (1.3), n=12	0.4[bc] (0.6), n=12

[a] No significant difference between substrates for all variables.
[b] Significantly different from the frequency of 0x, under the same extraction pH ($p<0.05$).
[c] Significantly different from the frequency of 2x, under the same extraction pH ($p<0.05$).
[d] n values lower than 12 refer to missing data due to insufficient amount of sample for analysis
For statistical analysis in pH 6.5 extraction, data for Ca and F were log-transformed and data for Pi were inversed transformed; In pH 5.5 and 4.5 extraction, data for Ca and Pi were log-transformed and data for F were elevated to 0.2; In acid extraction, data for Ca, Pi and F were log-transformed.

CONSIDERAÇÕES FINAIS

O fluido do biofilme representa o meio aquoso que está em íntimo contato com a superfície dentária quando processos de desmineralização estão ocorrendo. Dessa forma, não é difícil compreender porque o processo de desmineralização dental é regulado pelo estado de saturação do fluido do biofilme em relação aos íons Ca, P_i e F. Além disso, sua composição inorgânica difere da saliva (Moreno e Margolis, 1988), destacando sua singularidade e enaltecendo a importância de estudos que esclareçam o que ocorre no fluido do biofilme durante desafios cariogênicos.

Assim, estudos que analisaram o fluido do biofilme demonstraram uma clara relação entre aumento da concentração de Ca após queda de pH, sendo este, o achado mais confiável diante da falta de resultados consistentes para o P_i e F. A partir da constatação de que esses íons poderiam ser acumulados no biofilme dental, sob a forma de minerais, ou ligados a parede das bactérias e proteínas, foi possível sugerir que o aumento de Ca no fluido seria proveniente desses reservatórios, o que demonstraria sua importância em reduzir a perda mineral da estrutura dental, pois interferiria no estado de saturação do fluido. A concentração de minerais no biofilme dental demonstrou ter relação inversamente proporcional ao índice de cárie do indivíduo e frequência de exposição do biofilme a carboidrato fermentável, sugerindo que a solubilidade desses reservatórios e consequentemente a importância em interferir com o processo de desmineralização dental estaria vinculada a quantidade de Ca, P_i e F acumulada no biofilme.

Entretanto, a diferença da quantidade de reservatórios não foi refletida no fluido do biofilme, o que poderia decorrer tanto da solubilidade desses reservatórios no pH alcançado após fermentação de açúcar, como da desmineralização da própria estrutura dental, caracterizando-a como um fator confundidor; assim, tornou-se relevante compreender o papel desempenhado pelos reservatórios e pelo substrato na cinética dos íons Ca, P_i e F no biofilme dental.

Dessa forma, o primeiro estudo in situ foi justificado pela hipótese de que o grau de solubilidade do substrato seria evidenciado no fluido do biofilme, o qual demonstraria o processo de mobilização dos íons Ca, P_i e F em função da dissolução

do mineral da estrutura dental. Os resultados obtidos no primeiro estudo demonstraram que o fluido do biofilme, logo após queda de pH, não refletiu o grau de solubilidade da estrutura dental (esmalte e dentina). A maior solubilidade para dentina não foi explicada pelos resultados observados no fluido do biofilme naquela condição analisada (5 minutos apos exposição a glicose), parecendo ser mais importante o tempo que a mesma continuaria se dissolvendo até que o pH retornasse ao valor considerado crítico para sua desmineralização. Adicionalmente, esse estudo foi capaz de demonstrar a mobilização de Ca provenientes dos reservatórios para o fluido do biofilme mediante o resultado observado para o controle utilizado (substrato isento de minerais na sua composição).

Sabe-se que a quantidade dos reservatórios na parte sólida do biofilme dental é dependente das condições dada para o mesmo ser formado (freqüência e tipo de carboidrato), porém se desconhecia o potencial de liberação desses reservatórios com capacidade de interferir nos processos de desmineralização dental. A finalidade do segundo estudo foi verificar a solubilidade dos mesmos em tampões com pHs considerados importantes para desmineralização dental, além de estudar a influência da quantidade de reservatórios na cinética dos íons Ca, P_i e F no biofilme dental. Os resultados demonstraram que a solubilidade foi proporcional a quantidade de reservatórios, porém inversamente proporcional ao pH, sendo importantes somente aqueles reservatórios que apresentam solubilidade maior que a estrutura dental, já que liberariam íons para o fluido do biofilme antes do meio se tornar subsaturante para o mineral do substrato dental.

