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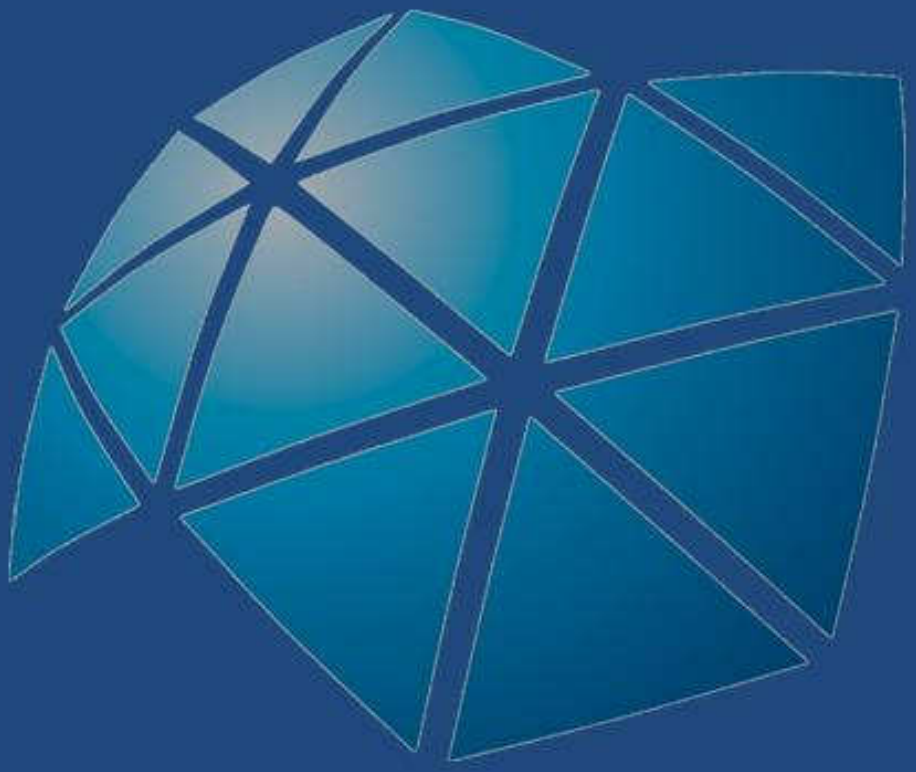

ARAÇATUBA
2009

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Tese apresentada à Faculdade de Odontologia da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Araçatuba, como parte dos requisitos para obtenção do título de Doutor em Odontopediatria

Orientadora: Profª Drª Marília Afonso Rabelo Buzalaf

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Pessan JP. Incorporação de flúor no biofilme dentário e fluido do biofilme após o uso de dentifrícios convencional e com concentração reduzida de flúor, em comunidades com diferentes níveis de flúor na água de abastecimento [tese]. Araçatuba: Universidade Estadual Paulista; 2009.

RESUMO

Avaliou-se a incorporação de flúor (F) no biofilme dentário e fluido do biofilme após o uso de dentifrícios convencional(DC) e com concentração reduzida de F(DCR). A hipótese do estudo foi de que o uso do DCR levaria a uma incorporação proporcionalmente mais alta no biofilme, principalmente nas camadas mais profundas do mesmo, e que estes aumentos seriam refletidos no fluido do biofilme. Na primeira etapa, foram comparadas as concentrações de F e cálcio (Ca) presentes no biofilme após o uso de dentifrícios contendo 0 (dentifrício placebo–DP), 513(DCR) e 1.072(DC) ppm F, em crianças residentes em comunidades contendo 0,04(A), 0,72(B) e 3,36(C) ppm F na água de abastecimento. Seguindo um protocolo duplo-cego e cruzado, as crianças escovaram seus dentes 2 vezes ao dia, durante 7 dias. Amostras de biofilme foram coletadas 1 e 12 horas após a última escovação. Na segunda e terceira etapas, conduzidas somente na comunidade B, avaliou-se a incorporação de F em secções seriais de biofilme formado utilizando "dispositivos *in situ* de Leeds", bem como a concentração de F no fluido do biofilme, respectivamente, empregando o protocolo descrito anteriormente. As análises de F foram realizadas após extração ácida (biofilme total), por cromatografia iônica (secções de biofilme) e após tamponamento com TISAB III (fluido do biofilme). As análises de Ca foram realizadas por espectrometria de absorção atômica (primeira e segunda etapas) e por método colorimétrico (terceira etapa). Os dados foram analisados por ANOVA, testes de Tukey e Bonferroni e Análise de Regressão Linear ($p<0,05$). Na primeira etapa, as concentrações de F estavam diretamente relacionadas às concentrações de F na água de abastecimento, independentemente do dentifrício utilizado. O uso dos dentifrícios fluoretados levou a aumentos significativos nas concentrações de F no biofilme 1h após o uso dos mesmos nas comunidades A e B apenas. Aumentos virtualmente idênticos foram observados 1h após a escovação com o DCR (*ca.*1,9 mmolF/kg) e DC (*ca.*2,4 mmolF/kg) nas comunidades A e B quando comparados ao DP; estes aumentos foram menos pronunciados na comunidade C. Para a segunda etapa, observou-se que o F se restringiu principalmente às camadas mais externas do biofilme para todos os dentifrícios, tendo sido observado um padrão semelhante para o Ca. Os valores obtidos para DC foram significativamente maiores que os obtidos para o DP. As concentrações de F e Ca estiveram positiva e significativamente relacionadas na maioria das secções do biofilme. Quanto à terceira etapa, as concentrações de F no biofilme total apresentaram um padrão semelhante ao observado para a primeira etapa; no entanto, estas não foram refletidas no fluido do biofilme. Houve uma correlação positiva e significativa entre as concentrações de flúor e cálcio no biofilme total para a maioria das situações analisadas. Os resultados confirmam a hipótese de que o uso do DCR leva a uma incorporação de F no biofilme proporcionalmente mais alta em comparação ao DC e também corroboram achados prévios sugerindo que a retenção de F no biofilme é dependente das concentrações de Ca no mesmo. Entretanto, a incorporação de F promovida pelo DCR não ocorre em camadas mais profundas em comparação ao DC.

Palavras-chave: Dentifrício fluoretado. Placa dentária. Biofilmes. Flúor. Cálcio.

Pessan JP. Fluoride uptake by dental biofilm and biofilm fluid after the use of conventional and low-fluoride dentifrices in communities with different fluoride levels in the drinking water [thesis]. Araçatuba: São Paulo State University; 2009.

ABSTRACT

This study evaluated fluoride (F) uptake by dental biofilm and biofilm fluid after the use of conventional (CD) and low-fluoride (LFD) dentifrices. The hypothesis of the study was that the use of LDF would lead to a proportionally higher F uptake by the biofilm, mainly in the deepest layers of it, and that such increases would be reflected in the biofilm fluid. In the first phase, F and calcium (Ca) concentrations in the biofilm were compared after the use of dentifrices containing 0 (placebo dentifrice–PD), 513 and 1,072 ppm F, in children living in communities containing 0.04(A), 0.72(B) and 3.36(C) ppm F in the drinking water. Following a double-blind, crossover protocol, children brushed their teeth twice daily, during seven days. Samples of biofilm were collected on the seventh day, 1 and 12 h after the last use of the dentifrices. In the second and third phases, which were conducted only in the community B, we evaluated F uptake in serial sections of biofilms formed using the "Leeds *in situ* devices", as well as in the biofilm fluid, respectively, following the same protocol described for the first phase. F analyses were carried out after acid extraction (whole biofilm), by ion chromatography (sections of biofilm) and after buffering with TISAB III (biofilm fluid). Analyses of Ca were done by atomic absorption spectrometry (first and second phases) and colorimetrically (third phase). Results were analyzed by ANOVA, Tukey's and Bonferroni's tests, by linear regression analysis and by Pearson's correlation ($p<0.05$). For the first phase, mean biofilm F concentrations were directly related to F concentrations in water, regardless of the dentifrice used. The use of the fluoridated dentifrices led to significant increases in F concentrations in the biofilm 1 h after their use, in the communities A and B only. Virtually identical increases were observed 1 h after brushing with CD (*ca*.1.9 mmolF/kg) and LFD (*ca*.2.4 mmolF/kg) in the communities A and B when compared to PD. These increases were less pronounced in the community C. For the second phase, it was observed that F was mostly restricted to the outer layers of biofilm for all the dentifrices tested, and a similar pattern was observed for Ca. The values obtained for CD were significantly higher than those observed for PD. F and Ca concentrations were positively and significantly correlated throughout most of biofilms' sections. For the third phase, F concentrations in whole biofilm showed a similar pattern to that observed for the first phase; however, F levels were not reflected in the biofilm fluid. Positive and significant correlations were verified among F and Ca concentrations in whole biofilm for most of the situations evaluated. The results confirm the hypothesis that the use of the LFD leads to a proportionally higher F uptake by the biofilm when compared to CD and also corroborate previous findings showing that F uptake by the biofilm is dependent on Ca concentrations. However, unlike our prediction, the use of LFD did not promote a higher F uptake in deeper layers of the biofilms when compared to CD.

Key-words: Fluoridated dentifrice. Dental Plaque. Biofilms. Fluoride. Calcium.

SUMÁRIO

Introdução Geral

INTRODUÇÃO GERAL

A manutenção dos níveis intrabucais de flúor exerce um papel fundamental na dinâmica da cárie dentária, uma vez que uma relação inversa entre as concentrações de flúor no biofilme dentário e na saliva e a prevalência e atividade de cárie tem sido demonstrada por vários estudos clínicos (Gaugler, Bruton, 1982[1]; Shields *et al.*, 1987[2]; Nobre dos Santos *et al.*, 2002[3]). Em diversos países ocidentais, as formas mais amplamente difundidas do uso dos fluoretos são a água, com concentrações usualmente em torno de 1 µg F/mL, e dentifrícios, tipicamente contendo 1.000-1.500 µg F/g.

Apesar da grande diferença entre as concentrações de flúor dos dois métodos, estudos epidemiológicos têm demonstrado um maior efeito da água fluoretada no controle da cárie dentária em comparação aos dentifrícios fluoretados (O´Mullane, 1990[4], Mellberg, 1990[5], Marinho *et al.*, 2003[6]). Embora diferenças quanto à frequência de exposição entre os dois métodos possam explicar em parte os resultados obtidos por estudos epidemiológicos, ainda não está claro o motivo pelo qual o consumo regular de água fluoretada resulta numa maior redução da cárie dentária quando comparado ao uso de dentifrícios fluoretados. Com base na premissa de que os processos de desmineralização do esmalte somente irão ocorrer na presença de biofilme dentário e considerando-se uma relação dose-resposta, seria esperado que o uso regular de dentifrícios fluoretados produzisse concentrações mais altas de flúor no biofilme dentário, o que, teoricamente, produziria um maior efeito cariostático durante os episódios de queda do pH do biofilme.

Esta questão foi levantada previamente, em estudos que demonstraram uma relação direta entre as concentrações de cálcio e flúor no biofilme dentário em comunidades com água

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[1] Gaugler RW, Bruton WF: Fluoride concentration in dental plaque of naval recruits with and without caries. Arch Oral Biol 1982; 27:269-72.

[2] Shields CP, Leverett DH, Adair S, Featherstone JDB: Salivary fluoride levels in fluoridated and non-fluoridated communities. J Dent Res 1987; 141 (Abstract No. 277).

[3] Nobre dos Santos M, Melo dos Santos L, Francisco SB, Cury JA: Relationship among dental plaque composition, daily sugar exposure and caries in the primary dentition. Caries Res 2002; 36:347-352.

[4] O'Mullane DM: The future of water fluoridation. J Dent Res 1990; 69 (Spec Issue):756-759.

[5] Mellberg JR: Evaluation of topical fluoride preparations. J Dent Res 1990; 69 (Spec Issue):771-779.

[6] Marinho VCC, Higgins JP, Sheiham A, Logan S: Fluoride toothpastes for preventing dental caries in children and adolescents. *The Cochrane Library 2005;* Issue 2, Oxford: Update Software.

de abastecimento fluoretada (Whitford *et al.*, 2002)[7] e não fluoretada (Whitford *et al.*, 2005)[8]. Em adição, verificou-se que as concentrações de flúor obtidas 12 horas após o uso de um dentifrício fluoretado permaneciam significativamente aumentadas em relação ao uso de um dentifrício placebo na comunidade não fluoretada, o que não ocorreu na comunidade fluoretada. Os autores sugeriram um limite na incorporação de flúor no biofilme dentário, o que seria determinado pelo número de sítios de cálcio disponíveis para ligação do flúor (Whitford *et al.*, 2002, 2005).

Uma vez que as diferenças entre as populações envolvidas nos estudos previamente mencionados (adultos norte-americanos residentes em uma área fluoretada e adolescentes brasileiros de uma área não fluoretada) não permitia uma comparação direta entre os resultados, Pessan *et al.* (2008)[9] conduziram um estudo com o mesmo protocolo utilizado por Whitford *et al.* (2002, 2005), mas com crianças brasileiras com mesma faixa etária, residentes em áreas com concentrações de flúor na água de abastecimento de 0,04, 0,85 e 3,5 ppm de flúor. Um dos principais achados do estudo de Pessan *et al.* (2008) foi que o dentifrício fluoretado produziu um aumento virtualmente idêntico nas concentrações de flúor no biofilme dentário 1 hora após seu uso (em torno de 6,5 mmol F/Kg, peso seco), para as 3 comunidades envolvidas, independentemente do que já havia sido incorporado devido ao consumo da água.

As razões deste fenômeno, também verificado em um estudo conduzido em indivíduos de faixa etária e nível socioeconômico semelhantes (Whitford *et al.*, 2005), ainda não estão claras. Considerando-se que a concentração de flúor no dentifrício é muito mais alta que a encontrada na água de abastecimento, era esperado que o uso de um dentifrício fluoretado pudesse igualar ou nivelar a quantidade de flúor retida no biofilme 1 hora após a escovação, independentemente da quantidade de flúor previamente retida no biofilme relacionada à exposição à água fluoretada, o que não ocorreu. A análise destes dados conjuntamente levou os investigadores a hipotetizar que o flúor em sua forma iônica (como presente na água) poderia penetrar mais profundamente no biofilme dentário quando comparado a uma fonte de flúor mais concentrada (como os dentifrícios), considerando-se que o uso destes produtos levaria a uma formação de compostos de CaF_2 ou CaF^+ na saliva, os quais migrariam

[7] Whitford GM, Wasdin JL, Shaffer TE, Adair SM: Plaque fluoride concentrations are dependent on plaque calcium concentrations. Caries Res 2002; 36:256-265.
[8] Whitford GM, Buzalaf MAR, Bijella MFB, Waller JA: Plaque fluoride concentrations in a community without water fluoridation: Effects of calcium and use of a fluoride or placebo dentifrice. Caries Res 2005; 39:100-107.
[9] Pessan JP, Silva SMB, Lauris JRP, Sampaio FC, Whitford GM, Buzalaf MAR: Fluoride uptake by plaque from water and from dentifrice. J Dent Res 2008; 87:461-465.

predominantemente para a superfície do biofilme dentário (Whitford *et al.*, 2005, Pessan *et al.*, 2008).

Para que o mecanismo proposto possa ser validado, seria interessante conduzir um estudo com o mesmo protocolo utilizado por Pessan *et al.* (2008), incluindo uma nova etapa, na qual um dentifrício com concentração reduzida de flúor (500-550 ppm) fosse utilizado. Visto que com o uso de um dentifrício contendo 1.100 ppm de flúor promoveu uma incorporação de flúor no biofilme constante para os voluntários das três comunidades estudadas, independentemente do nível basal de flúor presente, seria esperado que a utilização de um dentifrício com a metade da concentração de flúor promovesse uma incorporação também constante para as 3 comunidades, embora em menor magnitude.

Em acréscimo, a avaliação da concentração de flúor em secções seriais do biofilme dentário (Robinson *et al.*, 1997[10], Kato *et al.*, 1997[11], Watson *et al.*, 2005[12]) após o uso dos dentifrícios previamente mencionados ainda não foi realizada e seria de extrema importância na elucidação dos eventos ocorridos na incorporação de flúor no biofilme dentário. É possível que o dentifrício de baixa concentração de flúor permita uma incorporação de flúor em regiões mais profundas do biofilme, já que uma menor concentração de flúor na saliva reduziria a formação e precipitação de compostos de cálcio e flúor na superfície do biofilme. Uma vez que o uso destes dentifrícios ainda é controverso com relação à redução na prevalência da cárie dentária (Ammari *et al.*, 2003)[13], o presente estudo poderia trazer informações adicionais que viessem a contribuir na tentativa de se estabelecer a real participação dos mesmos no controle da cárie.

Finalmente, a avaliação das concentrações de flúor e cálcio no fluido do biofilme poderia trazer informações úteis sobre a disponibilidade dos mesmos no interior do biofilme e, consequentemente, o papel destes íons na dinâmica da desmineralização e remineralização do esmalte dentário após o uso de dentifrícios convencional e com concentração reduzida de

[10] Robinson C, Kirkham J, Percival R, Shore RC, Bonass WA, Brookes SJ, Kusa L, Nakagaki H, Kato K, Nattress B: A method for the quantitative site-specific study of the biochemistry within dental plaque biofilms formed in vivo. Caries Res 1997; 31:194-200.

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flúor. Visto que o fluido do biofilme é a fração que tem contato direto com a superfície do esmalte, fica evidente que o estudo de sua composição inorgânica se reveste de grande relevância do ponto de vista clínico, já que a concentração de flúor e cálcio no mesmo determina a saturação do mineral dentário na cavidade bucal (Margolis e Moreno, 1992[14]; Vogel *et al.*, 1990[15]). Desta forma, considerando-se que a maioria dos indivíduos não remove completamente o biofilme após a escovação e que a desmineralização do esmalte somente irá ocorrer na presença de biofilme, a quantidade de flúor retida neste pode exercer um papel importante no controle da cárie dentária. Desta maneira, o entendimento do mecanismo de incorporação do flúor no biofilme é fundamental para a elaboração de estratégias visando ao aumento da sua incorporação.

Diante do exposto, o presente estudo teve por objetivo avaliar a incorporação de flúor no biofilme dentário e fluido do biofilme após o uso de dentifrícios convencional e com concentração reduzida de F, em comunidades com 0,04, 0,72 e 3,36 ppm de F na água de abastecimento.

Para abordar o tema proposto, o estudo será apresentado em três capítulos distintos, conforme descrito abaixo:

- Capítulo 1: **"Effect of regular and low-fluoride dentifrices on plaque fluoride"** (artigo submetido ao periódico Journal of Dental Research[16,17]);

- Capítulo 2: **"Distribution of F and Ca in plaque formed in presence of F dentifrices"** (artigo preparado para submissão ao periódico Journal of Dental Research[16]).

- Capítulo 3: "**Plaque and plaque fluid fluoride concentrations associated to the use of conventional and low-fluoride dentifrices"**. (artigo preparado para submissão ao periódico European Journal of Oral Sciences[18]).

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[16] Anexo 1
[17] Anexo 2
[18] Anexo 3

Capítulo 1

"Effect of regular and low-fluoride dentifrices on plaque fluoride"

Effect of Regular and Low-Fluoride Dentifrices on Plaque Fluoride

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Number of cited references: 15

This study was based on a thesis to be submitted to Araçatuba Dental School, São Paulo State University (Brazil), in partial fulfillment of the requirements for the PhD degree in Pediatric Dentistry.

ABSTRACT

Previous studies indicate that the use of low-fluoride dentifrices (LF) could lead to proportionally higher plaque fluoride levels when compared to conventional dentifrices (C). This double-blind, randomized, crossover study determined the effects of placebo dentifrice, LF and C on plaque fluoride concentrations ([F]) in children living in communities with 0.04, 0.72 and 3.36 ppmF in the drinking water. Children used the toothpastes twice daily, for 1 week. Samples were collected 1 and 12h after the last use of dentifrices and were analyzed for fluoride and calcium. Results were analyzed by 3-way ANOVA and linear regression analysis. Similar increases were found 1h after brushing with LF (*ca.*1.9 mmolF/kg) and C (*ca.*2.4 mmolF/kg) in the 0.04 and 0.72 ppmF communities. Despite the increases were less pronounced in the 3.36 ppmF community, our results indicate that the use of a LF promotes a proportionally higher increase in plaque [F] when compared to C.

INTRODUCTION

A series of studies has been conducted to evaluate the effect of fluoridated toothpastes on fluoride uptake by plaque and its retention throughout the day in communities with sub-optimal (Whitford *et al.*, 2005), optimal (Whitford *et al.*, 2002; Pessan *et al.*, 2006) and above-optimal (Pessan *et al.*, 2008) water fluoride concentrations. Although small differences were found in the results among the studies, all of them agreed that intraoral fluoride levels were directly related to water fluoride concentrations regardless the dentifrice used, and that fluoride uptake by plaque is dependent on plaque calcium concentrations. Furthermore, because the latest study was conducted simultaneously in children from communities with 0.04, 0.85 and 3.5 μg F/mL in water, it was possible to observe for the first time that the increments in fluoride levels in plaque 1 h after brushing were virtually identical in each community.

The reasons for this phenomenon, also found in a study conducted in individuals of similar age range and socioeconomic background (Whitford *et al.*, 2005), are still not clear. Considering that fluoride levels in toothpaste are much higher than those found in water, it was expected that the use of fluoridated dentifrice could equalise the amount of fluoride retained in plaque 1 h after brushing, regardless the background fluoride exposure from water, but this did not happen. These findings led the investigators to hypothesize that fluoride in low concentration sources, as in water, penetrates deeper into plaque when compared to a high fluoride source, like dentifrice, since the use of these products could lead to the formation of salivary CaF^{+}/CaF_2 compounds, which would migrate predominantly onto plaque surface (Watson et al., 2005; Whitford *et al.*, 2005, Pessan *et al.*, 2008).

Considering this hypothesis, it would be possible that the use of a dentifrice with a lower fluoride concentration could lead to a proportionally higher fluoride uptake by plaque when compared to a dentifrice containing 1,000-1,100 mgF/Kg. Since there is no agreement about the clinical efficacy of low-fluoride dentifrices on caries control when compared to conventional dentifrices (Ammari *et al.*, 2003), the validation of this hypothesis could be useful for a better comprehension of the effects of such formulations on intraoral fluoride levels, which was demonstrated to be directly related to the clinical efficacy of topical fluoridated products (Duckworth *et al.*, 1992).

Thus, the present study was conducted following the same protocol of the study by Pessan *et al.* (2008) in order to verify if similar increments of fluoride would be found in plaque after the use of conventional and low-fluoride dentifrices, in communities with different fluoride

levels in water, and if the low-fluoride dentifrice would promote a proportionally higher fluoride retention in plaque in comparison to the conventional dentifrice.

MATERIALS & METHODS

Fifty-six 8-10-year-old Brazilian children from cities with different water fluoride concentrations, Pirajuí (0.04 ppm, *n*=19), Bauru (0.72 ppm, *n*=20) and Brejo das Freiras (3.36 ppm, *n*=17), participated. Water fluoride concentrations were determined weekly through analysis of 4 samples from volunteers' homes. Sample size was established at 15 subjects per group in order to obtain a power of 80% (α=0.05), based on a study conducted with the same protocol (Pessan *et al.*, 2008). As inclusion criteria, the children were permanent residents of their community and from similar socioeconomic backgrounds, attended the same public school where samples were collected, and drank tap water exclusively. The protocol was approved by the IRB of Bauru Dental School[1] and informed consent was obtained from children's parents[2].

The study began with a dental prophylaxis to remove all plaque and calculus. The protocol of the study, described in detail in previous publications (Whitford *et al.*, 2002, 2005, Pessan *et al.*, 2006, 2008), followed a double-blind, crossover design, in which the volunteers were randomly assigned (blocking stratification) to brush with a conventional fluoride dentifrice (Crest, 1,072 mgF/kg), a low-fluoride dentifrice (Crest, 513 mgF/Kg) or a fluoride-free placebo. The dentifrices' tubes were coded by a researcher not involved in the present study. They used these products for 1.0 min in the morning and at bedtime for one week and rinsed with 10 mL of water[3]. The abrasive system in the calcium-free products was hydrated silica.

During the sixth day, children brushed only the occlusal surfaces to allow plaque accumulation. After going to bed, they refrained from eating or drinking anything except water and did not brush their teeth until the following morning. On the following morning, plaque was collected from the right side of mouth; children then brushed the occlusal surfaces for 1.0 min and rinsed with 10 mL of tap water. One hour later plaque (left side) was again collected. Thus the samples were collected 1.0 hr and approximately 12 hr after the last use of the dentifrices. The entire protocol was then repeated using the dentifrices not previously used.

[1] Anexos 4 a 6
[2] Anexo 7
[3] Anexo 8

Plaque samples were dried at 95°C and weighed to the nearest microgram. Fluoride in plaque was determined after extraction with 0.5 M $HClO_4$ which was buffered with TISAB I (Orion Research)[4]. Calcium concentrations were determined by AAS using an aliquot of the $HClO_4$ digests of plaque. All analyses were done in duplicate and the coefficients of variability were 6.9% and 7.1%, respectively for fluoride and calcium.

The results are expressed as mean ± se. The outcomes were not normally distributed so log transformations were performed. A three-factor repeated measures ANOVA was then used to examine differences in each outcome. For pairwise comparisons, Tukey's and Bonferroni's tests were used, respectively for fluoride and calcium concentrations in plaque. The two fixed effect factors were fluoride dentifrice and time after toothbrushing and the independent factor was community. A significance level of 5% was selected.

RESULTS

Mean plaque fluoride concentrations were directly related to the water fluoride concentrations regardless the dentifrice used (Table 1). Significant interactions were observed between the factors communities and treatments ($p<0.05$), as well as between communities and time after brushing ($p<0.001$). For Pirajuí and Bauru, the use of the fluoridated pastes led to significant increases in plaque fluoride concentrations when compared to the placebo dentifrice 1h after brushing. No differences were verified between 1 and 12h after brushing with the fluoridated dentifrices, except for Pirajuí. For Brejo das Freiras, no significant differences were observed regardless of the dentifrice used and time after brushing.

Although the values obtained for Bauru were numerically higher than those from Pirajuí (Table 1), a similar trend was observed for fluoride uptake by plaque (1h after brushing) in these communities (Figure 1). The increments in fluoride concentration 1h after brushing with the 513 mgF/Kg toothpaste were virtually identical (around 1.9 mmol F/kg). For the 1,072 mgF/Kg dentifrice the same trend was verified, with a mean increase around 2.4 mmolF/Kg 1h after brushing. Regarding the differences between fluoride concentrations in plaque 1 and 12h after brushing with the fluoride dentifrices (plaque fluoride clearance), plaque fluoride concentrations in Pirajuí and Bauru decreased about 43% 12h after brushing compared to the values found 1h after brushing for the 513 mgF/Kg dentifrice (Figure 2). For the 1,072 mgF/Kg dentifrice, the % decrease was less uniform (47% and 30%, respectively, for Pirajuí

[4] Anexo 9

and Bauru). For Brejo das Freiras, however, both the increases and decreases in plaque fluoride concentrations were considerably lower when compared to the other communities.

Regarding calcium concentrations in plaque, there was an interaction between the factors community and time of sample collection ($p<0.001$), so that no pattern could be observed (Table 2). Overall, the data observed in Bauru were numerically higher than those observed in the other communities.

DISCUSSION

The studies conducted by Whitford *et al.* (2002, 2005) led to the hypothesis that the high fluoride levels in saliva after the use of a dentifrice would promote the formation of CaF^{+}/CaF_2 compounds. These would be less likely to migrate into the deeper layers of plaque when compared to the ionic form of fluoride present in water, but instead accumulate mainly in the outer layers (Watson *et al.*, 2005; Whitford *et al.*, 2005). Although this hypothesis was not fully tested, the results of the study by Pessan *et al.* (2008) provided additional evidence for it. In their study, an average increase of 6.5 mmol F/Kg was found in plaque 1 h after brushing with a 1,030 mgF/Kg dentifrice and these decreased at a similar rate (around 50%) for all communities, regardless the baseline fluoride levels in plaque associated with the regular consumption of water. In order to better understand this phenomenon, the present study was conducted using the same protocol described in the previous studies, but this time including a low-fluoride dentifrice, to allow dose-response observations.

Plaque fluoride concentrations were directly related to fluoride concentrations in the drinking water, for all dentifrices used and times of sample collection, confirming the findings obtained by Pessan *et al.* (2008). However, the increments in plaque fluoride concentrations were about 2-fold lower than those previously observed. The reasons for this are not evident. Both in the present study and in that by Pessan *et al.* (2008) samples were collected in the same season. Thus, variations due to differences in water consumption may not be the case. Additionally, care was taken in order to reproduce as much as possible the study by Pessan *et al.* (2008), so children from the same communities and same age range were selected and fluoride concentrations in the conventional dentifrices were virtually identical. The only difference between the studies was the volume of water to rinse the mouth after brushing, reduced from 30 mL (Pessan *et al.*, 2008) to 10 mL, which is more adequate for children (Issa and Toumba, 2004); thus at least similar (if not higher) plaque fluoride levels could be expected. The only factor that might help to explain the lower values found in the present work is the amount of

sodium lauryl sulphate (SLS) in the dentifrices. This anionic surfactant has strong affinity for calcium, which could affect fluoride uptake by plaque from a fluoride dentifrice (Whitford, personal communication). However, as the same source of the dentifrices was used in the present study and the other studies, this possibility does not seem to be the only explanation for the observed difference.

Despite the unexpected lower values found in the present study, it was still possible to verify similar increments in plaque fluoride concentrations 1h after the use of low-fluoride (*ca.*1.9 mmolF/kg) and conventional (*ca.* 2.4 mmolF/kg) dentifrices, for the communities of Pirajuí and Bauru, when comparing these values with the placebo levels, although these increases were considerably lower in the 3.36 ppmF community. It is noteworthy that considering the 2-fold difference between fluoride concentrations in both toothpastes, a 100% increment of that obtained for the low-fluoride dentifrice would be expected after using the conventional dentifrice (around 3.8 mmol F/kg), but these were only around 63% higher (*ca.* 2.4 mmol F/kg). These results are in agreement with the hypothesis that the use of low-fluoride dentifrices promotes a proportionally higher uptake of fluoride by plaque when compared to conventional dentifrices. The lower fluoride concentration in the 513 mgF/Kg dentifrice would promote less formation of CaF_2 and CaF^+ compounds in saliva immediately after brushing, when compared to the 1,072 mgF/Kg dentifrice. Thus, it is reasonable to assume that a proportionally higher amount of fluoride in the ionic form would be present in saliva, which would be attracted as counterions to calcium bound to fixed acidic groups in plaque (Rølla, 1977; Rølla and Bowen, 1977) and bacterial cell surfaces (Rose *et al.*, 1993, 1994, 1996 and 1997). This mechanism is in agreement with the study by Watson *et al.* (2005) which demonstrated that fluoride is restricted mainly to the outer layers of dental plaque after exposure to a NaF solution.

Another point that deserves attention regards the kinetics of fluoride clearance from dental plaque after the use of fluoride dentifrices, which seems to be different for low-fluoride and conventional dentifrices and also depends on the fluoride levels present in plaque from other sources than dentifrice (*i.e.*, water). This is evident when we observe Figure 2. For the 513 mgF/Kg dentifrice, ~ 40% of the fluoride incorporated in dental plaque at 1h was removed from plaque after 12h in Pirajuí and Bauru. However, in Brejo das Freiras, the clearance was much lower (~26%). Moreover, when a dentifrice with twice the fluoride concentration was used, the clearance remained unaltered for Pirajuí, but was lower for Bauru (~30%), while in Brejo das Freiras practically there was no clearance. These findings suggest that in conditions

of low fluoride supply (from water and/or dentifrice), the fluoride clearance from plaque is slower, while conditions of high supply may lead to faster plaque fluoride clearance. This may be due to the fact that in Brejo das Freiras the frequent exposure to water may have replenished the plaque fluoride reservoirs.

According to a recent meta-analysis, no conclusion could be taken regarding the effectiveness of 500-550 mgF/Kg dentifrices against conventional formulations due to the lack of randomized controlled trials addressing this subject (Ammari *et al.*, 2003). Although the present study did not investigate the effectiveness of low-fluoride dentifrices on caries prevalence, intraoral fluoride retention has been directly related to the clinical efficacy of topical products (Duckworth *et al.*, 1992). Thus, considering that the low-fluoride formulation led to a proportionally higher retention of fluoride in plaque, it is possible that the differences between the clinical performances of low-fluoride and conventional toothpastes are lower than expected. This concept is supported by a recent randomized clinical trial that demonstrated that the use of low-fluoride toothpastes was as effective as the conventional formulation in preventing caries in caries-inactive, but not in caries-active children (Lima *et al.*, 2008). Thus, based on caries-risk considerations, the use of low-fluoride formulations could constitute in a good alternative for caries control children, while minimizing the risk of dental fluorosis.

The results of the present study provide additional information on the mechanism of fluoride uptake by plaque after the use of dentifrices with different fluoride concentrations, indicating that a proportionally higher amount of fluoride is retained in plaque after the use of low-fluoride dentifrices compared to conventional formulations. Future studies, however, are needed in order to fully address this question. Information on the fluoride concentration in plaque fluid and in serial sections of dental plaque could help to clarify this mechanism.

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Table 1. Fluoride concentrations and ranges in dental plaque (mmol/kg, dry weight) at 1 and 12h after the last use of the placebo, low-fluoride and conventional dentifrices

Communities (fluoride concentration in water)	**Time after brushing with placebo dentifrice**		**Time after brushing with 513 mgF/Kg dentifrice**		**Time after brushing with 1,072 mgF/Kg dentifrice**	
	1h	**12h**	**1h**	**12h**	**1h**	**12h**
Pirajuí (0.04 ppm)	1.1±0.1 ^{A,ac} (0.5-2.1)	0.9±0.1 ^{A,a} (0.3-2.3)	2.9±0.3 ^{A,b} (0.7-5.7)	1.6±0.4 ^{A,ac} (0.4-7.5)	3.5±0.4 ^{A,b} (0.6-6.5)	1.8±0.3 ^{A,c} (0.6-6.2)
Bauru (0.72 ppm)	1.3±0.2 ^{A,a} (0.5±3.2)	1.3±0.2 ^{AB,a} (0.5-3.2)	3.2±0.5 ^{A,b} (0.5-8.0)	1.9±0.2 ^{A,ab} (0.8-3.4)	3.6±0.4 ^{A,b} (0.8-7.2)	2.5±0.3 ^{A,b} (0.6-5.7)
Brejo das Freiras (3.36 ppm)	2.9±0.5 ^{B,a} (0.5-7.7)	2.8±0.7 ^{B,a} (0.3-8.3)	3.5±0.6 ^{A,a} (1.1-6.9)	2.5±0.3 ^{A,a} (1.1-4.9)	4.0±0.5 ^{A,a} (0.9-7.9)	4.1±0.7 ^{A,a} (1.1-8.2)

Mean ± se (n=19 in Pirajuí, n=20 in Bauru and n=17 in Brejo das Freiras). Columns with the same upper case superscript letter are not significantly different regarding communities. Values in the same row with the same lower case superscript letter are not significantly different ($p>0.05$). Comparison made by three-way, repeated measures ANOVA on the natural log of the outcome and Tukey´s post hoc test.

Table 2. Calcium concentrations and ranges in dental plaque (mmol/kg, dry weight) at 1 and 12h after the last use of the placebo dentifrice or fluoride dentifrice

Communities (fluoride concentration in water)	**Time after brushing with placebo dentifrice**		**Time after brushing with 513 mgF/Kg dentifrice**		**Time after brushing with 1,072 mgF/Kg dentifrice**	
	1h	**12h**	**1h**	**12h**	**1h**	**12h**
Pirajuí[A] (0.04 ppm)	189.8±27.7[a] (17.8-412.1)	291.0±65.6[b] (79.4-1173.7)	167.0±26.5[a] (33.8-508.1)	240.1±48.6 [b] (42.6-944.2)	208.0±33.4[a] (32.9-524.6)	240.5±36.9[b] (55.9-619.6)
Bauru[B] (0.72 ppm)	454.7±127.9 (80.3-1853.9)	374.4±75.6 (79.6-1452.4)	316.4±47.7 (111.0-779.9)	342.1±36.8 (115.4-705.3)	432.8±70.9 (109.6-1151.2)	342.9±66.6 (73.9-1169.9)
Brejo das Freiras[AB] (3.36 ppm)	294.4±43.2 (74.7-585.7)	226.6±54.5 (30.0-656.6)	294.8±55.2 (73.7-775.3)	229.1±27.8 (76.5-578.3)	296.9±58.0 (103.5-1103.1)	260.1±58.0 (97.9-1042.8)

Mean ± se (n=19 in Pirajuí, n=20 in Bauru and n=17 in Brejo das Freiras). Different upper case superscript letters indicate significant differences among communities 1h after brushing only. Different lower case superscript letters in the same row indicate significant differences between 1 and 12h after brushing, for Pirajuí only ($p<0.05$). Comparison made by three-way, repeated measures ANOVA on the natural log of the outcome and Bonferroni´s post hoc test.