Considera-se que os resultados gerados por esses estudos foram importantes para o melhor entendimento da cinética dos íons Ca, P_i e F no biofilme dental, gerando novos questionamentos a respeito da relação temporal entre mobilização desses íons em função da queda de pH.

CONCLUSÃO

Tendo em vista os resultados dos estudos feitos, conclui-se que:

Os reservatórios presentes no biofilme dental desempenham um papel importante de mobilização de Ca para o fluido do biofilme, a qual é função inversa do pH e direta da concentração de Ca nos reservatórios do biofilme. Entretanto o aumento de Ca observado no fluido do biofilme após queda de pH (5,0) devido a fermentação de açucar não tem relação com a concentração de Ca na parte sólida do biofilme, assim como não explica a maior suscetibilidade da dentina a desmineralização quando comparada ao esmalte. Os reservatórios sólidos de Ca do biofilme só teriam importância como fonte de Ca para o fluido diante de quedas extremas de pH e a diferença de maior suscetibilidade a cárie da dentina seria melhor explicada pelo maior tempo que o fluido fica subsaturante em relação ao pH critico para dentina em comparação com o esmalte.

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[1] De acordo com a norma da UNICAMP/FOP, baseada na norma do International Committee of Medical Journal Editors – Grupo de Vancouver. Abreviatura dos periódicos em conformidade com o Medline.

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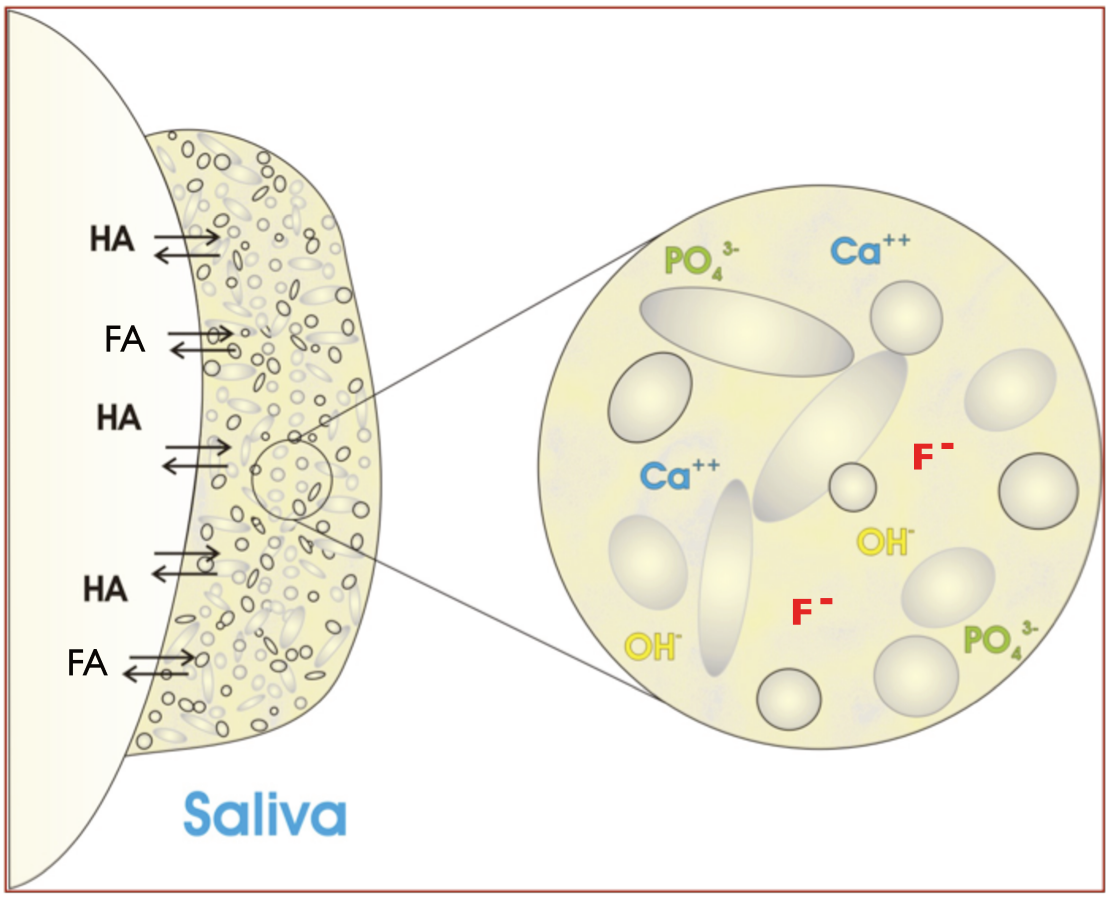
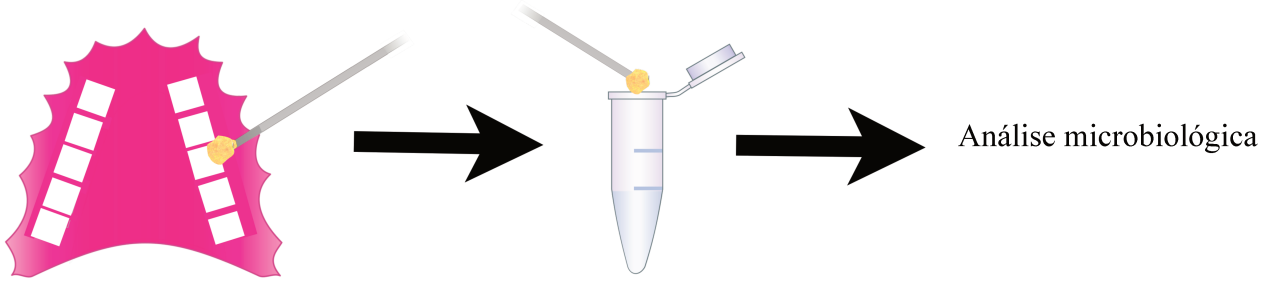
APÊNDICE 1 – Ilustração da porção aquosa do biofilme (fluido)