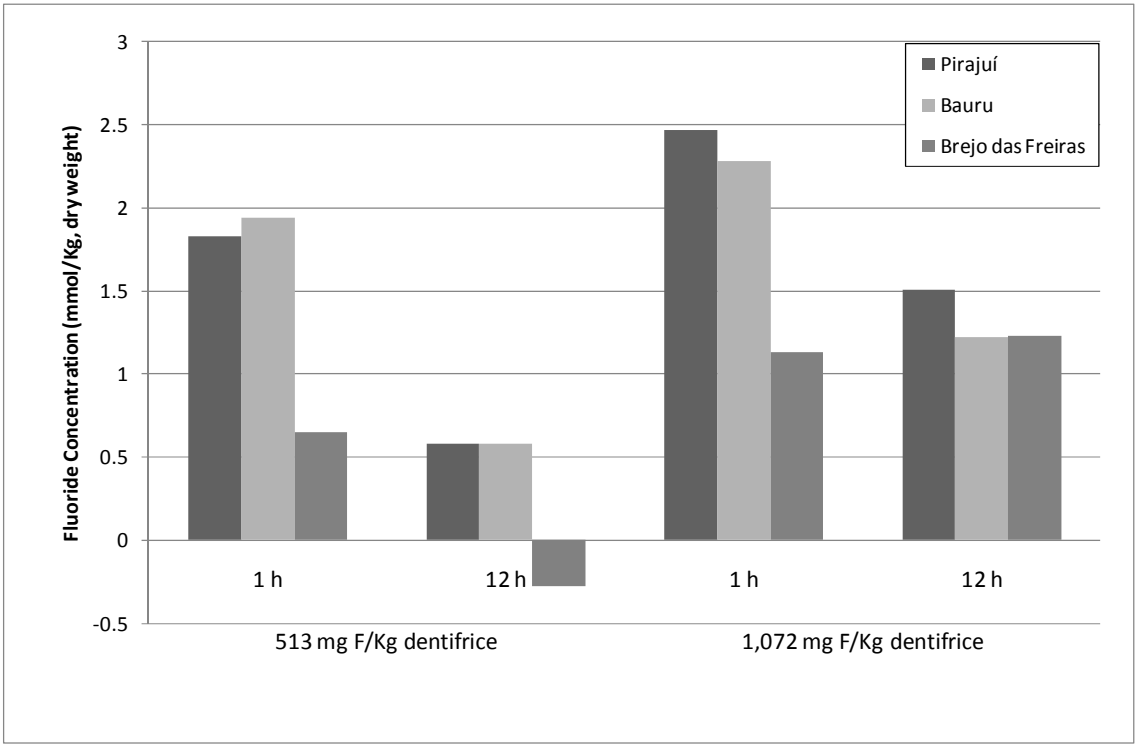

Figure 1. Mean increments in plaque fluoride levels (mmol/kg, dry weight) after brushing with 513 and 1,072 ppm F toothpastes, when compared to placebo values. The increments were calculated by subtracting the mean values obtained for the placebo dentifrices (1.02, 1.30 and 2.82 mmol F/kg for Pirajuí, Bauru and Brejo, respectively) from those found for low-fluoride and conventional dentifrices 1 and 12h after brushing.

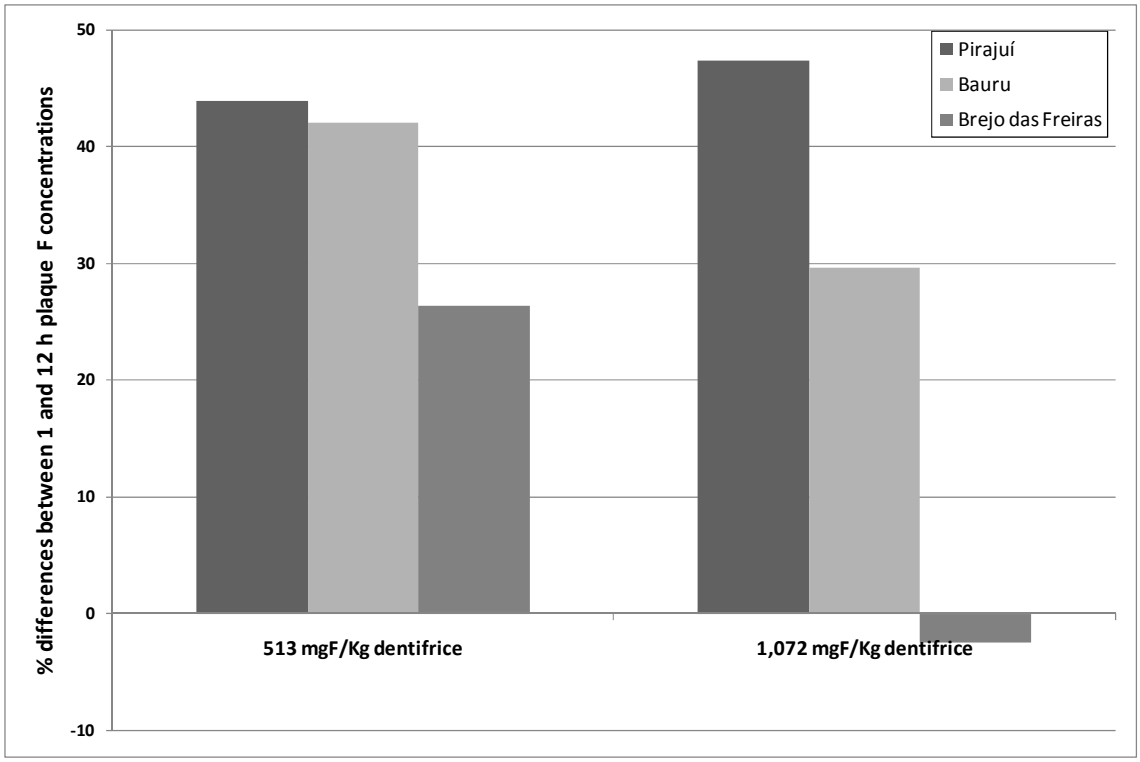

Figure 2. Differences (%) between 1 and 12h plaque fluoride concentrations after brushing with 513 and 1,072 ppm F toothpastes. The values were obtained by considering the values obtained 1h after brushing with the dentifrices as 100% and calculating the percent corresponding to the values obtained 12h after brushing.

Capítulo 2

"Distribution of F and Ca in plaque formed in presence of F dentifrices"

Distribution of F and Ca in Plaque Formed in Presence of F Dentifrices

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This study was based on a thesis to be submitted to Araçatuba Dental School, São Paulo State University (Brazil), in partial fulfillment of the requirements for the PhD degree in Pediatric Dentistry.

ABSTRACT

There is evidence that low-fluoride dentifrices (LFD) promote a proportionally higher plaque fluoride uptake when compared to conventional dentifrices (CD). We hypothesized that fluoride would be deposited predominantly in outer layers of plaque after exposure to CD, whereas it would migrate into deeper layers during exposure to LFD. This randomized, double-blind study evaluated fluoride and calcium distribution in sections of plaque biofilms generated *in situ*. Children brushed with a placebo dentifrice (PD), LFD and CD for 1 week (crossover design) and intact biofilms were collected 1 and 12 h after brushing. Fluoride and calcium were mostly restricted to the outer layers of biofilms for all dentifrices tested, and these ions were directly related throughout most of biofilm's layers. Results for CD were significantly higher than those for PD, but did not differ from those obtained for LFD. Unlike our hypothesis, the use of LFD did not promote a higher F uptake in inner layers of biofilms.

INTRODUCTION

Recent data has indicated that use of a fluoridated dentifrice (~1,000 µg F/g) resulted in virtual identical increases in plaque fluoride concentrations, which decreased at a similar rate, regardless of exposure to different local levels of fluoride in the drinking water (Pessan *et al.*, 2008). This raises questions concerning the control of fluoride uptake by dental plaque in relation to exposure to exogenous fluoride. Whitford *et al.* (2005) proposed that high levels of salivary fluoride during exposure to a conventional dentifrice (1,000-1,100 µg F/g) could lead to the deposition of CaF_2/CaF^+ compounds at the plaque/saliva interface, effectively forming some kind of a partial barrier at plaque surfaces to limit ionic fluoride diffusion into deeper regions. However, based on the solubility product for CaF_2, this effect would be attenuated with low fluoride sources because smaller amounts of insoluble calcium compounds would be formed. It would be reasonable to expect, therefore, that a low-fluoride dentifrice would lead to a lower formation of CaF_2/CaF^+ and, as a result, ionic fluoride could penetrate more easily into deeper layers of plaque biofilms.

There is evidence that the use of a low-fluoride dentifrice promotes a proportionally higher fluoride uptake by plaque when compared to a conventional dentifrice. The use of a dentifrice containing 513 µg F/g led to an increase around 1.9 mmol F/Kg in plaque fluoride concentrations 1 h after brushing, in communities with 0.04 and 0.72 ppm F in water. Considering a dose-response relationship, when a dentifrice containing 1,072 µgF/g was used the authors expected values twice higher than those obtained for the low-fluoride dentifrice (around 3.8 mmolF/Kg), but the increases observed were around 2.4 mmolF/Kg (Pessan *et al.*, personal communication). This study add to the body of evidence supporting the hypothesis described above. The localization of fluoride in plaque after using conventional and low-fluoride dentifrices, however, is unclear. In plaque biofilms exposed to a 1,000 ppm NaF solution, fluoride is restricted mainly in the outer layers (Watson *et al.*, 2005), but no direct information is available for dentifrices, which are the most widespread form of topical use of fluorides.

With regard to calcium, there is a positive relationship between fluoride and calcium in whole plaque (Whitford *et al.*, 2002, 2005). Like fluoride, calcium tends to accumulate in the outer regions of plaque after exposure to fluoridated solutions (Robinson *et al.*, 1997) but there is no information available on calcium concentrations in different layers of biofilm after the use of fluoride dentifrice.

Based on the mechanism proposed by Whitford *et al.* (2005) and on the information presented above, we have investigated the possibility that after exposure to a conventional dentifrice, fluoride would be retained predominantly in outer layers of plaque whereas it would migrate to deeper layers when a low-fluoride dentifrice was used. We also investigated the relationship between calcium and fluoride after such exposures.

MATERIALS & METHODS

Study protocol

Eleven 8-10-year-old Brazilian children from an optimally fluoridated community (Bauru, 0.72 ppm in the public water supply) participated. As inclusion criteria, children were from similar socioeconomic backgrounds, attended the same public school where samples were collected, and only drank tap water. The protocol was approved by the IRB of Bauru Dental School[1] and informed consent was obtained from parents prior to the beginning of the study[2]. Plaque biofilms were generated using the 'Leeds *In Situ* Device', constructed as described by Robinson *et al.* (1997), except that devices' rings were made by composite-resin instead of nylon[3] (for sterilization procedures, please see Watson *et al.*, 2004). Following a double-blind, crossover design, the volunteers were randomly assigned (blocking stratification) to brush with a conventional fluoride dentifrice (Crest, 1,072 mgF/kg), a low-fluoride dentifrice (Crest, 513 mgF/Kg) or a fluoride-free placebo for one week. After initial prophylaxis, one device was bonded to the buccal surface of each first upper molar by composite resin and worn for one week. During this time, children were instructed to brush twice daily, preferably avoiding the areas in which the devices had been bonded. They used approximately 0.5 g of the dentifrice supplied and rinsed their mouth with 10 mL of water after brushing[4]. After 7 days, devices were de-bonded and recovered intact 1 and approximately 12 hours after the last brushing episode. The devices with undisturbed plaque *in situ* were immediately frozen in liquid nitrogen. The entire protocol was then repeated using the dentifrices not previously used.

Sample Embedding and Sectioning

The protocol described by Watson *et al.* (2005) was followed. After overnight lyophilization (-50°C, 50 mbar vacuum), the devices were transferred to polyethylene capsules, impregnated

[1] Anexos 4 a 6
[2] Anexo 10
[3] Anexo 11
[4] Anexo 12

with methacrylate[5] (24% v/v methylmethacrylate, 75% v/v butylmethacrylate, 1% v/v benzoyl peroxide) under vacuum for 30 min, which was then polymerized by overnight incubation (60°C)[6]. Embedded plaque was serially sectioned in a plane parallel to the underlying enamel substratum (starting on the uppermost surface of the resin ring), using an ultramicrotome (Reichert, Vienna, Austria). The sectioning regime was: 5×5 µm sections, followed by 1×4 µm section and a further 5×5 µm sections. Each group of ten 5 µm sections was collected in an Eppendorf tube pooled to give a single plaque fluoride and calcium measurement. The 4 µm sections were dried on glass slides for image analysis and determination of biomass fraction. This sectioning regime was repeated throughout biofilm's depth until the enamel surface was reached[7].

Determination of Fluoride and Calcium Concentrations in Plaque Sections

Chloroform (50 µL) was used to dissolve and disperse methacrylate from plaque sections in each Eppendorf tube. Tubes were then centrifuged (20,000 rpm, 30 min) and subsequently kept with lids open, allowing the chloroform to evaporate. Fluoride was extracted by overnight immersion in water (Duckworth *et al.*, 1994) and analyzed by ion chromatography[8]. Calcium was analyzed by atomic absorption spectrometry (Kato *et al.*, 1997).

Determination of Fluoride and Calcium Concentrations in Biomass of Plaque Sections

Sections of 4 µm were stained with 0.1% aqueous toluidine blue to reveal plaque biomass. Images were captured by a video camera (JVC 3-CCD) and analyzed with Zeiss KS300 Imaging Software (Zeiss, Jena, Germany), allowing the calculation of the area occupied by stained biomass[9]. Since the section thickness was known, plaque biomass volume within the section could be calculated. From these data, we were able to estimate fluoride and calcium concentrations in the plaque biomass, by dividing the total fluoride in each plaque section by the measured biomass fraction (Watson *et al.*, 2005).

Data (log transformed) were analyzed by two- and three-factor repeated measures ANOVA, respectively for sections analyzed together (sum of all sections for each device) and

[5] Anexo 13
[6] Anexo 14
[7] Anexo 15
[8] Foi utilizado um aparelho de cromatografia iônica (Metrohm UK Ltda, Herisau, Suíça) e o *software* Metrohm IC (versão 2.3). Foi utilizada uma coluna para ânions – Hamilton PrPx 110 (dimensões da coluna = 150 × 4.1 mm, tamanho das partículas = 7 µm, , Hamilton, Estados Unidos) e o eluente empregado foi uma solução de $Na(CO_3)_2$ 2 mM / $NaCO_3$ 2,4 mM / Tiocianato de Sódio 0,05 mM, a um fluxo de 1,5 mL/minuto.
[9] Anexo 16

separately, and by Tukey's test. Linear regression analysis was used to verify the relationship between F and Ca in plaque. A significance level of 5% was selected.

RESULTS

Mean biofilm thickness was 848 (±101) µm, and ranged from 513 to 945 µm. Individual graphs for each device were plotted before performing ANOVA, revealing that both F and Ca were mainly restricted to the outer layers of plaque. Since an equal number of observations is needed to perform ANOVA, data were arranged taking the biofilm/saliva interface as the starting point for all the devices, so all the layers deeper than 513 µm (10^{th} layer) were not included in this first statistical analysis. To determine whether there were any differences among the treatments close to dental surface (where de/remineralization processes occur), second statistical analysis was then performed. This second analysis was performed with enamel surface as the starting point for the 4 inner layers. Thus, ANOVA was performed for both the 10 outer layers and for the 4 inner layers of the plaque biofilms.

For fluoride data, significant differences among treatments ($F=6.547$, $p=0.007$) and depths ($F=51.631$, $p<0.00001$) were observed, with no interactions among the factors evaluated ($p>0.05$). The use of the conventional dentifrice resulted in significantly higher fluoride levels in the biofilms than those obtained for the placebo ($p = 0.005$), regardless the time of sample collection. However, no differences were observed between placebo and low-fluoride or between low-fluoride and conventional dentifrices.

Regarding biofilm depth, fluoride concentrations were significantly higher in the 3 outer layers when compared to the 7 deeper layers (Figure 1). Considering data arranged from the enamel surface, no significant differences were observed for the 4 inner layers ($p>0.05$).

For calcium concentrations, significant differences were verified among the layers ($F=150.738$, $p<0.00001$), with significant interactions between the factors biofilm depth, treatments and time of sample collection ($F=1.807$, $p=0.027$). The four outer layers had significantly higher calcium concentrations when compared to the 6 inner layers (Figure 2). For the second ANOVA (data arranged from enamel surface), as observed for fluoride, no significant differences were observed among the layers.

A moderate correlation was found between fluoride and calcium concentrations in each section of the biofilms collected. When combining all the 660 sections together (11 volunteers, 10 outer sections, 3 treatments, 2 times after sample collection), the correlation coefficient was $r = 0.49$ ($p<0.001$). Tables 1 and 2 show the individual coefficients of

determination when isolating the factors and for the 10 outer sections of the biofilms, respectively.

Finally, data were also arranged considering each device as a whole unit (the sections were not taken into account), allowing comparisons with previous studies conducted with a similar protocol. The results from the 2-factor repeated measures ANOVA revealed significant differences between treatments only ($F=8.888$, $p=0.002$); no difference was detected between times after sample collection ($F=1.579$, $p=0.238$), nor interactions between the two factors analyzed ($F=0.797$, $p=0.464$). The values obtained for the placebo dentifrice were significantly lower when compared to the low-fluoride ($p=0.04$) and the conventional ($p=0.002$) dentifrices, but no difference was verified between the fluoridated dentifrices ($p=0.286$).

DISCUSSION

The study of fluoride distribution throughout plaque biofilms is important in elucidating the diffusion and retention of fluoride and the interactions between plaque, saliva and the underlying dental tissues (Robinson et al 1997, Kato *et al.*, 1997). To date, the most suitable method for studying the distribution of ions and other chemical agents (*e.g.* triclosan) within plaque is the Leeds *in situ* device. Due to the complex methodology involved, only 3 studies evaluating fluoride distribution in the biofilm have been reported, and all of them agreed that fluoride is mainly restricted to the outer layers of biofilm after exposure to a fluoridated solution (Robinson *et al.*, 1997, Kato *et al.*, 1997, Watson *et al.*, 2005).

Fluoride distribution in sections of biofilms after the use of a fluoridated dentifrice had still not been described, although it would be expected that a viscous vehicle might further limit penetration. This assumption was confirmed by the results of the present study. While fluoride was restricted mainly to the outer layers of biofilms (Figure 1), the concentrations of the ion were much lower than the reported in the above-mentioned studies.

Some factors may explain the differences between the results obtained for fluoridated solutions and dentifrices. First, the dentifrice's viscosity may reduce fluoride permeability into the biofilm, since biomass density increases from plaque/saliva interface inwards and the distribution of channels and voids decrease in this direction (Robinson et al., 2006). Moreover, studies with non dental-biofilms demonstrated that solute transfer through channels is more rapid than transfer through dense biomass, because of lower diffusion resistance (Zhang and Bishop, 1994). These studies together seem to explain the decrease in biofilm fluoride concentration from plaque/saliva interface towards enamel after exposure to a fluoridated

solution (Kato *et al.*, 1997, Robinson *et al.*, 1997, Watson *et al.*, 2005). An additional possibility is that fluoride interacts with the surface of plaque biomass. Surface area/plaque biomass volume decreases from salivary interface towards the enamel and mirrors fluoride distribution (Robinson and Watson 2005). Second, children washed their mouth after brushing with the dentifrice, which considerably reduces oral fluoride concentrations (Duckworth *et al.*, 1991). Third, other components in the dentifrice composition, such Sodium Lauryl Sulphate (SLS), may compete with fluoride for calcium binding sites (Whitford, personal communication), besides producing alterations in biofilm's structure. These would be induced either by removal of structural material (especially proteins) by the detergent, and by SLS binding to plaque constituents, which would cause mutual repulsion of endogenous components, thus altering the structure (Robinson *et al.*, 2006). Fourth and last, samples were collected at least 1 h after exposure to the dentifrices, so no immediate analysis on fluoride distribution was carried out.

Calculations on the fluoride diffusion coefficient in dental plaque illustrate that plaque thickness is an extremely important determinant of fluoride penetration. In plaque biofilms 100 µm and 500 µm thick, exposure to a fluoridated solution for 20 min would deliver, respectively, 91% and 0.14% of the applied fluoride concentration to the tooth surface beneath the biofilm (Stewart, 2005). In the present study, biofilms were relatively thick (~ 850 µm), simulating plaque biofilms at caries prone sites (Sissons *et al.*, 1992; Dibdin, 1993). These biofilms therefore are different from those obtained in the studies by Whitford *et al.* (2002, 2005) and Pessan *et al.* (2008). Thus, when considering biofilm's thickness along with dentifrice's viscosity, mouth washing with water after brushing and dentifrice's composition (especially SLS), it is not surprising to observe that fluoride concentrations in deeper layers of biofilms were not numerically different, regardless the dentifrice used.

It is noteworthy that when each device was considered a whole unit, the fluoride concentrations followed the same pattern observed for a previous study when whole plaque was collected *in vivo* (Pessan, personal communication). However, this pattern was different when the biofilm's layers were considered separately. This may have been due to the fact that biofilm samples in the *in vivo* study were collected from buccal and lingual surfaces, being therefore much thinner than samples collected with the devices. Based on the diffusion coefficients mentioned above (Stewart, 2005), and given that both studies followed the same treatment protocol, fluoride diffusion into *in vivo* plaque possibly occurred in a higher percentage of plaque when compared to the thick biofilms generated using the Leeds *in situ* device. Additional studies should be conducted in order to evaluate fluoride distribution in

serial sections of plaque biofilms with a thickness compatible to biofilms formed *in vivo* in order that our hypothesis could be more precisely addressed.

Plaque fluoride and calcium concentrations have been demonstrated to be positive and significantly correlated in whole plaque biofilms (Whitford *et al.*, 2002, 2005; Pessan *et al.*, 2006, 2008), suggesting that plaque fluoride and calcium concentrations are interdependent. Our data shows that fluoride and calcium distribution profiles through biofilm's depth were quite similar for all the dentifrices tested (Figures 1 and 2), supporting previous findings obtained in biofilms exposed to a fluoridated solution (Kato *et al.*, 1997). In addition, these ions are positively and significantly correlated when all data are combined, especially for samples collected 1h after brushing (Table 1), and the coefficients of determination were significant for most layers when considered separately (Table 2). These results support the view that fluoride is retained in plaque mainly through calcium-binding sites.

As fluoride concentrations in the biofilm have been reported to be inversely associated with caries increments and activity (Duckworth *et al.*, 1992; Gaugler and Bruton, 1982), the development of new formulations able to penetrate fluoride into deeper layers of dental biofilm is extremely desirable. Based on the premise that fluoride retention in plaque is mediated by calcium-binding sites, it is possible that increasing the availability of calcium in plaque would increase fluoride uptake. Rinses, dentifrices and chewing gums containing calcium in different formulations and concentrations have been used for such purposes, but with inconsistent results (Whitford *et al.*, 2005, Vogel *et al.*, 2006, 2008a, 2008b, Pessan *et al.*, 2006), so the hypothesis still remains to be proved. Other alternatives include dentifrices with a reduced pH and with a lower viscosity. The use of a dentifrice containing 550 µg F/g, at pH 4.5 led to significantly higher fluoride concentrations in whole biofilm when compared to a conventional dentifrice (1,100 µg F/g, pH 7.0) (Buzalaf *et al.*, 2009). In addition, the use of a liquid dentifrice promoted higher fluoride concentrations in whole biofilm when compared to toothpastes, at same pH and fluoride concentrations (Buzalaf *et al.*, 2009). For both dentifrice's pH and consistency, however, the distribution of fluoride within biofilm layers remains unclear.

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Table 1. Relationship between fluoride and calcium concentrations in plaque biofilm sections, according to fluoride concentration in the dentifrices and the time after brushing

Fluoride concentration in dentifrices (µg F/g)		Time after brushing		
		12h	1h	1 and 12 h
0	r	0.4665	0.4904	0.4733
513	r	0.3706	0.592	0.4685
1,072	r	0.4665	0.5378	0.542

$p<0.001$ for all correlation coefficients

Table 2. Relationship between fluoride and calcium concentrations in the 10 outer plaque sections (closer to plaque/saliva interface)

Mean distance from plaque/saliva interface (µm)*	r	p
27	0.178	0.153
81	0.289	0.019
135	0.326	0.008
189	0.371	0.002
243	0.182	0.114
297	0.29	0.018
351	0.444	0.0002
405	0.346	0.005
459	0.483	<0.0001
513	0.366	0.003

*Linear regression analysis was performed considering all data collected for each volunteer simultaneously (3 dentifrices, 2 times after brushing)

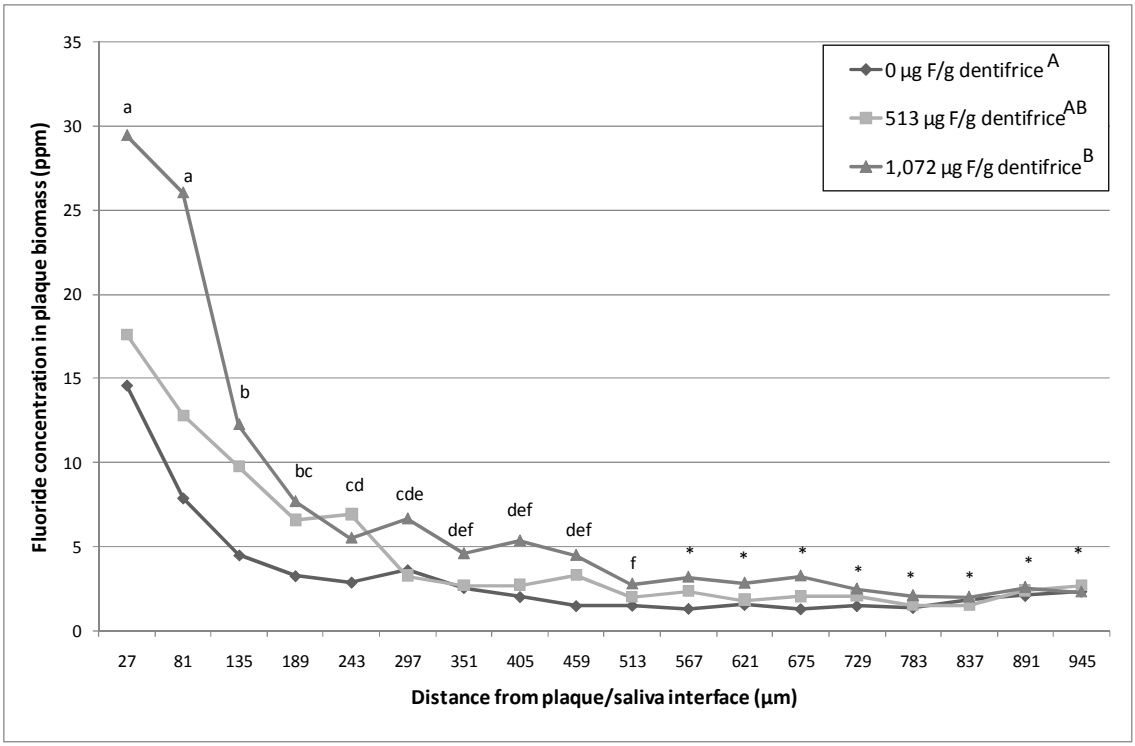

Figure 1. Mean biomass-associated fluoride concentration profiles (ppm) in serial sections of plaque biofilms generated *in situ*, after exposure to dentifrices containing 0, 513 and 1,072 µg F/g. Each point refers to the mean of the 11 volunteers (values obtained for 1 and 12 h after brushing analyzed together). Different lower case letters indicate significant differences among the sections for all dentifrices tested. Different upper case letters indicate significant differences among the dentifrices for all the sections. Comparison made by three-way, repeated measures ANOVA on the natural log of the outcome, and Tukey's post hoc test ($p<0.05$). Samples obtained from layers 594 to 972 µm (indicated by an asterisk) were not included in the statistical analysis.

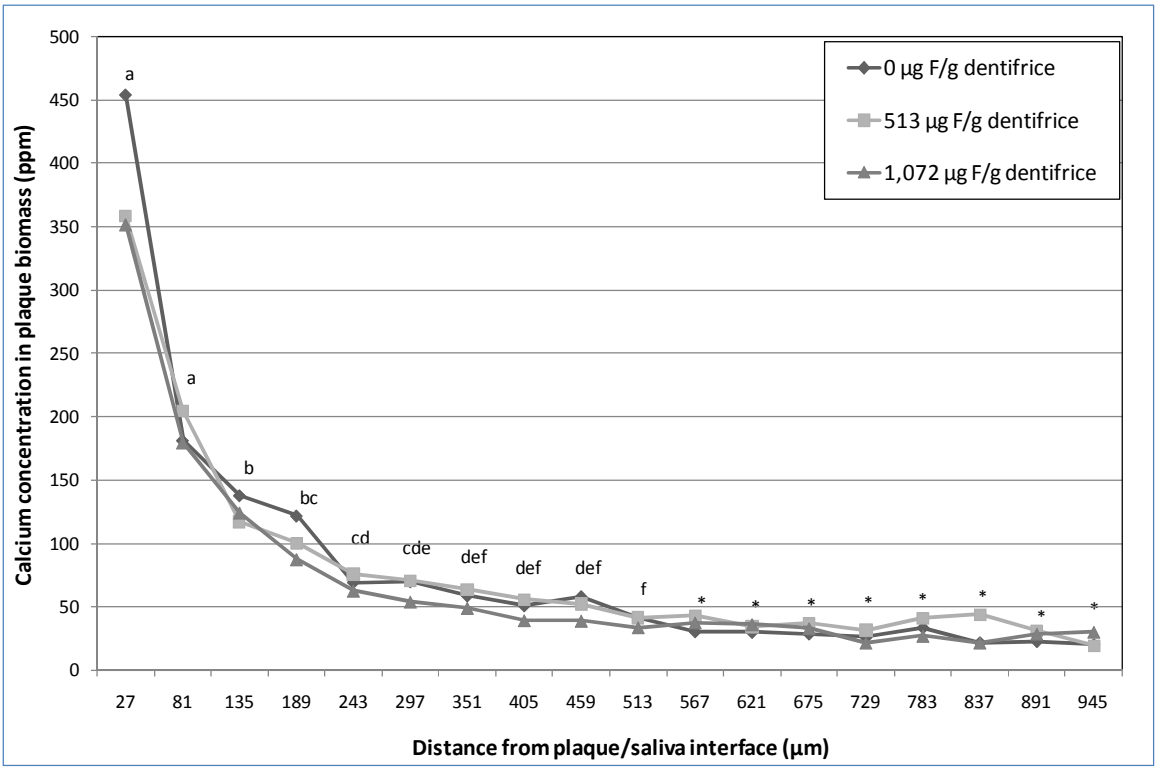

Figure 2. Mean biomass-associated calcium concentration profiles in serial sections of plaque biofilms generated *in situ*, after exposure to dentifrices containing 0, 513 and 1,072 µg F/g. Each point refers to the mean of the 11 volunteers (values obtained for 1 and 12 h after brushing analyzed together). Different lower case letters indicate significant differences among the sections for all dentifrices tested. Comparison made by three-way, repeated measures ANOVA on the natural log of the outcome, and Tukey's post hoc test ($p<0.05$). Samples obtained from layers 594 to 972 µm (indicated by an asterisk) were not included in the statistical analysis.

Capítulo 3

"Plaque and plaque fluid fluoride concentrations associated to the use of conventional and low-fluoride dentifrices"

Plaque and plaque fluid fluoride concentrations associated to the use of conventional and low-fluoride dentifrices

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Running title: Plaque fluid [F] after F dentifrices use

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Pessan JP, Italiani FM, Kobayashi CAN, Lauris JRP, Vogel GL, Whitford GM, Buzalaf MAR. Plaque and plaque fluid fluoride concentrations associated to the use of conventional and low-fluoride dentifrices. *Eur J Oral Sci.*

Abstract

There is evidence that the use of a low-fluoride dentifrice (LFD) leads to a proportionally higher plaque fluoride concentration ([F]) when compared to a conventional dentifrice (CD), but no information is available on plaque fluid [F]. This double-blind, crossover study evaluated whole plaque and plaque fluid [F], as well as whole plaque calcium concentrations ([Ca]) after brushing with a placebo dentifrice (PD), LFD and CD. Children (n=20) were randomly assigned to brush twice daily with one of the dentifrices, during 7 days. Samples were collected at 1 and 12 h after brushing. The use of the fluoridated dentifrices significantly increased plaque [F]s 1 h after brushing when compared to PD. Plaque fluid [F]s were not affected by the [F] in the dentifrice, but were significantly higher 1 h after brushing; a similar trend was observed for plaque [Ca]. Positive and significant correlations were found between plaque [F] and [Ca] under most of the conditions evaluated. The mean increase in plaque [F] observed 1 h after brushing with the CD were about 47% higher than those obtained for the LFD, supporting the assumption that the use of a LFD promotes proportionally higher increases in plaque [F] when compared to a CD.

Key words: Fluoride, Calcium, Dental Plaque, Plaque fluid, Toothpaste

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Introduction

The use of fluoridated toothpastes has been regarded as one of the most effective means for caries control worldwide (1), since it combines the mechanical removal or disruption of dental plaque with the caries-protective effect of fluoride (2). Although the contribution of fluoridated toothpastes on caries reduction is well documented in both developed and developing countries, the use of these products has also been associated to an increase in the prevalence of dental fluorosis (3), due to the ingestion of high amounts of dentifrice during toothbrushing, especially by young children (4).

Among the strategies to minimize fluoride intake from this source, the use of low-fluoride dentifrices has been recommended, although the evidences for the clinical efficacy of such formulations are still controversial when compared to dentifrices containing of 1,000-1,100 µg F/g. (5). Considering that dental caries is a plaque-dependent disease and that demineralization of tooth structure will occur only on surfaces underneath dental plaque, the amount of fluoride retained at this compartment is of paramount importance from a clinical perspective, since fluoride can be released from plaque during a cariogenic challenge, delivering the ion to the plaque fluid and, therefore, to enamel under plaque (6).

A recent study demonstrated that fluoride uptake by dental plaque at both 1 and 12 h was not significantly different after the use of dentifrices containing 513 and 1,072 µg F, suggesting that the use of the low-fluoride formulation is able to promote a proportionally higher fluoride uptake when compared to the conventional dentifrice (Pessan *et al.*, IADR Meeting, 2009). The bioavailability of fluoride in the plaque fluid under clinical conditions, however, is still now known. The only study described so far used an *in situ* protocol, with a monospecific artificial test plaque, so the results cannot be directly extrapolated to *in vivo* conditions (7).

As the plaque fluid is the site at which fluoride influences de- and remineralization processes, the aim of the present study was to assess fluoride uptake by dental plaque and plaque fluid, using the same protocol described by Pessan *et al.* (IADR Meeting, 2009). Plaque calcium concentrations were also evaluated, as it has been reported that plaque fluoride uptake is dependent on plaque calcium concentrations (8-10).

Material and Methods

Twenty 8-10-year-old Brazilian children, residents in an optimally fluoridated community (Bauru, 0.72 ppm F in the public water supply), participated. Sample size was established at 15 subjects per group in order to obtain a power of 80% (α=0.05), based on a study conducted with the same protocol (10). As inclusion criteria, children presented good oral health status, were not using medicines during the experimental protocol and drank tap water exclusively. The protocol was approved by the IRB of Bauru Dental School[1] and informed consent was obtained from children's parents[2].

The study began with a dental prophylaxis to remove all accessible plaque and calculus. The protocol of the study, described in detail in previous publications (2,8-10), followed a double-blind, crossover design, in which the volunteers were randomly assigned (blocking stratification) to brush with a conventional fluoride dentifrice (Crest, 1,072 mgF/kg, Proctor & Gamble, Cincinnati, Ohio, United States), a low-fluoride dentifrice (Crest, 513 mgF/Kg) or a fluoride-free placebo. The dentifrices' tubes were coded by a researcher not involved in the present study. They used these products for 1.0 min in the morning and at bedtime for one week and rinsed with 10 mL of water[3]. The abrasive system in the calcium-free products was hydrated silica.

[1] Anexos 4 a 6
[2] Anexo 7
[3] Anexo 8

During the sixth day, children brushed only the occlusal surfaces to allow plaque accumulation. After going to bed, they refrained from eating or drinking anything except water and did not brush their teeth until the following morning. On the following morning, plaque was collected from the right side of mouth; children then brushed the occlusal surfaces for 1 min and rinsed with 10 mL of tap water. One hour later plaque (left side) was again collected. Thus the samples were collected 1 h and approximately 12 h after the last use of the dentifrices. The entire protocol was then repeated using the dentifrices not previously used.

Plaque samples were collected from all accessible surfaces using a Hollenback carver, immediately transferred to plastic strips and then placed inside oil-filled centrifuge tubes (11). After determination of the sample weight (± 10 µg), the tubes were centrifuged for 5 min (14,000 rpm) at 4°C to separate the fluid from the plaque solids. The fluid was recovered with oil-filled capillary micropipettes, which were used to transfer the fluid to the fluoride electrode for analysis, as described below[4]. After fluid extraction, plaque solids were kept frozen until the extraction of acid-soluble whole plaque F and Ca[5]. The tip of the centrifuge tube was cut (11), and the remaining plaque was centrifuged into a 2.0 mL microcentrifuge tube containing 0.5 M HCl (500 µL/10 mg of plaque, wet weight) (6). The samples were agitated by being rotated at 30 rpm for 3 h at room temperature and subsequently centrifuged. The supernatant was collected and neutralized with 1 M NaOH (250 µL/10 mg of plaque, wet weight) and kept frozen until analyses. TISAB III (Thermo Orion, Beverly, Massachusetts, United States) was added (1:10) just before analysis. Fluoride analysis of all the samples was done on the surface of an oil-covered inverted F electrode (9409, Thermo Orion, Beverly, Massachusetts, United States) with the use of a microscope and micro-reference electrode, held in a micromanipulator, to close the circuit (11). Plaque fluid samples were diluted with TISAB III (1:10) on the surface of the F electrode by means of micropipettes (11). Calcium

[4] Anexo 17
[5] Anexo 18

concentrations were determined colorimetrically in triplicate at 650 nm (FLUOstar OPTIMA, BMG Labtech, Germany). Preparation of the samples for measuring calcium concentration was conducted with the Arsenazo III method (6,12). For the analyses of total Ca in whole plaque, the standards also contained TISAB III.