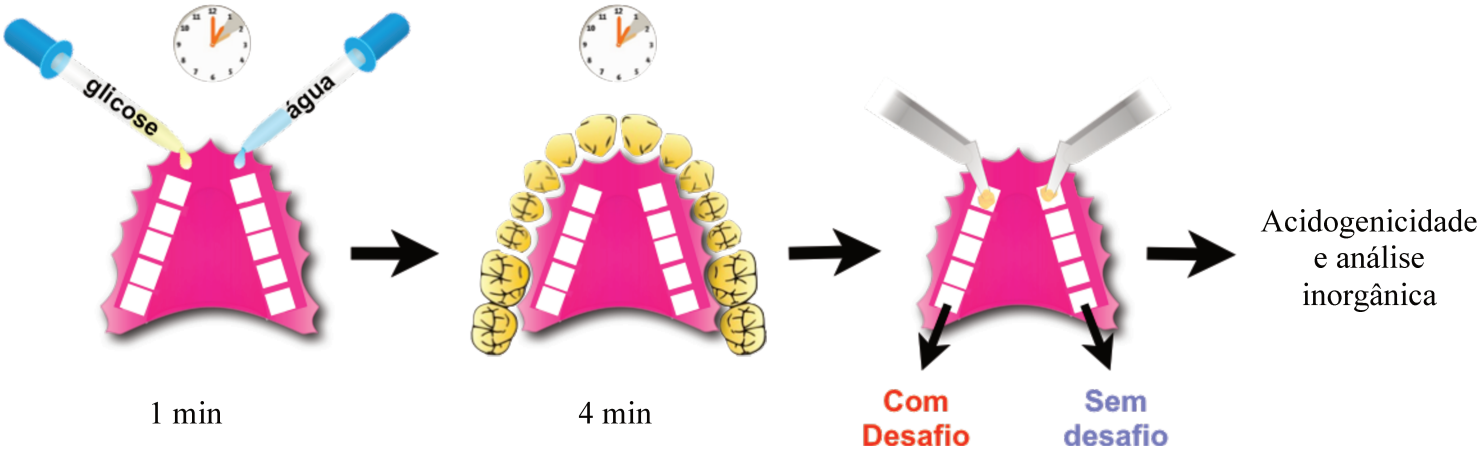
Esquema ilustrando a porção aquosa do biofilme, denominada fluido, o qual governa a ocorrência dos processos de desmineralização e remineralização. Esses processos são modulados pelo grau de saturação desse meio em relação aos íons cálcio, fosfato e fluoreto.