Data passed normality (Kolmogorov-Smirnov) and homogeneity (Levene) tests and were then analyzed by two-way, repeated-measures ANOVA. Tukey's test was used as the *post hoc* test for ANOVA. Pearson's correlation was used to verify the relationships between fluoride concentrations in whole plaque and plaque fluid, as well as between fluoride and calcium concentrations in whole plaque. The significance level was set at 5%.

Results

Table 1 shows mean concentrations of fluoride in whole plaque and plaque fluid, as well as calcium concentrations in whole plaque. A dose-response relationship could be observed for mean whole plaque fluoride concentrations, when considering each time of sample collection separately. The values obtained at 12 h after brushing were 0.88, 1.09 and 0.96 µmol/g and 1 h after brushing were 1.06, 1.64 and 1.96 µmol/g, respectively for the placebo, 513 µg F/g and 1,030 µg F/g dentifrices. No significant differences, however, were observed among the dentifrices ($F=2.69$, $p=0.08$). Significant differences were observed between times of sample collection ($F=17.99$, $p<0.001$), as well as for the interaction between treatments and times of sample collection ($F=3.37$, $p<0.05$).

Plaque fluid fluoride concentrations at 1 h after brushing were significantly higher than the correspondent values for 12 h after brushing ($F=19.72$, $p<0,001$). No significant differences were observed among the dentifrices ($F=0.22$; $p=0.81$). For calcium concentrations in whole plaque, a similar trend was observed, with significant differences

between times of sample collection ($F=5.98$, $p<0.05$) and no differences among the dentifrices ($F=0.06$; $p=0.94$).

Plaque fluoride and calcium concentrations were positively and significantly correlated for most of the situations tested. The highest coefficients of determination were observed for the low-fluoride ($r=0.67$, $p=0,001$) and conventional dentifrices ($r=0.72$, $p<0.0001$), both at 1 h after brushing (Table 2). When all values were analyzed together, the coefficient of determination was $r=0.53$ ($p<0.0001$). No relationship between plaque fluid and whole plaque fluoride concentrations could be observed for any of the situations tested individually. When all data were considered together, the coefficient of determination was $r=0.27$ ($p=0.77$).

Discussion

The study of the mineral composition of dental plaque is of great importance from a clinical perspective, since plaque fluoride concentrations are inversely related to caries incidence and activity (13,14), and directly related to the clinical efficacy of topical fluoride formulations (15). The results of the present study demonstrated that, in an area with optimally fluoridated water, plaque fluoride concentrations 1 h after brushing with a conventional or a low-fluoride dentifrice are not significantly differently from each other. In addition, the values obtained for the conventional dentifrice were only 47% higher than those obtained for the placebo dentifrice 1 h after brushing, confirming previous findings that the use of the low-fluoride formulation leads to a proportionally higher plaque fluoride uptake when compared to a conventional dentifrice (Pessan *et al.*, IADR Meeting, 2009). It is important to point out that the data reported by Pessan *et al.* (IADR Meeting, 2009) were obtained using a different method of fluoride analysis ($HClO_4$ extraction, dry plaque) than the

one employed in the present study (HCl extraction, wet plaque under oil), which gives additional credibility to the data obtained.

The above-mentioned findings seem to be supported by a recent randomized clinical trial, which concluded that the anticaries effect of a low-fluoride dentifrice was similar to the conventional dentifrice when used by caries-inactive, but not caries-active children, residents in an area with low-fluoride concentrations in the drinking water (16). Considering the similarity of the values obtained 1 h after brushing with both toothpastes in the present study (conducted in an optimally fluoridated area), it could suggested that the differences in the clinical performances of conventional and low-fluoride formulations seem to be smaller than currently believed (5). This would occur especially in an optimally fluoridated area, where intraoral fluoride reservoirs can be replenished at a higher frequency from water besides the use of the fluoridated dentifrice. It must be emphasized, however, that the present protocol did not evaluate fluoride concentrations immediately after toothbrushing. It is possible that the higher fluoride levels in plaque shortly after exposure to a conventional toothpaste may have a great impact on the remineralization of early carious lesions in caries-active individuals, when compared to the use of a low-fluoride formulation. Time-course studies could bring additional information on fluoride uptake and clearance from both the solid and fluid phases of plaque after the use of conventional and low-fluoride dentifrices. Therefore, as concluded by Lima *et al.* (16), caries activity may be taken into account when recommending a low-fluoride dentifrice.

The significant increases in plaque fluoride concentrations after the use of the fluoridated dentifrices were not reflected in the fluid phase of plaque (Table 1), what could be explained by several factors, mainly the lag time between the brushing episode and the first plaque sample collection, along with considerations on oral fluoride clearance. However, the present results are not in agreement with those described in a recent *in situ* study, which

demonstrated a clear dose-response relationship in plaque fluid fluoride concentrations 30 min after exposure to conventional and low-fluoride dentifrices, as well as 45 min after plaque exposure to a sucrose solution (75 min after toothbrushing) (7). Although the study by Tenuta *et al.* (7) provided additional information about plaque fluid fluoride concentrations after the use of a low-fluoride dentifrice, some differences between their protocol and that used in the present study must be addressed. In the study by Tenuta *et al.* (7), enamel blocks were pre-treated with slurries of dentifrices for 5 minutes, the volunteers did not spit out the dentifrice's foam after brushing, a monospecific artificial test plaque was used and the contact area with saliva and plaque was too small when compared to plaque present in smooth surfaces (as in the present study), what may have substantially reduced salivary clearance in their study. In contrast, in the present report there was no pre-treatment of enamel surfaces, children spat the foam out after brushing and rinsed their mouths with water, a natural plaque was assessed and the contact area between plaque and saliva was substantially higher. Therefore, the limitations of the protocol used by Tenuta *et al.* (2009) do not allow a direct extrapolation of the results to *in vivo* conditions.

It was noteworthy that plaque fluid fluoride concentrations observed at 1 h after brushing were significantly higher than those obtained at 12 h, regardless the dentifrice used. Interestingly, a similar pattern was observed for whole plaque calcium concentrations (Table 1), what has already been observed in previous studies using the same research protocol (2,10). Considering that all the dentifrices were silica-based (calcium-free), the only reasonable explanation seems to be the influence of Sodium Lauryl Sulphate (SLS) on plaque structure (Whitford *et al.*, AADR Meeting, 2008). It was demonstrated that SLS is able to produce alterations in plaque structure induced either by removal of structural material (especially proteins), and by SLS binding to plaque constituents, which would cause mutual repulsion of endogenous components (17). It is reasonable to assume that the expansion in

plaque structure would increase the mobility of ions in plaque and, therefore, in its fluid phase, reason by which increases in both plaque fluid fluoride concentrations and in whole plaque calcium concentration were seen 1 h after brushing even for the placebo dentifrice. Such hypothesis deserves to be further investigated, since it may have significant implications on the fluoride uptake by dental plaque after the use of dentifrices.

Plaque fluoride concentrations were positively and significantly correlated to plaque calcium concentrations under most of the conditions of the study, confirming previous findings that plaque fluoride concentrations are dependent on plaque calcium concentrations (2,8-10). As a completely plaque-free situation is impossible in a clinical situation, the amount of fluoride retained in plaque may be determinant on the fate of the enamel beneath plaque, since fluoride can be released from this compartment during a cariogenic challenge, influencing on the remineralization process. Therefore, strategies to increase plaque fluoride concentrations should be based on methods to increase the fraction of plaque Ca capable of reacting with and retaining F (2).

This is the first clinical study that evaluated plaque fluid fluoride concentrations after the use of conventional and low-fluoride dentifrices. Although the results for the whole plaque confirm previous findings that the use of a low-fluoride dentifrice promotes a proportionally higher plaque fluoride uptake 1 h after brushing when compared to a conventional dentifrice, such increases are not reflected in the plaque fluid. The reason for this is possibly the establishment of an equilibrium between the fluoride in the oral fluids (saliva and plaque fluid), which are highly influenced by the salivary clearance. SLS also seems to influence plaque and plaque fluid fluoride concentrations, which deserves to be further investigated.

Acknowledgements

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Table 1. Fluoride concentrations in whole plaque and plaque fluid and calcium concentrations in whole plaque 1 and 12 h after the use of placebo, 513 µg F/g and 1.072 µg F/g dentifrices

	Fluoride concentration in the dentifrices					
	Placebo		513 µg F/g		1.072 µg F/g	
Time after brushing	12 h ^{A}	1 h ^{B}	12 h ^{A}	1 h ^{B}	12 h ^{A}	1 h ^{B}
Plaque fluid [F] (µM)	17.3 (±1.9)	24.4 (±2.6)	19.5 (±1.8)	23.2 (±2.0)	17.2 (±1.8)	22.5 (±2.2)
Whole plaque [F] (µmol/g, wet weight)	0.88^{a} (±0,10)	1.06^{ab} (±0,10)	1,09^{ab} (±0,13)	1,64^{bc} (±0,14)	0,96^{a} (±1,16)	1.96^{c} (±0,42)
Whole plaque [Ca] (µmol/g, wet weight)	36.7 (±7.7)	41.7 (±5.1)	34.4 (±4.7)	46.9 (±4.0)	33.2 (±4.8)	45.0 (±7.5)

Mean ± se (n=20). Different upper case superscript letters indicate significant differences between times of sample collection for fluoride concentrations in plaque fluid and in whole plaque. Different lower case superscript letters in the same row indicate significant differences among whole plaque samples ($p<0.05$). Comparison made by two-way, repeated measures ANOVA and Tukey´s post hoc test.

Table 2. Relationship between fluoride and calcium concentrations in whole plaque samples, collected at 1 and 12 h after the use of placebo, 513 µg F/g and 1.072 µg F/g dentifrices

	Fluoride concentration in the dentifrices						
	Placebo		513 µg F/g		1.072 µg F/g		All dentifrices
Time after brushing	12 h	1 h	12 h	1 h	12 h	1 h	1 and 12 h
r	0.62	0.07	0.35	0.67	0.48	0.72	0.53
p	0,004	0,780	0,129	0,001	0,032	<0,0001	<0.0001

Coefficients of determination calculated by Pearson's correlation (n=20)

Anexos

ANEXO 1: Instruções aos autores – Journal of Dental Research

MANUSCRIPTS

Prepare manuscript, tables, legends, and footnotes as double-spaced text (a minimum of 6 mm between lines) formatted for 8-1/2 x 11-inch paper. Top, bottom, and side margins should be one inch, with no indented paragraphs. Figures and tables should not exceed 8-1/2 x 11 inches. Both Macintosh (Framemaker, MacWrite, Word, WordPerfect, Works WP, or WriteNow) and IBM PC (DCA-RFT, FrameMaker, MultiMate, Office Writer, Text, Word for Windows, WordPerfect, WordStar, Works WP, or XYWrite) files will be accepted. Manuscripts should be "clean", i.e., free of tabs and codes. Bold and italic type should appear exactly as they will appear on the printed page. Italicize items that will appear in italics; this will include the genus and species of an organism, g (for gravitational force), Latin words and abbreviations (for example, e.g., i.e., in vitro, in vivo, et al.), and journal names in the References section. Tabs should be used to separate columns within tables. **Do not use elaborate table formatting.**

Use a standard font such as Times New Roman or Arial to avoid misrepresentation of your data on different computers that do not have the unusual or foreign language fonts.

Title and Section Headings

Bold type should be used for the title on page 1. Use upper- and lower-case letters. First-level headings, which include ABSTRACT, INTRODUCTION, MATERIALS & METHODS, RESULTS, DISCUSSION, ACKNOWLEDGMENTS, and REFERENCES should be bold type, all upper-case letters, as shown. Second-level subheads should be bold type, upper- and lower-case. Third-level subheads should be bold type, upper- and lower-case, with a paragraph indent. Any lower-ranked subheads should be italicized, and in upper- and lower-case. Please type no more than 10 characters per inch. Authors are reminded to include their complete mailing addresses, telephone, FAX, and e-mail addresses, as available. Copies of "in press" and "submitted" manuscripts that will provide essential information for the referees should also be enclosed.

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Authors should remember that they are writing to communicate to often-uninformed readers. Here are a few suggestions: Show a clear chronological progression and logic to the development of your ideas throughout the manuscript and within paragraphs and sentences. Speak to the reader in a direct and straightforward voice. Tell the reader your purpose, then provide background, data, and conclusions. You will make your point most effectively by illustrating with a well-chosen example, rather than providing an encyclopedic discourse. In each paragraph and sentence, stick to the subject. For example, if the subject is "biophysical properties", don't write sentences in that paragraph that change the subject to the names of cited contributors. Each sentence should contain only one thought. Write short and simple sentences. Choose the best word so that you say what you mean. To make your information accessible to the widest possible audience, avoid jargon, acronyms, and needless words. Before submission, contributors must review their manuscripts with (i) computer grammar and spelling tools/filters and (ii) a colleague who is expert in English language grammar and syntax. Manuscripts may be returned without review or rejected on the basis of poor English or accepted standards of style. Check to ensure that all listed references, figures, and tables are cited in the text and that all cited references, figures, and tables are presented in appropriate sections. The Editor reserves the right to make changes to improve the clarity of the text. All such changes will be subject to contributors' approval before publication.

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All revisions must be accompanied by a cover letter signed by all authors to the Editor. The letter must (i) detail on a point-by-point basis the contributors' disposition of each of the referees' comments, and (ii) certify that all contributors approve of the revised content and that the manuscript complies with stipulations 'i' through 'iv' in "General Policy". Responses to separate reviewers should be on separate pages. Also include a copy of the revision with all changes highlighted.

RANDOMIZED CLINICAL TRIALS

Effective January 2004, manuscripts reporting a randomized clinical trial should follow the CONSORT guidelines as published in the Annals of Internal Medicine (Ann Int Med 134:657-662, 2001). Click here to download the checklist. This completed checklist file should be uploaded as Supplemental Material.

The *Journal* encourages authors to register their clinical trials in a public trials registry, and we ask authors of manuscripts describing such studies to submit the name of the registry and the study registration number prior to publication. The International Committee of Medical Journal Editors plans to consider clinical trials for

publication only if they have been registered (see *N Engl J Med* 2004;351:1250-1 - http://content.nejm.org/cgi/content/full/351/12/1250). The following registries meet these requirements: http://prsinfo.clinicaltrials.gov and http://controlled-trials.com/isrctn/submission/.

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Prior to submission, the *Journal* asks that novel gene sequences be deposited in a public database (GenBank, http://www.ncbi.nlm.nih.gov/Genbank/submit.html, EMBL, http://www.ebi.ac.uk/embl/Submission/index.html, or DDBJ http://www.ddbj.nig.ac.jp/sub-e.html), and the accession number provided to the JDR. Manuscript submissions including microarray data should (a) include the information recommended by the MIAME http://www.mged.org/Workgroups/MIAME/miame.html guidelines in their submission, and/or (b) identify the submission details for the experimental details to one of the publicly available databases (ArrayExpress http://www.ebi.ac.uk/arrayexpress/ or GEO http://www.ncbi.nlm.nih.gov/geo/).

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The components of a manuscript should be: 1) title page, 2) abstract, 3) introduction,4) materials and methods, 5) results, 6) discussion, 7) acknowledgments, 8) references, 9) tables, and 10) figure legends. The complete manuscript should be arranged in that order. Number all pages consecutively in the top right-hand corner, including the title page. Label figures clearly. Each figure label must indicate the number corresponding to the citation in the text, an arrow indicating the top, and contributors' abbreviated names.

1) Title Page (page 1)

Type in bold type with only the first letters of the main words capitalized. The title should be brief (not to exceed 60 characters) and illustrative of the key finding. Also type the contributors' initials and last names in upper- and lower-case letters. Use superscript numbers to relate contributors to different departments or institutions, or to indicate a change in address. For the corresponding author who will receive reprint requests, provide the full postal (including ZIP or Postal Code) and e-mail addresses, telephone and FAX numbers, as available. If the corresponding author is not the first author, indicate by a number superscript, and use the phrase "corresponding author", and that individual's e-mail address. The following information must be included on the cover page: 1) a short title (running head) of up to 45 characters; 2) three to five key words; 3) the number of words in the abstract; 4) the number of words in the abstract and the text (excluding tables, figure legends, acknowledgments, and cited references); 5) the number of tables and figures; and 6) the number of cited references. If applicable, include source footnotes on page 1 to indicate prior preliminary publication. For example, state that the work was "Based on a thesis submitted to the graduate faculty, Azimuth University, in partial fulfillment of the requirements for the PhD degree" or that a preliminary report was presented at, or published in... Report all sources of funding in a later section, "Acknowledgments".

2) Abstract (page 2)

A self-standing summary of the text, this section should not exceed one typed page (about 150 words). Concisely describe the (i) background and rationale, (ii) hypothesis or study objective, (iii) design and key methods, (iv) essential results, and (v) conclusions. Avoid abbreviations. The abstract will be re-published separately by information retrieval services.

3) Introduction (page 3)

Briefly and clearly describe the background and rationale for the stated hypothesis to be tested or objective to be studied. Sufficient detail must be provided to permit the interdisciplinary reader to evaluate the results without review of earlier publications. Describe and cite only the most relevant earlier studies; avoid presentation of an exhaustive review of the field. Do not include a summary of the results presented in the manuscript.

4) Materials & Methods

To provide sufficient technical information so that the experiments can be repeated, the (i) experimental or study design, (ii) specific procedures, and (iii) type of statistical analysis must be described clearly and carefully. Use section subheadings in a logical order to title each category or method. Previously published methods should be named (e.g., "ultrasonic treatment" rather than mention of the cited contributors' names) and cited. New methods must be described completely. Present the data that validate the new method. A method used for only part of one experiment may be described briefly in the "Results" section, table footnote, or figure legend. Present descriptive information about large numbers of experimental reagents, microbes, test materials, primer sequences, in tabular form with a brief explanation in the text. Proprietary names and sources of supply of all commercial products must be given in parentheses in the text (name and model of product, company, city, and state or country). Report generic names and terms wherever possible. For protocols involving the use of human subjects or

specimens, indicate succinctly that subjects' rights have been protected by an appropriate institutional review board and informed consent was granted. When laboratory animals are used, indicate the level of institutional review and assurance that the protocol ensures humane practices.

5) Results

This section serves only to introduce data in the (i) text, (ii) tables, and (iii) figures and to call attention to their significant parts. Report results concisely, using tables and figures to present important differences or similarities that cannot otherwise be presented or summarized in the text. The rationale and design of experiments should be made clear in the previous sections of the manuscript. Reserve subjective comments, interpretation, or reference to the previous literature for the "DISCUSSION". Number tables and figures in the order in which they are described and cited in the text. All tabular data should identify and report (i) either standard deviation values or standard errors of the means, (ii) the number of replicate determinations or human or animal subjects, and (iii) probability values and name(s) of statistical test(s) for reported differences. Restrict presentation of photo- and electron micrographs to those essential to the results. If essential to the results, color can be published at the discretion of the Editor. (The cost for color in reprints, however, must be borne by the author. For cost estimates, contact the Central Office at 703-548-0066 , or FAX 703-548-1883, e-mail publications@iadr.org.)

6) Discussion

Explain and interpret the results with a scientifically critical view of the previously published work in the field. Highlight the advances made by the new data. Indicate the limitations of the findings. State the conclusions of the report, and explain why they are merited by the data. This is the only proper section for subjective comments.

7) Acknowledgments

Recognize individuals who provided assistance to the project. Report all sources of grant and other support for the project or study, including funds received from contributors' institutions and commercial sources, and do not refer to a study being only partially funded by the cited sources. Consultancies and funds paid directly to investigators must also be listed, with statements such as "This investigation was supported in part by USPHS Research Grant DE-0000-00 from the National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892".

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Examples of correct forms of references are listed below. They are single-spaced here for illustration but should be double-spaced in the manuscript.

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(List all authors, but if the number exceeds six, give six authors' names followed by *et al*.) West DJ, Snavely DB, Zajac BA, Brown GW, Babb CJ (1990). Development and persistence of antibody in a high-risk institutionalized population given plasma-derived hepatitis B vaccine. *Vaccine* 8:111-114.

2. Organization as author

The Royal Marsden Hospital Bone-Marrow Transplantation Team (1977). Failure of syngeneic bone-marrow graft without preconditioning in post-hepatitis marrow aplasia. *Lancet* 2:742-744.

3. No author given Coffee drinking and cancer of the pancreas (editorial) (1981). *Br Med J* 283:628.

4. Article in a foreign language

Massone L, Borghi S, Pestarino A, Picini R, Gambini C (1987). Localisations palmaires purpuriques de la dermatite herpetiforme. *Ann Dermatol Venereol* 114:1545-1547.

5. Volume with supplement

Magni F, Rossoni G, Berti F (1988). BN-52021 protects guinea pig from heart anaphylaxis. *Pharmacol Res Commun* 20(Suppl 5):75-78.

6. Issue with supplement

Gardos G, Cole JO, Haskell D, Marby D, Paine SS, Moore P (1988). The natural history of tardive dyskinesia. *J Clin Psychopharmacol* 8(4 Suppl):31S-37S.

7. Volume with part

Hanly C (1988). Metaphysics and innateness: a psychoanalytic perspective. *Int J Psychoanal* 69(Pt 3):389-399.

8. Issue with part

Edwards L, Meyskens F, Levine N (1989). Effect of oral isotretinoin on dysplastic nevi. *J Am Acad Dermatol* 20(2 Pt 1):257-260.

9. Issue with no volume

Baumeister AA (1978). Origins and control of stereotyped movements. *Monogr Am Assoc Ment Defic* (3):353-384.

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Danoek K (1982). Skiing in and through the history of medicine. *Nord Medicinhist Arsb*:86-100.

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Ronne Y (1989). Ansvarsfall. Blodtransfusion till fel patient. *Vardfacket* 13:XXVI-XXVII.

12. Type of article indicated as needed

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9) Tables and **10) Figures** (maximum, four total)

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Type one table per page. In the order of mention in the text, number each table consecutively with Arabic numerals in the heading. In the heading, follow the table number with a brief descriptive title, generally highlighting the key result. Design tables to highlight key results and comparisons. Make every effort to make the presentation of data clear, simple, and uncluttered. As column headings, use accurate descriptors instead of symbols, acronyms, and abbreviations. To avoid overlong titles and cumbersome tables, use explanatory footnotes whenever possible. In the table or title, indicate the order of footnotes with superscript a,b,c,d,e,f, ... If needed in footnotes, cite the short form of references in parentheses. In tabular columns and the text, decimals less than unity must have the decimal point preceded by a zero. To ensure that the presentation is clear, report only the number of significant digits appropriate to the sensitivity and discrimination of the measure and the differences to be illustrated. Column headings should be simple and clear so that tables will be understandable without consultation of the text. Generally, column headings identify dependent variables, while independent variables are identified by row descriptors on the left. Tables will usually be printed either 3-1/4 or 7 inches wide.

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The **Random House Dictionary of the English Language** (Unabridged) will be used as the authority for spelling of non-medical terms. Where two plural forms are provided, the American English form will be used. For anatomical nomenclature, **Nomina Anatomica** (5th ed.) and **Dorland's Illustrated Dictionary** will be considered authoritative.

NOMENCLATURE

Authors should refer to the *International System of Units* (SI), D.T. Goldman and R.J. Bell, Eds., NBS Special Publication 330 (1981). This booklet is available from the US Dept. of Commerce, National Institute of Standards and Technology, Washington, DC 20234. Use of correct symbols includes m for milli-, for micro-, and L for liter (as in mL, L, etc.). Express grams as g, hours as hr, seconds as sec, and centrifugal force as g (e.g., 10,000 g). Use nm rather than Angstroms. Concentrations should be expressed as mol/L or mmol/L, etc. Insert leading zeros in all numbers less than 1.0 in the text, tables, and figures.

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The complete names of individual teeth must be given in full in the text of articles (e.g., "permanent upper right first premolar"). In Tables, these names may be abbreviated by Viohl's Two-digit System. As approved by the International Standards Organization, the first digit indicates the quadrant and the second digit the type of tooth within the quadrant. Starting at the upper right side and rotating clockwise, quadrants are assigned the digits 1 to 4 for the permanent and 5 to 8 for the deciduous teeth; within the same quadrant, teeth from the midline backward are assigned the digits 1 to 8 (deciduous teeth, 1 to 5). For example, the permanent lower right first molar is designated '46' and the deciduous upper left canine, '63'.

ANEXO 2: Comprovante de submissão ao periódico Journal of Dental Research

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Current Revision #	1
Other Version	08-0482
Submission Date	2009-03-05
Current Stage	Searching for Reviewers
Title	Effect of Regular and Low-Fluoride Dentifrices on Plaque Fluoride
Running Title	Effect of fluoride dentifrices on plaque F
Manuscript Type	Research Report
Special Section	N/A
Category	Clinical
Manuscript Comment	Number of words in the abstract: 150 Number of words in the abstract and the text: 2,542 Number of tables and figures: 4 Number of cited references: 15
Corresponding Author	Marília Buzalaf (Bauru Dental School/University of São Paulo)
Contributing Authors	Juliano Pessan , Karina Alves , Irene Ramires , Marcel Taga , Fabio Sampaio , Gary Whitford
Abstract	Previous studies indicate that the use of low-fluoride dentifrices (LF) could lead to proportionally higher plaque fluoride levels when compared to conventional dentifrices (C). This double-blind, randomized, crossover study determined the effects of placebo dentifrice, LF and C on plaque fluoride concentrations ([F]) in children living in communities with 0.04, 0.72 and 3.36 ppmF in the drinking water. Children used the toothpastes twice daily, for 1 week. Samples were collected 1 and 12h after the last use of dentifrices and were analyzed for fluoride and calcium. Results were analyzed by 3-way ANOVA and linear regression analysis. Similar increases were found 1h after brushing with LF (ca.1.9 mmolF/kg) and C (ca.2.4 mmolF/kg) in the 0.04 and 0.72 ppmF communities. Despite the increases were less pronounced in the 3.36 ppmF community, our results indicate that the use of a LF promotes a proportionally higher increase in plaque [F] when compared to C.
Associate Editor	Assigned
Key Words	Fluoride, Calcium, Dental Plaque, Fluoride dentifrice
Author Disclosure	• Material and Methods Section properly reports animal or specimens protocols - no.

ANEXO 3: Instruções aos autores – European Journal of Oral Sciences

European Journal of Oral Sciences
Official publication of NOF - the Scandinavian Division of the International Association for Dental Research
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Current Volume: 117 / 2009
ISI Journal Citation Reports® Ranking: 2007: 13/51 (Dentistry, Oral Surgery & Medicine)
Impact Factor: 2.071

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Review and Focus Articles should include a Title page, an Abstract page and a Reference list as regular Original Research Articles. Although a Review article (particularly following a systematic review) may adhere to the format of the Original Research Article, Review and Focus articles need not contain Materials and Methods, Results or Discussion sections, and may instead employ other headings as relevant for the topic addressed.

5.4. References
Number references consecutively in the order in which they are first mentioned in the text. Identify references in texts, tables, and legends by Arabic numerals (within parenthesis). Check to ensure that all listed references are cited in the text. If an author's name is mentioned in the text, small capital letters should be used. Non-refereed material and, if possible, non-English publications should be avoided. Congress abstracts, unaccepted papers, unpublished observations, and personal communications may not be placed in the Reference list. References to 'unpublished findings' and to 'personal communication' (provided explicit consent has been given by the sources) may be inserted in parentheses in the text. Unpublished articles should be referred to only

if proof can be given that they are accepted for publication. Copies of such articles may be requested for evaluation of the manuscript submitted.
Authors are urged to study the examples of correct reference formats given below. For abbreviations of journals, consult the *List of the Journals Indexed* in Index *Medicus*. List all authors; do not use *et al.* in the Reference list. Avoid issue numbers in journal articles. Give first and last page of references in full.

Journals
Standard journal article:
JERNVALL J, THESLEFF I. Reiterative signaling and patterning during mammalian tooth morphogenesis. *Mech Dev* 2000; **92:** 19-29.
Article in supplement or special issue:
MUNDY GR. Cellular and molecular regulation of bone turnover. *Bone* 1999; **24** (Suppl): 35S-38S.
Corporate (collective) author:
WHO COLLABORATING CENTRE FOR ORAL PRECANCEROUS LESIONS. Definition of leukoplakia and related lesions: an aid to studies on oral precancer. *Oral Surg Oral Med Oral Pathol* 1978; **46:** 518-539.
Unpublished article:
FLEISCHMANNOVA J, MATALOVA E, TUCKER AS, SHARPE PT. Mouse models of tooth abnormalities. *Eur J Oral Sci* 2008; **116:** in press.

Books and other monographs:
Personal author(s):
PINDBORG JJ. *Atlas of diseases of the oral mucosa*, 5th ed. Copenhagen: Munksgaard, 1992; 50-66.
Chapter in book:
RUCH JV. Tooth morphogenesis and differentiation. In: LINDE A, ed. *Dentin and dentinogenesis*. Vol. I. Boca Raton, FL: CRC Press, 1984; 47-79.
No author given:
International statistical classification of diseases and related health problems. 10th revision, 2nd Ed, Vol 1. Geneva: World Health Organization, 2005; 550-564.

5.5. Tables, Figures and Figure Legends

Tables: Tables should be numbered consecutively with Arabic numerals. Each table should include a compulsory, concise explanatory title and an explanatory legend. A table should be organized with due regard for the proportion of the printed column/page. Specifically, tables which are too wide must be avoided, as these have to be printed vertically.

Figure Legends: Include Figure Legends after the reference section of the Main Text.

Figures: Articles will not be published unless the Figures fulfill journal quality criteria in terms of scientific information, general style, legibility of text and numbers, as well as electronic format and resolution. Double documentation of data in text, tables or figures is not acceptable. Always consider whether data might be better given in the text or in a table. All graphs, drawings, and photographs are considered Figures and should be numbered in sequence with Arabic numerals. Each figure should have a legend (number and list legends after the reference section of the main text). Figures should be planned to fit the proportions of the printed page or one column's width. Authors are encouraged to arrange micrographs into multipanel montages. Magnifications should be indicated by scale bars. Text on photographs should be in capitals. To provide appreciable diagrams, lettering should be kept to a minimum and be large enough to sustain reduction in printing. Unnecessary colour, grey shades and 3D character should be avoided in graphs.

Preparation of Electronic Figures for Publication: Print publication requires high quality images to prevent the final product being blurred or unclear. Submit EPS (line art), TIFF (halftone/photographs) or PDF (line art/photographs) files only. MS PowerPoint and images embedded in MS Word files are not acceptable for reproduction. Halftone images in colour or greyscale (TIFF only) should have a resolution of 300 dpi in relation to the reproduction size (see below), while line drawings should have a resolution of 600 to 1200 dpi. EPS files should be saved with fonts embedded (and with a TIFF preview if possible). For scanned images, the scanning resolution (at final image size) should be as follows: line art: >600 dpi; half-tones (including gel photographs): >300 dpi; figures containing both halftone and line images: >600 dpi.
Further information can be obtained at Blackwell Publishing's guidelines for figures: www.blackwellpublishing.com/bauthor/illustration.asp
You can check here whether your electronic artwork fulfill criteria before submitting it:

www.blackwellpublishing.com/bauthor/eachecklist.asp

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Colour Charges: Any figure submitted in colour will be reviewed and processed with the understanding that it will be published in colour. Colour illustrations are printed free of charge if their use contributes significantly to the scientific value of the article.

6. AFTER ACCEPTANCE

Upon acceptance of a paper for publication, the manuscript will be forwarded to the Production Editor, who is responsible for the production of the journal. Please note that an article can not be printed unless all electronic files are up to standards, including specifically resolution and file formats of illustrations. Likewise, a completed Exclusive License Form must have been received by the Journal prior to publishing. The Production Editor may contact authors in these respects.

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The corresponding author will receive an e-mail alert containing a link to a web site. A working e-mail address, which is checked daily, must therefore be provided for the corresponding author. The proof can be downloaded as a PDF (portable document format) file from this site.
A software capable of reading PDF files, such as Acrobat Reader, will be required. Acrobat Reader can be downloaded (free of charge) from Adobe. This will enable the file to be opened, read on screen, and printed out in order for any corrections to be added. Further instructions will be sent with the proof. Hardcopy proofs will be posted if no e-mail address is available; in your absence, please arrange for a colleague to access your e-mail to retrieve the proofs. Proofs must be returned to the Production Editor within three days of receipt. As changes to proofs are costly, we ask that you only correct typesetting errors. Excessive changes made by the author in the proofs, excluding typesetting errors, will be charged separately. Other than in exceptional circumstances, all hardcopy figures are retained by the publisher. Please note that authors are responsible for all statements made in their published work, including changes made by the Copy Editor.

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Paper offprints may be ordered online. Please click on the following link, fill in the necessary details and ensure that you type information in all of the required fields: http://offprint.cosprinters.com/cos/bw/main.jsp?SITE_ID=bw&FID=USER_HOME_PG
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Note to NIH Grantees: Pursuant to NIH mandate, Wiley-Blackwell will post the accepted version of contributions authored by NIH grant-holders to PubMed Central upon acceptance. This accepted version will be made publicly available 12 months after publication. For further information, see www.wiley.com/go/nihmandate

ANEXO 4: Parecer do Comitê de Ética em Pesquisa

Universidade de São Paulo
Faculdade de Odontologia de Bauru
Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – CEP 17012-901 – C.P. 73
PABX (0XX14)3235-8000 – FAX (0XX14)3223-4679

Comitê de Ética em Pesquisa (3235-8356)

Processo nº 116/2005

Bauru, 02 de dezembro de 2005.

Senhora Professora,

Informamos que após o envio da documentação solicitada referente ao projeto de pesquisa encaminhado a este Comitê de Ética em Pesquisa **"Incorporação de flúor no biofilme dentário e fluído do biofilme após o uso de dentifrícios convencionais e com concentração reduzida de flúor em comunidades com diferentes níveis de flúor na água de abastecimento"** de autoria de Juliano Pelim Pessan, sob sua orientação foi novamente analisado e considerado **APROVADO** por este Comitê em reunião realizada no dia **30 de novembro de 2005**.

Informamos ainda, que após o envio do trabalho concluído, este Comitê enviará o parecer final, que será utilizado para publicação do trabalho.

Atenciosamente,

Prof. Dr. José Henrique Rubo
Coordenador

Ilmª Srª **Profª Drª Marília Afonso Rabelo Buzalaf**
DD. Docente do Departamento de Ciências Biológicas

ANEXO 5: Parecer do Comitê de Ética em Pesquisa após parecer da Comissão Nacional de Ética em Pesquisa (CONEP)

Universidade de São Paulo
Faculdade de Odontologia de Bauru
Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – Brasil – CEP 17012-901 – C.P. 73
PABX (0XX14)3235-8000 – FAX (0XX14)3223-4679

Comitê de Ética em Pesquisa (3235-8356)

Of.nº CEP/09 2006/FOB

Reg. CONEP nº 12757

REF: PROC. CEP-FOB/USP Nº 116/2005

Bauru, 10 de agosto de 2006.

Senhora Professora,

Encaminhamos a V.Sª PARECER Nº 807/2006 emitido pela Comissão Nacional de Ética em Pesquisa - CONEP, referente ao projeto de pesquisa **"Incorporação de flúor no biofilme dentário e fluído do biofilme após o uso de dentifrícios convencionais e com concentração reduzida de flúor em comunidades com diferentes níveis de flúor na água de abastecimento"** de autoria de Juliano Pelim Pessan, a ser desenvolvido sob sua orientação, tendo sido considerado **APROVADO**.

Solicitamos que ao término do trabalho V.Sª envie um relatório para parecer final o qual será utilizado para publicações científicas.

Atenciosamente,

Prof. Dr. José Henrique Rubo
Coordenador

Ilmª Srª Profª Drª **Marília Afonso Rabelo Buzalaf**
DD. Docente do Departamento de Ciências Biológicas

ANEXO 6: Parecer da Comissão Nacional de Ética em Pesquisa (CONEP)

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MINISTÉRIO DA SAÚDE
Conselho Nacional de Saúde
Comissão Nacional de Ética em Pesquisa - CONEP

PARECER Nº 807/2006

Registro CONEP: 12757 (Este nº deve ser citado nas correspondências referentes a este projeto)

Registro CEP: 116/2005 **Processo nº 25000.023152/2006-13**
Projeto de Pesquisa: *"Incorporação de flúor no biofilme dentário e fluido do biofilme após o uso de dentifrícios convencionais e com concentração reduzida de flúor em comunidades com diferentes níveis de flúor na água de abastecimento".*
Pesquisador Responsável: Dr. Juliano Pelim Pessan
Instituição: Faculdade de Odontologia de Bauru-USP
Área Temática Especial: Cooperação estrangeira
Patrocinador: CNPq

Ao se proceder à análise do projeto de pesquisa em questão, em resposta ao Parecer CONEP nº 389/2006, cabem as seguintes considerações:

a) Foi apresentada a atividade de cada colaborador internacional do estudo. As cartas de aceite para a realização dos exames estão adicionadas ao protocolo.
b) O orçamento detalhado foi apresentado e informado os materiais de doação.
c) A nova versão do Termo de Consentimento Livre e Esclarecido contém as solicitações enunciadas no parecer anterior.
d) As informações enviadas atendem aos aspectos fundamentais da Res. CNS 196/96; o projeto foi aprovado pelo Comitê de Ética em Pesquisa da instituição supracitada.