APÊNDICE 2 - Esquema da coleta do biofilme do primeiro estudo in situ

Primeiro procedimento



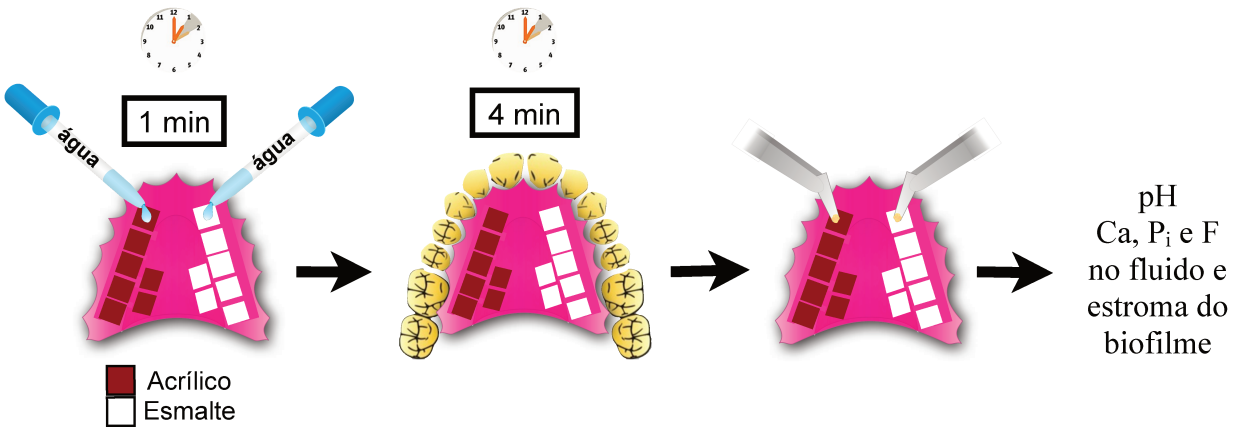
Segundo procedimento



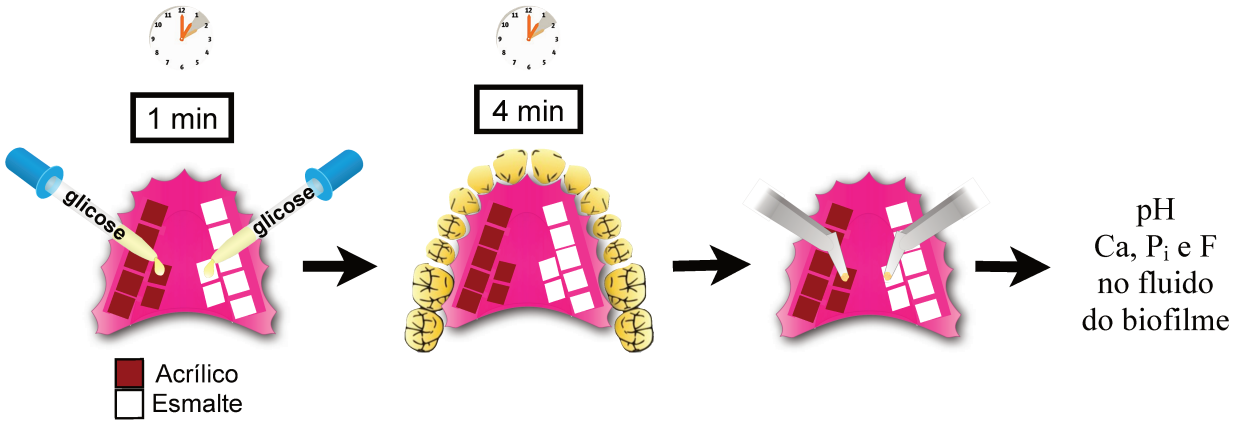
Após a coleta de uma alíquota do biofilme para análise microbiológica, os biofilmes restantes foram coletados simulando as condições antes (baseline) e após exposição a glicose. Para isso, nos biofilmes formados sobre os blocos de um lado do aparelho foi gotejado água (controle) enquanto que nos do lado oposto foi gotejado glicose a 20%. Um minuto após o gotejamento extra-oral, o voluntário recolocava o aparelho na cavidade bucal, removendo-o 4 minutos depois e coletando o biofilme nessas duas condições.

APÊNDICE 3 - Esquema da coleta do biofilme do segundo estudo in situ

Primeiro procedimento – gotejamento de água
(em 5 blocos de cada substrato)