Diante do exposto, a Comissão Nacional de Ética em Pesquisa – CONEP, de acordo com as atribuições definidas na Res. CNS 196/96, manifesta-se pela aprovação do projeto de pesquisa proposto.

Situação: Protocolo aprovado.

Brasília, 25 de julho de 2006.

WILLIAM SAAD HOSSNE
Coordenador da CONEP/CNS/MS

ANEXO 7: Carta de informação ao sujeito da pesquisa e Termo de consentimento livre e esclarecido (Capítulos 1 e 3)

Universidade de São Paulo
Faculdade de Odontologia de Bauru
Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – CEP 17012-901 – C.P. 73
PABX (0XX14)235-8000 – FAX (0XX14)223-4679

1ª Via

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (1ª e 2ª ETAPAS)

O uso de pastas de dente com flúor é muito importante para prevenir a cárie dentária. Além de ajudar na limpeza dos dentes com a escova, a pasta serve para depositar flúor nos dentes e até mesmo na placa bacteriana que não foi removida durante a escovação. Vimos, por meio desta, convidar o seu filho para participar de uma pesquisa onde será avaliada a quantidade de flúor presente na placa bacteriana do mesmo, após o uso de 3 pastas de dente com diferentes concentrações de flúor. No início do estudo, será feita uma limpeza dos dentes do seu filho, para remover placa bacteriana e tártaro. Então seu filho receberá um tubo de pasta de dente e uma escova nova e deverá escovar seus dentes duas (2) vezes por dia, durante uma semana (7 dias). No sexto dia da semana, seu filho receberá uma instrução especial para escovar seus dentes, conforme será explicado pessoalmente. Também no sexto dia, após a última escovação (antes de dormir), seu filho não poderá comer ou beber mais nada, mas poderá beber água à vontade. No sétimo dia da semana, serão coletadas amostras de placa bacteriana e saliva às 8 e às 9 horas da manhã. Após isto, será oferecido um lanche para o seu filho e será feita nova limpeza dos dentes. Então estes procedimentos serão repetidos mais 2 vezes, com as duas pastas de dente que ainda não foram usadas, completando 3 semanas de estudo. É importante lembrar que a coleta de placa bacteriana e de saliva não traz desconforto e não fazem mal algum para o seu filho. Além disso, as pastas de dente utilizadas neste trabalho são produtos comerciais, já testados e aprovados pelos órgãos competentes (ANVISA e Ministério da Saúde), não havendo risco de nenhuma natureza para o seu filho. Também ressaltamos que o acúmulo de placa sobre os dentes por 24 horas (6º dia do estudo) não produz nenhum dano aos dentes e à gengiva. Quanto aos benefícios oferecidos ao seu filho, no início do estudo será feito um exame clínico do mesmo, em relação às condições bucais e o resultado deste exame será prontamente informado a você. Além disso, no final do estudo, serão dadas instruções sobre higiene bucal, por escrito e verbalmente. Todas as pastas de dente, além de outros gastos envolvidos no trabalho, ficarão por conta dos pesquisadores. Os pesquisadores estarão sempre à disposição para o esclarecimento de quaisquer dúvidas sobre o trabalho de pesquisa ***(Telefone: 3235-8246, Laboratório de Bioquímica da FOB/USP, falar com Juliano Pessan ou Professora Marília Buzalaf).*** Além disso, você poderá entrar em contato com o Comitê de Ética da FOB/USP a qualquer momento, para eventuais críticas ou reclamações ***(Telefone 3235-8356).***

Pelo presente instrumento que atende às exigências legais, o Sr. (a) _____, portador da cédula de identidade _____, após leitura minuciosa do TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO, devidamente explicado pelos profissionais em seus mínimos detalhes, ciente dos serviços e procedimentos aos quais seu filho será submetido, não restando quaisquer dúvidas a respeito do lido e explicado, firma seu **CONSENTIMENTO LIVRE E ESCLARECIDO** concordando em permitir a participação de seu filho _____ nesta pesquisa.

Fica claro que os pais do sujeito da pesquisa ou seu representante legal podem, a qualquer momento, retirar seu **CONSENTIMENTO LIVRE E ESCLARECIDO** e o sujeito da pesquisa poderá deixar de participar deste trabalho. Além disso, todas as informações prestadas serão consideradas confidenciais e guardadas por força de sigilo profissional (Art. 9º do Código de Ética Odontológica).

Por estarem de acordo assinam o presente termo.

Bauru-SP, _____ de _____ de _____.

_____ Assinatura do Pai ou Responsável

_____ Juliano Pelim Pessan
Pesquisador responsável

ANEXO 8: Ficha de instruções aos pais e aos voluntários (Capítulos 1 e 3)

Instruções para os pais e para as crianças

Escovar os dentes 2 vezes por dia (manhã e noite), da seguinte maneira:

- Usar somente a pasta e a escova fornecida, utilizando um **tanto de pasta que cubra todas as cerdas da escova** (ver figura).

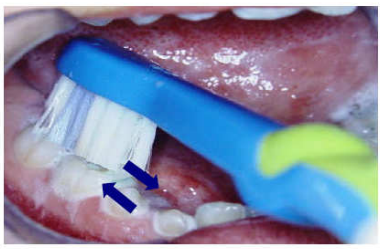
- Após a escovação, enxaguar a boca com água da torneira, **durante 5 segundos**. Utilizar o **copinho de plástico** para medir o tanto de água para lavar a boca (**observar a marcação preta, ver figura**).

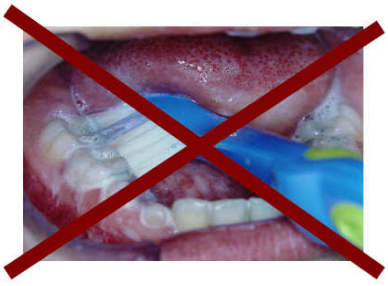
- Colar o selo verde no lugar de cada escovação. Caso esqueça de escovar por algum motivo, não colar o selo e marcar no campo correspondente o motivo.

	Terça-feira	Quarta-feira	Quinta-feira	Sexta-feira	Sábado	Domingo
Escovação da Manhã	XXXXXXXXX					
Escovação da Noite						

Na **SEGUNDA-FEIRA**, somente escovar a parte de cima dos dentes do fundo, (manhã e noite)

	Segunda-feira
Escovação da Manhã	
Escovação da Noite	





Depois do jantar, na **SEGUNDA-FEIRA**, não comer ou beber mais nada. Só pode beber água.

Na **TERÇA-FEIRA**, a criança deve ir à Escola às **7:30** horas da manhã. **NÃO TOMAR CAFÉ DA MANHÃ E NÃO ESCOVAR OS DENTES.**

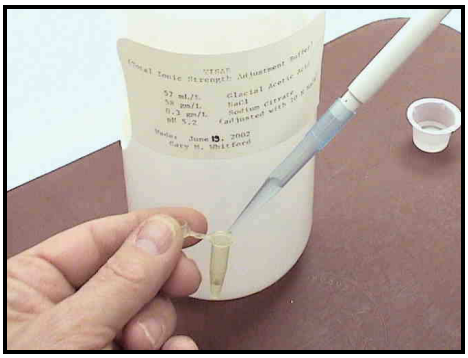
Levar para a escola: Escova e pasta de dente, copinho do bochecho, água da torneira e esta ficha. Após as coletas de saliva e placa, as crianças receberão um lanche.

No caso de qualquer dúvida, entrar em contato nos telefones:

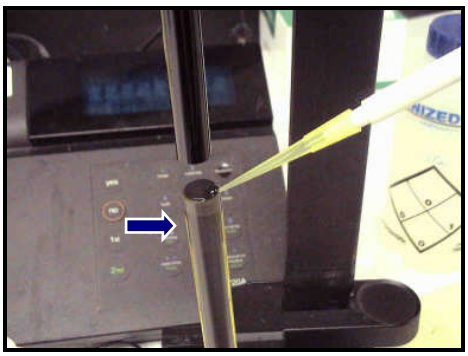
(14) 3235-8246 – Faculdade de Odontologia de Bauru (USP)
(14) 9715-7800 – Juliano (14) 3227-3615 – Juliano residência

ANEXO 9: Análise de flúor nas amostras de biofilme após extração por ácido perclórico

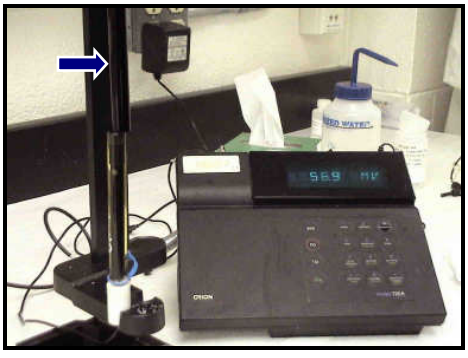
a. Adição de 75 µL de $HClO_4$ 0,5 M ao tubo contendo biofilme dentário, após secagem e pesagem



b. Adição de 375 µL de TISAB I, para tamponamento da amostra



c. Colocação da solução contendo flúor sobre o eletrodo de referência (seta), o qual permanece em posição invertida



d. Posicionamento do eletrodo íon-específico (seta) em contato com a solução, previamente colocada sobre o eletrodo de referência

ANEXO 10: Carta de informação ao sujeito da pesquisa e Termo de consentimento livre e esclarecido (Capítulo 2)

Universidade de São Paulo
Faculdade de Odontologia de Bauru
Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – CEP 17012-901 – C.P. 73
PABX (0XX14)235-8000 – FAX (0XX14)223-4679

1ª Via

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (3ª ETAPA)

O uso de pastas de dente com flúor é muito importante para prevenir a cárie dentária. Além de ajudar na limpeza dos dentes com a escova, a pasta serve para depositar flúor nos dentes e até mesmo na placa bacteriana que não foi removida durante a escovação. Vimos, por meio desta, convidar o seu filho para participar de uma pesquisa onde será avaliada a quantidade de flúor presente na placa bacteriana do mesmo, após o uso de 3 pastas de dente com diferentes concentrações de flúor. No início do estudo, será feita uma limpeza dos dentes do seu filho, para remover placa bacteriana e tártaro. Em seguida, será utilizado um pequeno aparelho circular, semelhante a uma lentilha (medindo apenas 6 mm de diâmetro). O seu filho utilizará 2 aparelhos, os quais serão colados em 2 dentes do fundo (molares) com cola especial para esta finalidade. Então seu filho receberá um tubo de pasta de dente e uma escova nova e deverá escovar seus dentes duas (2) vezes por dia, durante uma semana (7 dias), tomando o cuidado para não escovar em cima do aparelho. No sexto dia da semana, após a última escovação (antes de dormir), seu filho não poderá comer ou beber mais nada, mas poderá beber água à vontade. No sétimo dia da semana, os aparelhos serão retirados dos dentes às 8 e às 9 horas da manhã. Após isto, será oferecido um lanche para o seu filho e será feita nova limpeza dos dentes. Então estes procedimentos serão repetidos mais 2 vezes, com as duas pastas de dente que ainda não foram usadas, completando 3 semanas de estudo. É importante lembrar que a colagem destes aparelhos e o seu uso por 7 dias não trazem desconforto e não fazem mal algum para os dentes ou para a gengiva do seu filho. Além disso, as pastas de dente utilizadas neste trabalho são produtos comerciais, já testados e aprovados pelos órgãos competentes (ANVISA e Ministério da Saúde), não havendo risco de nenhuma natureza para o seu filho. Quanto aos benefícios oferecidos ao seu filho, no início do estudo será feito um exame clínico do mesmo, em relação às condições bucais e o resultado deste exame será prontamente informado a você. Além disso, no final do estudo, serão dadas instruções sobre higiene bucal, por escrito e verbalmente. Todas as pastas de dente, além de outros gastos envolvidos no trabalho, ficarão por conta dos pesquisadores. Os pesquisadores estarão sempre à disposição para o esclarecimento de quaisquer dúvidas sobre o trabalho de pesquisa ***(Telefone: 3235-8246, Laboratório de Bioquímica da FOB/USP, falar com Juliano Pessan ou Professora Marília Buzalaf).*** Além disso, os pais dos sujeitos da pesquisa poderão entrar em contato com o Comitê de Ética da FOB/USP a qualquer momento, para eventuais críticas ou reclamações ***(Telefone 3235-8356).***

Pelo presente instrumento que atende às exigências legais, o Sr. (a) _____, portador da cédula de identidade _____, após leitura minuciosa do TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO, devidamente explicado pelos profissionais em seus mínimos detalhes, ciente dos serviços e procedimentos aos quais seu filho será submetido, não restando quaisquer dúvidas a respeito do lido e explicado, firma seu **CONSENTIMENTO LIVRE E ESCLARECIDO** concordando em permitir a participação de seu filho _____ nesta pesquisa.

Fica claro que os pais do sujeito da pesquisa ou seu representante legal podem, a qualquer momento, retirar seu **CONSENTIMENTO LIVRE E ESCLARECIDO** e o sujeito da pesquisa poderá deixar de participar deste trabalho. Além disso, todas as informações prestadas serão consideradas confidenciais e guardadas por força de sigilo profissional (Art. 9º do Código de Ética Odontológica).

Por estarem de acordo assinam o presente termo.

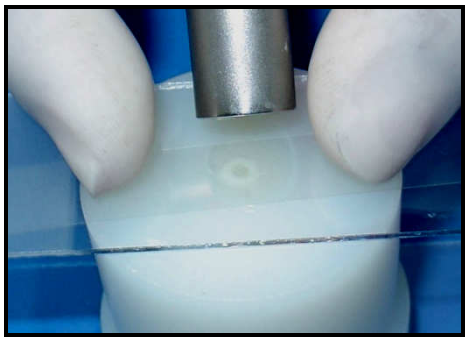
Bauru-SP, _____ de _____ de _____.

_____ Assinatura do Pai ou Responsável

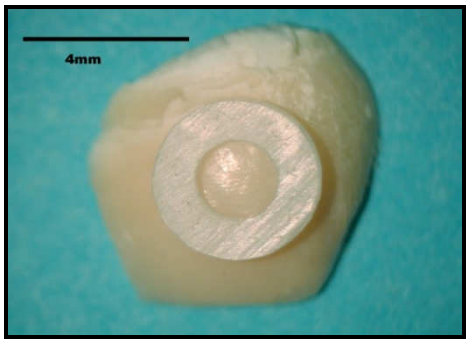
_____ Juliano Pelim Pessan
Pesquisador responsável

ANEXO 11: Preparo dos "Dispositivos *in situ* de Leeds"

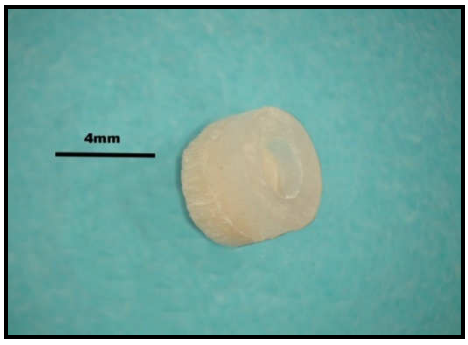
a. Corte da face vestibular de um molar, utilizando disco diamantado em uma cortadeira automática (Isomet, Buehler).



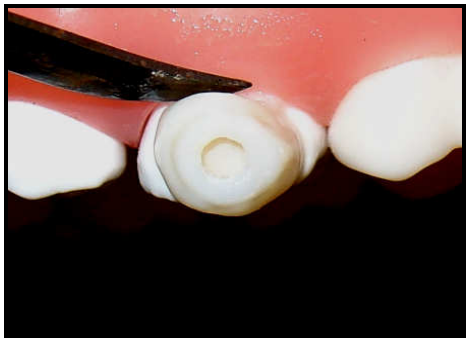
b. Confecção do anel de resina composta. A resina é condensada no interior de uma matriz de teflon, comprimida com lâmina de vidro (protegida por tira de poliéster) e fotopolimerizada.



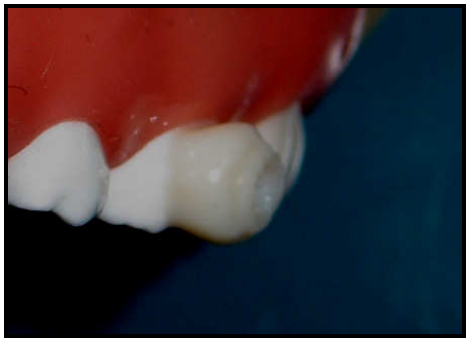
c. Colagem do anel de resina sobre o disco de esmalte. O anel de resina é pressionado firmemente contra o esmalte para se evitar penetração do cianoacrilato no interior do anel. (Cortesia de H. Abudiak)



d. Vista lateral do dispositivo *in situ* de Leeds após remoção dos excessos. O disco de esmalte tem tamanho ligeiramente maior que o anel de resina composta. (Cortesia de H. Abudiak)



e. Previamente à polimerização da resina composta, faz-se um nicho de retenção nas áreas cervical e oclusal, de forma a facilitar a apreensão do dispositivo pelo removedor de braquete (durante a remoção do mesmo).



f. Vista lateral do dispositivo (notar nichos nas regiões cervical e oclusal. O excesso de resina ao redor do dispositivo confere maior proteção quanto aos esforços mastigatórios e promove lisura de superfície, resultando em maior conforto para o voluntário.

ANEXO 12: Ficha de instruções aos pais e aos voluntários (Capítulo 2)

Instruções para os pais e para as crianças

Escovar os dentes 2 vezes por dia (manhã e noite), da seguinte maneira:

- Usar somente a pasta e a escova fornecida, utilizando um **tanto de pasta que cubra todas as cerdas da escova** (ver figura).

- Após a escovação, enxaguar a boca com água da torneira, **durante 5 segundos**. Utilizar o **copinho de plástico** para medir o tanto de água para lavar a boca (**observar a marcação preta, ver figura**).

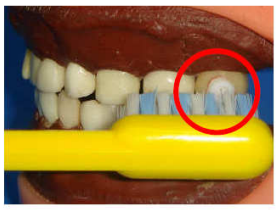
- Colar o selo verde no lugar de cada escovação. Caso esqueça de escovar por algum motivo, não colar o selo e marcar no campo correspondente o motivo.

	Quarta-feira	Quinta-feira	Sexta-feira	Sábado	Domingo	Segunda-feira	Terça-feira
Escovação da Manhã	XXXXXXXXX						
Escovação da Noite							

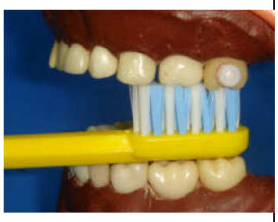
IMPORTANTE:

- Não escovar em cima do aparelho (ver figuras);
- Não colocar o dedo no aparelho;
- Não comer alimentos duros;
- Não comer balas duras, nem chiclete;
- Fazer um bochecho forte com água após comer qualquer alimento.

ERRADO



CERTO



Depois do jantar, na **TERÇA-FEIRA**, não comer ou beber mais nada. Só pode beber água.

Na **QUARTA-FEIRA**, a criança deve ir à Escola às **7:30** horas da manhã. **NÃO TOMAR CAFÉ DA MANHÃ E NÃO ESCOVAR OS DENTES.**

Levar para a escola: Escova e pasta de dente, copinho do bochecho, água da torneira e esta ficha. Após as coletas de saliva e placa, as crianças receberão um lanche.

No caso de qualquer dúvida, entrar em contato nos telefones:

(14) 3235-8246 – Faculdade de Odontologia de Bauru (USP)
(14) 9715-7800 – Juliano (14) 9707-2139 – Flávia (14) 8141-3113 – Karina

ANEXO 13: Preparo da mistura de metacrilato para embebição dos dispositivos

A mistura de metacrilato usada para embebição dos dispositivos é composta de metil metacrilato (24,8% volume/volume), butil metacrilato (74,2%, volume/volume) e peróxido de benzoíla (1% peso/volume).

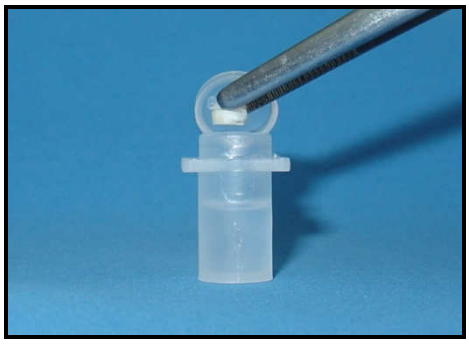
A mistura foi preparada conforme descrito abaixo:

i. A hidroquinona, presente no metil e butil metacrilato como estabilizador, foi removida por extração com água. Para tanto, volumes iguais de metacrilato e NaOH 2,5 M foram colocados em um funil de separação, sendo a mistura agitada vigorosamente por 2 minutos e, sem seguida, mantidos em repouso, permitindo a separação de fases. A fração inferior (aquosa) foi descartada e o sobrenadante (metacrilato), retido. Para ambos metil e butil metacrilato, o procedimento de extração foi realizado 2 vezes.

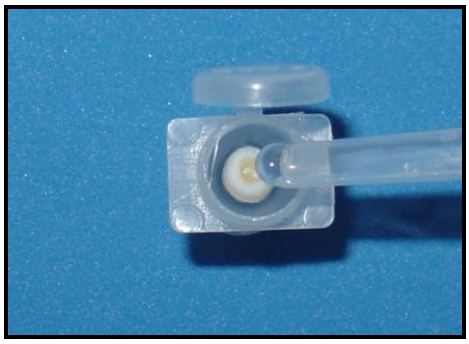
ii. A água foi removida do metil e butil metacrilato, adicionando-se excesso de $CaCl_2$ (anidro) ao metacrilato. Com base no produto de solubilidade do $CaCl_2$ anidro a 0 ºC (36,7%), considerou-se como excesso 40 g de $CaCl_2$ para cada 100 mL de metacrilato. A suspensão foi agitada vigorosamente por 2 minutos. Após armazenamento *overnight* a -20 ºC, as suspensões foram filtradas 2 vezes (Filtro nº1, Whatman, 0,2 µm de poro). Os filtrados resultantes foram decantados em frascos previamente secos. Estes reagentes foram armazenados a -20 ºC, até requeridos para uso.

iii. A mistura de metacrilato foi completada pela adição de peróxido de benzoíla. Após atingirem temperatura ambiente, o butil metacrilato e o metil metacrilato foram misturados em uma proporção de 75:24. A esta mistura, foi adicionado peróxido de benzoíla, a uma concentração de 1% (peso/volume). Esta mistura final foi aquecida até se tornar transparente, armazenada em frascos de vidro de 2 mL e então armazenada a -20 ºC até requerida para uso.

ANEXO 14: Embebição dos "Dispositivos *in situ* de Leeds" em metacrilato

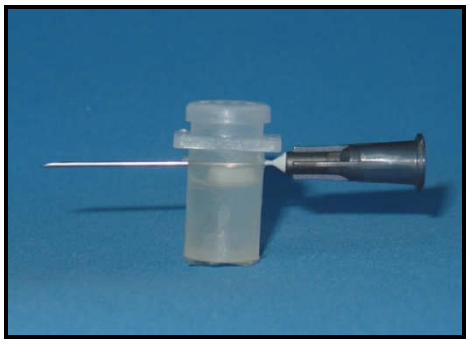


a. Colocação do dispositivo *in situ* de Leeds após liofilização sobre o bloco de metacrilato pré-polimerizado

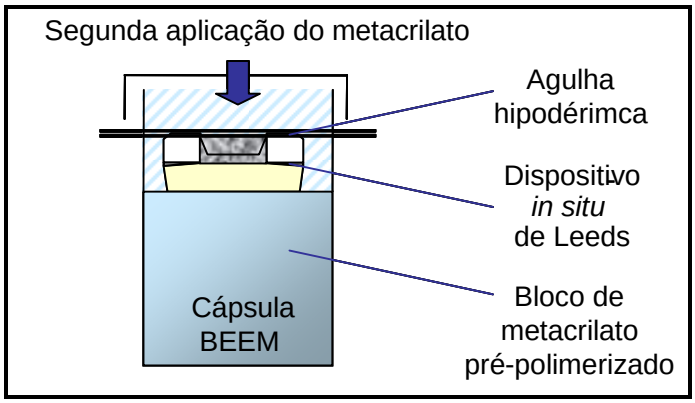


b. Colocação de mistura de metacrilato sobre o biofilme liofilizado. Deve-se encher até próximo da borda, devido à contração de polimerização .

c. Colagem do anel de resina sobre o disco de esmalte. O anel de resina é pressionado firmemente contra o esmalte para se evitar penetração do cianoacrilato no interior do anel. (Cortesia de H. Abudiak)



d. Vista lateral do dispositivo *in situ* de Leeds após remoção dos excessos. O disco de esmalte tem tamanho ligeiramente maior que o anel de resina composta. (Cortesia de H. Abudiak)

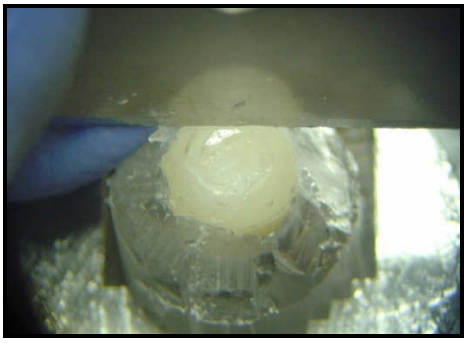


e. Ilustração do procedimento revisado para embebição da amostra, adotado para se prevenir movimentação da amostra de placa no interior do dispositivo. Modificado de Watson *et al.* (2005).

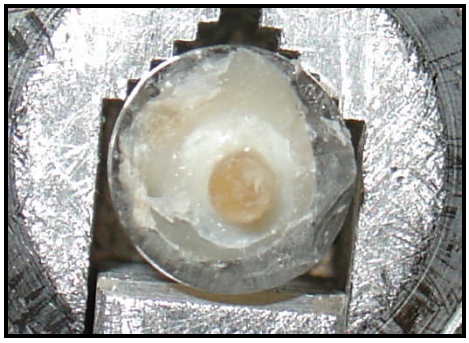
ANEXO 15: Remoção do metacrilato e secção das amostras de biofilme

a. Remoção do bloco de metacrilato, no qual está embebida a amostra de biofilme.

b. Remoção inicial dos excessos de metacrilato com lâmina metálica, realizada até as proximidades da amostra embebida.

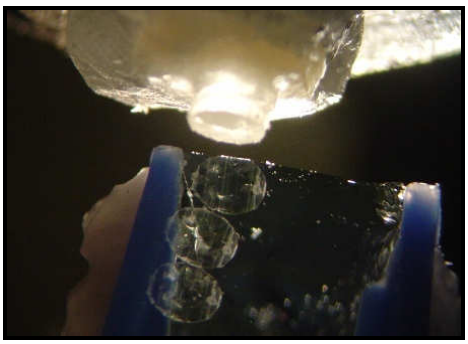


c. Remoção do anel de resina composta, utilizando-se a mesma lâmina metálica empregada para remoção dos excessos de metacrilato.



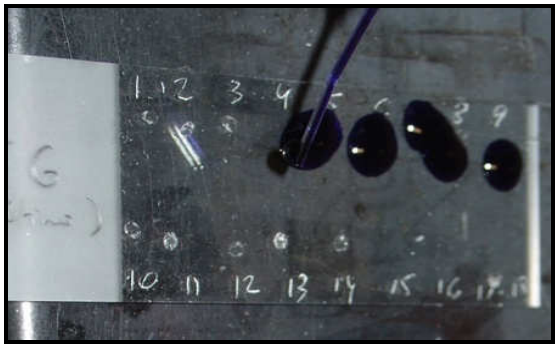
d. Aspecto final (vista superior) da amostra de biofilme, embebida em metacrilato, após remoção do anel de resina composta.

e. Aspecto final (vista lateral) da amostra de biofilme, embebida em metacrilato, após remoção do anel de resina composta.

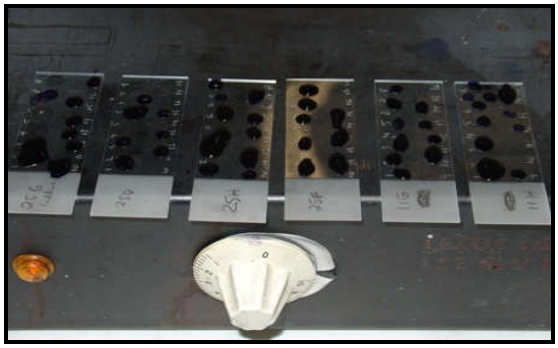


f. Secção de amostra de biofilme (vista aproximada).

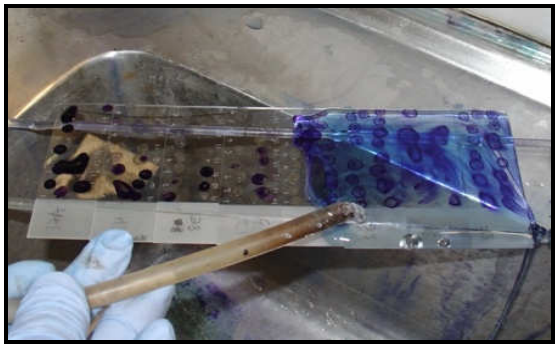
ANEXO 16: Obtenção das imagens das secções de biofilme após pigmentação por Azul de Toluidina



a. Aplicação de solução de Azul de Toluidina sobre as secções do biofilme.

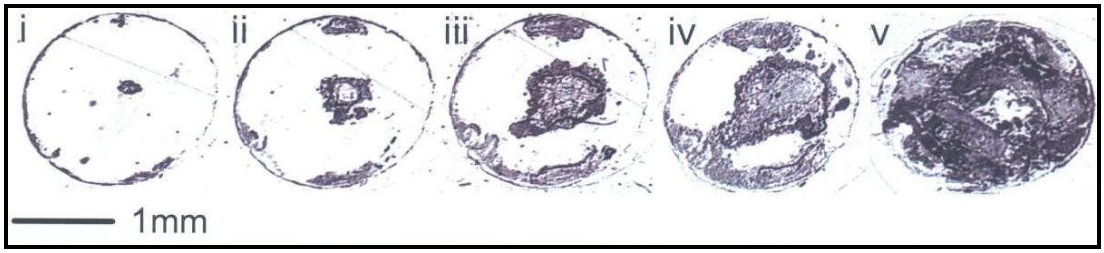


b. Aquecimento das lâminas de vidro contendo as secções de biofilme, permitindo a adequada pigmentação das secções e a subsequente evaporação do reagente de cor.



c. Lavagem das lâminas de vidro em água corrente. Toma-se o cuidado para não se utilizar um jato muito forte, o qual pode remover as secções de seu lugar original.

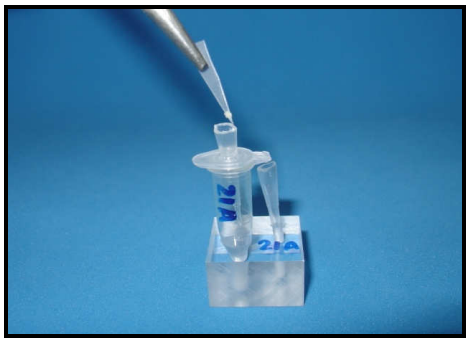
d. Câmara de vídeo (JVC 3-CCD) acoplada ao microscópio, utilizada na captura das imagens das secções de biofilme .



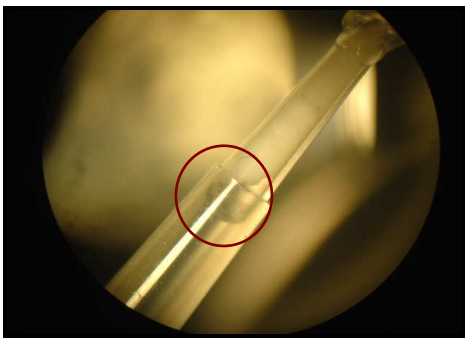
e. Secções de um biofilme típico pigmentadas por azul de toluidina. Secções i a v se referem a distâncias da interface com a saliva de 27, 135, 243, 351 e 621 µm, respectivamente. (Cortesia de P. S. Watson)

ANEXO 17: Coleta de biofilme, extração da fase fluida e análise de flúor

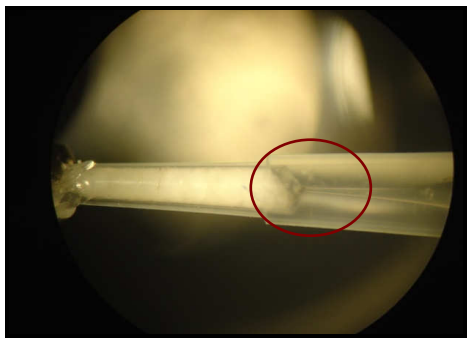
a. Tubo para microcentrífuga (esquerda) com tubo para coleta de biofilme, previamente preenchido com óleo mineral. À direita observa-se a ponta coletora de biofilme em seu suporte.



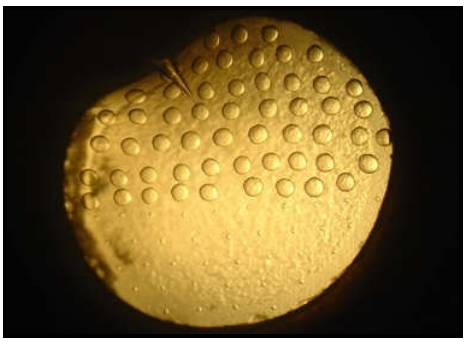
b. Imersão da ponta plástica (contendo biofilme) em óleo mineral, imediatamente após coleta, evitando-se a perda de umidade.



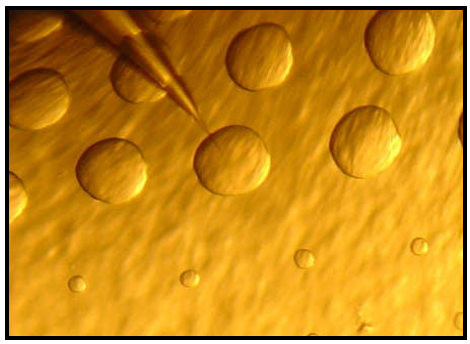
c. Separação do fluido do biofilme após centrifugação. Sob microscópio torna-se possível a separação visual do óleo mineral e do fluido do biofilme (detalhe).



d. Extração do fluido do biofilme, utilizando uma micropipeta de vidro (previamente preenchida com óleo mineral). No detalhe, observa-se a ponta da micropipeta no interior do fluido do biofilme.

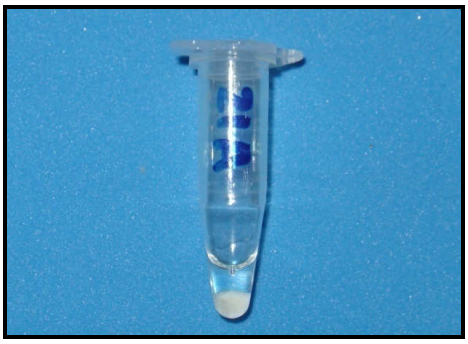


e. Amostras de fluido do biofilme tamponadas com TISAB III (acima) e gotas de TISAB III sem amostras (abaixo), sobre a superfície do eletrodo Orion 9409 (sob óleo mineral). O tamanho das gotas deve ser o mesmo, para que a proporção 1:10 (TISAB:amostra) seja mantido durante as leituras.



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

ANEXO 18: Extração e análise de flúor e cálcio do biofilme total

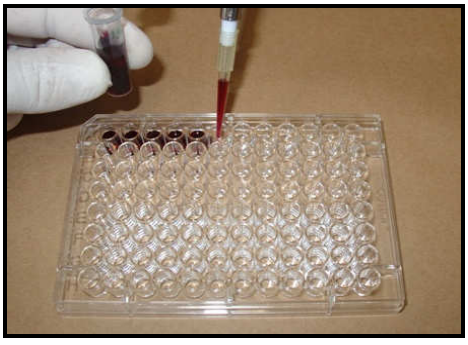


a. Fase sólida do biofilme na base do tubo, após extração do fluido, sob óleo mineral.

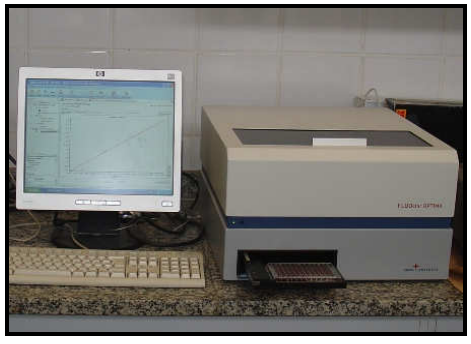
b. Adição de HCl 0,5 M (500 µL/10 mg de biofilme, peso úmido), homogenização em vórtex e agitação por 3 horas (30 rpm) para extração de flúor e cálcio do biofilme.

c. Tamponamento das amostras com NaOH 1 M (250 µL/10 mg de biofilme, peso úmido). Em seguida, as amostras são centrifugadas para coleta do sobrenadante, para análise de flúor e cálcio.

d. Colocação das amostras (2 µL), previamente tamponadas com TISAB III (1:10) nos poços da microplaca, aos quais previamente foram adicionados 100 µL de água deionizada.



e. Adição de Arsenazo III (100 µL) às amostras.



f. Análise das amostras em leitor de microplacas (FLUOstar OPTIMA, BMG Labtech, Alemanha) a 650 nm.

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