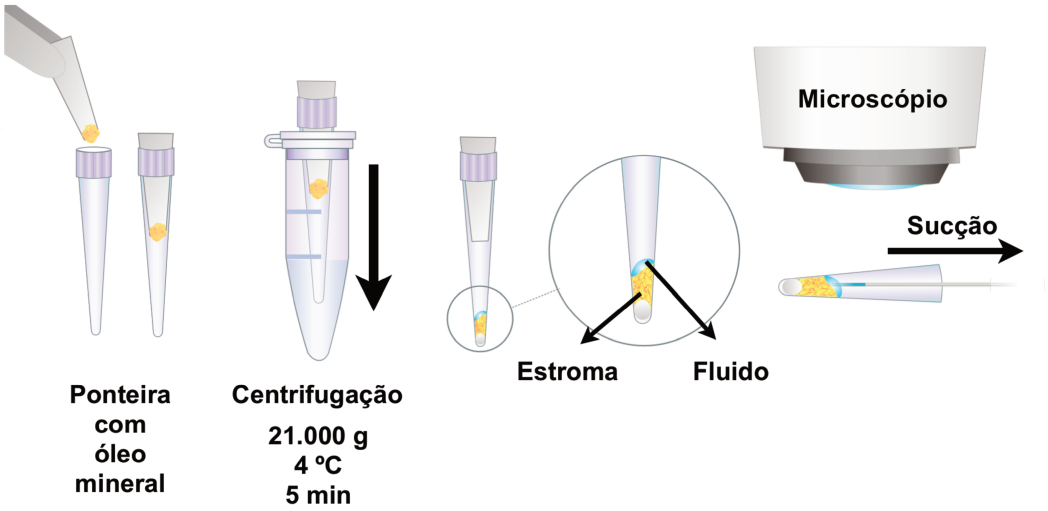


Segundo procedimento – gotejamento da solução de glicose
(em 2 blocos de cada substrato)



O biofilme foi coletado em duas condições: após gotejamento de água (baseline) e de glicose a 20%. Assim, o biofilme formado sobre 5 blocos de cada substrato foi coletado 5 minutos após exposição a água. Em seguida, o biofilme formado sobre os 2 blocos restantes de cada substrato, posicionados separadamente dos demais, foi exposto a solução de glicose a 20%, sendo o mesmo coletado 5 minutos após essa exposição.

APÊNDICE 3 – Esquema da extração do fluido do biofilme



O biofilme foi colocado em uma ponteira de 10 µL contendo óleo mineral para evitar evaporação do fluido. Após a centrifugação em dispositivo adaptado, 3 fases são formadas na ponteira: precipitado (parte sólida ou estroma do biofilme), fluido do biofilme e óleo mineral. O fluido foi coletado com auxílio de micropipeta, no qual foi analisado o pH e concentração de cálcio, fósforo e fluoreto.

APÊNDICE 4 – Esquema da análise do pH no fluido do biofilme

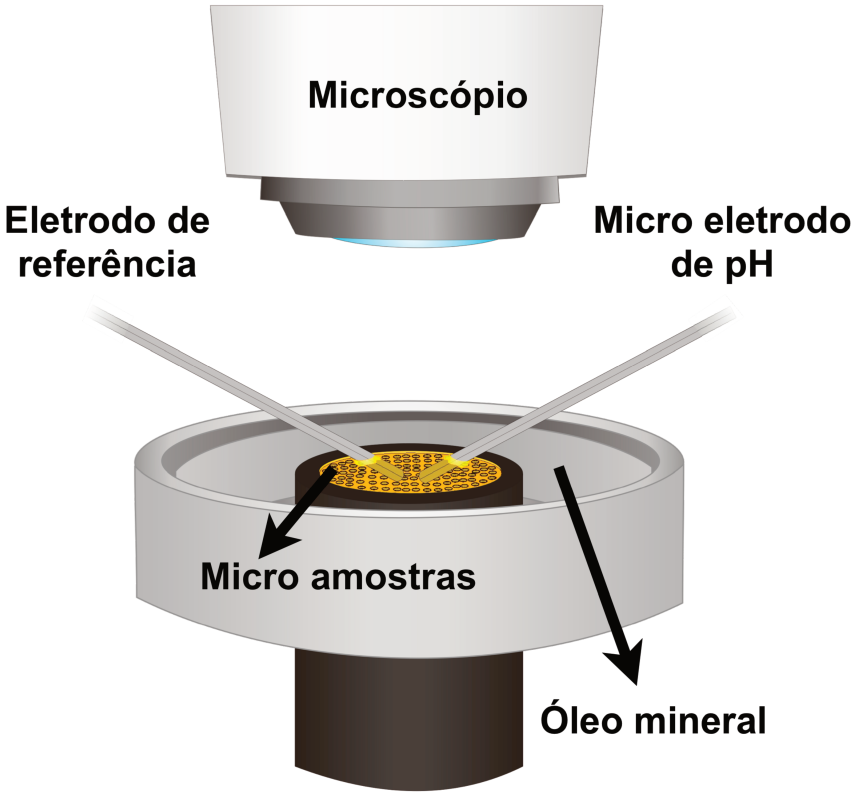


Ilustração da determinação do pH. As amostras do fluido do biofilme foram posicionadas sobre o cristal do eletrodo de flúor invertido (funcionando somente como apoio), sob óleo mineral saturado com 4% de CO_2. Com o auxílio de um microscópio, o microeletrodo de pH e o de referência foram posicionados no interior das amostras, e ao fechar o circuito, o pH era determinado.

APÊNDICE 6 – Esquema de análise do flúor no fluido do biofilme

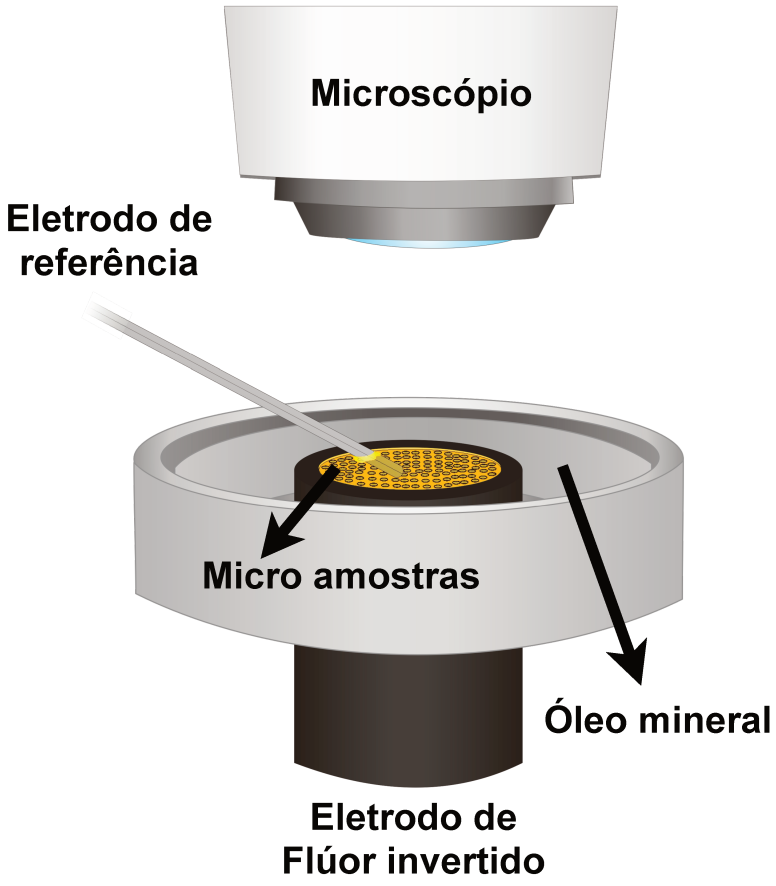
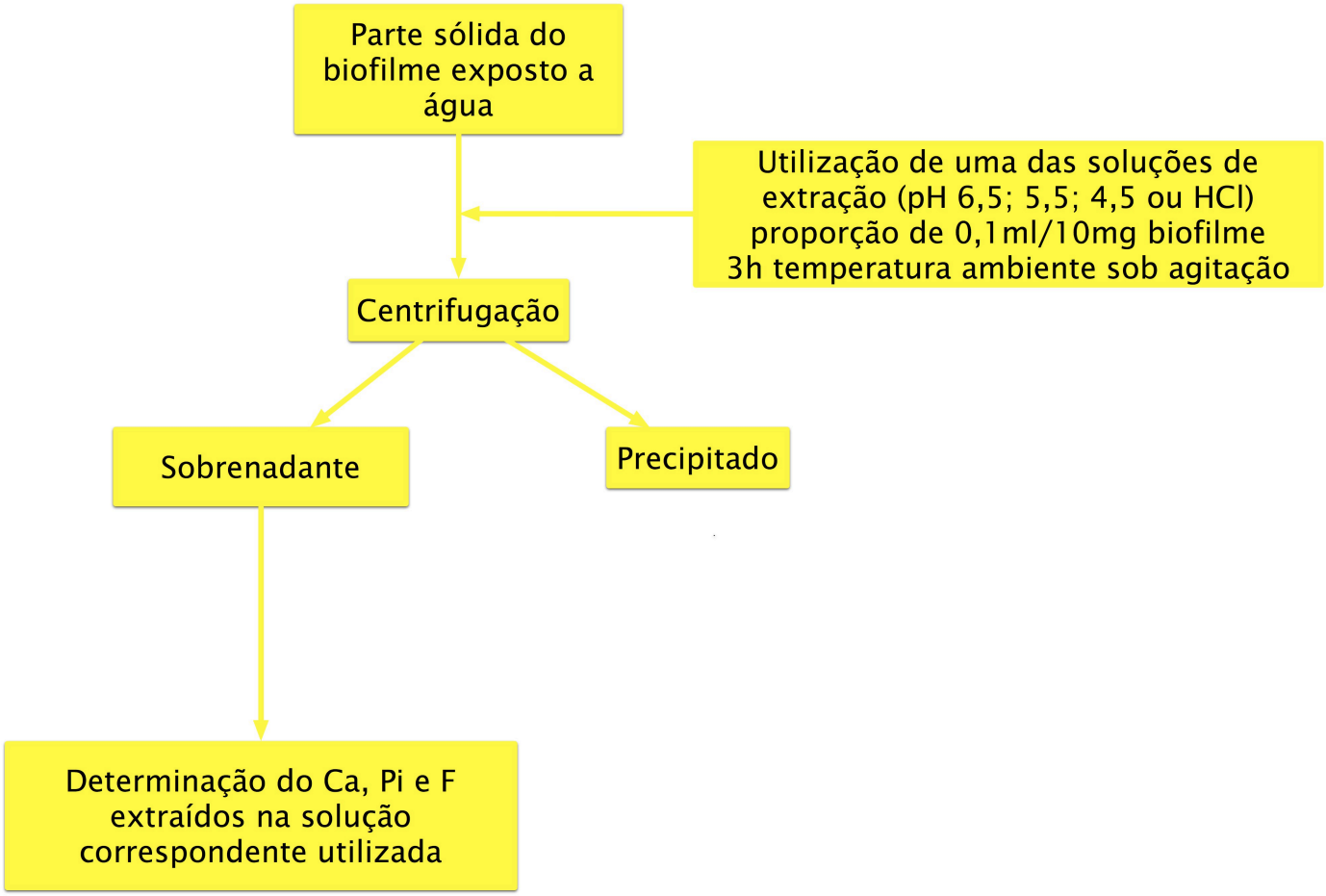


Ilustração do aparelho utilizado para microanálise de fluoreto. As amostras foram posicionadas na superfície do cristal íon-específico do eletrodo de flúor invertido, sob óleo mineral, e diluídas com TISAB III (10:1). Com o auxílio de um microscópio, o microeletrodo de referência foi posicionado em contato com as amostras, fechando o circuito e permitindo a determinação da concentração de fluoreto através de um potenciômetro.

APÊNDICE 7 – Fluxograma da extração da parte sólida do biofilme de acordo com a solução utilizada no segundo estudo in situ



ANEXO 1 – Deliberação da defesa em formato alternativo

INFORMAÇÃO CCPG/OO2/06[6]

Tendo em vista a necessidade de revisão da regulamentação das normas sobre o formato e a impressão das dissertações de mestrado e teses de doutorado e com base no entendimento exarado no Parecer PG n° 1985/96, que trata da possibilidade do formato alternativo ao já estabelecido, a CCPG resolve:

Artigo 1º - O formato padrão das dissertações e teses de mestrado e doutorado da UNICAMP deverão obrigatoriamente conter:

I. Capa com formato único ou em formato alternativo que deverá conter informações relativas ao nível (mestrado ou doutorado) e à Unidade de defesa, fazendo referência à Universidade Estadual de Campinas, sendo o projeto gráfico das capas definido pela PRPG.
II. Primeira folha interna dando visibilidade à Universidade, a Unidade de defesa, ao nome do autor, ao título do trabalho, ao número de volumes (quando houver mais de um), ao nível (mestrado ou doutorado), a área de concentração, ao nome do orientador e co-orientador, ao local (cidade) e ao ano de depósito. No seu verso deve constar a ficha catalográfica.
III. Folha de aprovação, dando visibilidade à Comissão Julgadora com as respectivas assinaturas.
IV. Resumo em português e em inglês (ambos com no máximo 500 palavras).
V. Sumário.
VI. Corpo da dissertação ou tese dividido em tópicos estruturados de modo característico à área de conhecimento.
VII. Referências, formatadas segundo normas de referenciamento definidas pela CPG da Unidade ou por critério do orientador.
VIII. Todas as páginas deverão, obrigatoriamente, ser numeradas, inclusive páginas iniciais, divisões de capítulos, encartes, anexos, etc... As páginas iniciais poderão ser numeradas utilizando-se algarismos romanos em sua forma minúscula.
IX. Todas as páginas com numeração "impar" serão impressas como "frente" e todas as páginas com numeração "par" serão impressas como "verso".

§ 1º - A critério do autor e do orientador poderão ser incluídos: dedicatória; agradecimento; epígrafe; lista de: ilustrações, tabelas, abreviaturas e siglas, símbolos; glossário; apêndice; anexos.

§ 2º - A dissertação ou tese deverá ser apresentada na língua portuguesa, com exceção da possibilidade permitida no artigo 2° desta Informação.

§ 3º - As dissertações e teses cujo conteúdo versar sobre pesquisa envolvendo seres humanos, animais ou biossegurança, deverão apresentar anexos os respectivos documentos de aprovação.

Artigo 2º - A critério do orientador e com aprovação da CPG da Unidade, os capítulos e os apêndices poderão conter cópias de artigos de autoria ou de co-autoria do candidato, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, escritos no idioma exigido pelo veículo de divulgação.

§ único - O orientador e o candidato deverão verificar junto às editoras a possibilidade de inclusão dos artigos na dissertação ou tese, em atendimento à legislação que rege o direito autoral, obtendo, se necessária, a competente autorização, deverão assinar declaração de que não estão infringindo o direito autoral transferido à editora.

Artigo 3º - Dependendo da área do conhecimento, a critério do orientador e com aprovação da CPG da Unidade, a dissertação ou tese poderá ser apresentada em formato alternativo, desde que observados os incisos I, II, III IV, V e VII do artigo 1º.

Artigo 4º - Para impressão, na gráfica da Unicamp, dos exemplares definitivos de dissertações e teses defendidas, deverão ser adotados os seguintes procedimentos:

§ 1º - A solicitação para impressão dos exemplares de dissertações e teses poderá ser encaminhada à gráfica da Unicamp pelas Unidades, que se responsabilizarão pelo pagamento correspondente.

§ 2º - Um original da dissertação ou tese, em versão definitiva, impresso em folha tamanho carta, em uma só face, deve ser encaminhado à gráfica da Unicamp acompanhado do formulário "Requisição de Serviços Gráficos", onde conste o número de exemplares solicitados.

§ 3º - A gráfica da Unicamp imprimirá os exemplares solicitados com capa padrão. Os exemplares solicitados serão encaminhados à Unidade em, no máximo, cinco dias úteis.

§ 4º - No formulário "Requisição de Serviços Gráficos" deverão estar indicadas as páginas cuja reprodução deva ser feita no padrão "cores" ou "foto", ficando entendido que as demais páginas devam ser reproduzidas no padrão preto/branco comum.

§ 5º - As dissertações e teses serão reproduzidas no padrão frente e verso, exceção feita às páginas iniciais e divisões de capítulos; dissertações e teses com até 100 páginas serão reproduzidas no padrão apenas frente, exceção feita à página que contém a ficha catalográfica.

§ 6º - As páginas fornecidas para inserção deverão ser impressas em sua forma definitiva, ou seja, apenas frente ou frente/verso.

§ 7º - O custo, em reais, de cada exemplar produzido pela gráfica será definido pela Administração Superior da Universidade.

Artigo 5º - É obrigatória a entrega de dois exemplares para homologação.

Artigo 6º - Esta Informação entrará em vigor na data de sua publicação, ficando revogadas as disposições em contrário, principalmente as Informações CCPG 001 e 002/98 e CCPG/001/00.

Campinas, 13 de setembro de 2006

Profa. Dra. Teresa Dib Zambon Atvars
Presidente
Comissão Central de Pós-Graduação

ANEXO 2 – Certificado de aprovação do Comitê de Ética

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 "**Potencial anti-cárie dos reservatórios de cálcio, fosfato e fluoreto do biofilme dental**", protocolo nº **090/2007**, dos pesquisadores **JAIME APARECIDO CURY, LIVIA MARIA ANDALÓ TENUTA e MARÍLIA FERREIRA CORREIA**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 14/11/2007.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project "**Anticaries potential of calcium, phosphate and fluoride reservoirs of dental biofilm**", register number **090/2007**, of **JAIME APARECIDO CURY, LIVIA MARIA ANDALÓ TENUTA and MARÍLIA FERREIRA CORREIA**, 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 at 14/11/2007.

Profa. Cínthia Pereira Machado Tabchoury
Secretária
CEP/FOP/UNICAMP

Prof. Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

ANEXO 3 – Comprovante de submissão de artigo referente ao primeiro capítulo

Submission Confirmation

Thank you for submitting your manuscript to *Journal of Dental Research*.

Manuscript ID:	JDR-10-0055
Title:	Kinetics of Ca, Pi and F in enamel and dentine biofilms during sugar challenge
Authors:	Correia, Marilia Tenuta, Livia Maria Del Bel Cury, Altair Cury, Jaime
Date Submitted:	13-Apr-2010

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