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Universidade Estadual de Campinas

Faculdade de Odontologia de Piracicaba

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

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

Orientadora: Profa. Dra. Cínthia Pereira Machado Tabchoury

Este exemplar corresponde à versão final da dissertação defendida pela aluna Marilia Mattar de Amoêdo Campos Velo e orientada pela Profa. Dra. Cínthia Pereira Machado Tabchoury.

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Profa. Dra. MARINES NOBRE DOS SANTOS UCHOA

ABSTRACT

The effect of fluoridated toothpaste on dental caries prevention in primary dentition is concentration-dependent. However, there is no pH-cycling model validated to assess the dose-response to fluoride (F) in human primary teeth enamel. Thus, this study validated a pH-cycling model in terms of dose-response to F to evaluate primary teeth enamel demineralization, and later, the model was tested with commercial toothpastes with different F concentrations. Two independent studies were conducted and primary teeth enamel slabs (3 X 3 X 2 mm), selected according to the surface hardness, were used in both. To validate the model, dental slabs (n=12/group) were submitted to the pH-cycling model and treated twice a day with: purified water (negative control) and solutions containing 62.5; 125; 250 and 375 µg F/mL. These F concentrations were chosen to simulate the dilution (1:3 w/w) that occurs in the oral cavity when dentifrices containing 250, 500, 1000 and 1500 µg F/g, respectively, are used. The model lasted 10 days and the slabs remained daily for 6 h in a demineralizing solution and 18 h in a remineralizing solution at 37°C. After validation, the model was tested with commercial toothpastes and enamel slabs (n=15/group) were treated with: no F toothpaste, 500 µg F/g, as low F toothpaste, 1100 and 1450 µg F/g, as standard toothpastes. The percentage of surface microhardness loss (%SMHL) and the carious lesion area (ΔS) were determined. The dose-response effect to F was analyzed by quadratic regression and the toothpaste effect by Tukey's test. The model showed dose-response relationship between F concentration and %SMHL ($R^2=0.7047$; $p<0.01$) and ΔS ($R^2=0.4465$; $p<0.01$). When the pH-cycling model was used to evaluate the anticaries potential of commercial toothpastes, the mean (±SD) of %SMHL and ΔS for the group treated with 500 µg F/g was 36.6±8.0 and 6298.5±1221.3, respectively, which were significantly higher than those treated with 1100 (25.2±8.7 and 4565.7±1122) and 1450 µg F/g toothpastes (24.2±5.2 and 2339.1±879.7), respectively. In conclusion, this validated model is able to differentiate the anticaries potential of toothpaste with low F concentration (500 μg F/g) from those with standard concentrations (1000-1500 μg F/g).

Keywords: Dental Caries. Dentifrices. Fluoride. Primary Enamel.

RESUMO

O efeito de dentifrícios fluoretados na prevenção da cárie dentária na dentição decídua é concentração-dependente. No entanto, não há um modelo de ciclagens de pH validado em termos de dose-resposta ao fluoreto (F) para esmalte de dente decíduo humano. Assim, esse estudo validou um modelo de ciclagens de pH em termos de dose-resposta ao F para avaliar a desmineralização do esmalte de dente decíduo, e posteriormente, o modelo foi testado com dentifrícios comerciais com diferentes concentrações de F. Dois estudos independentes foram realizados e blocos de esmalte de dente decíduo (3 X 3 X 2 mm), selecionados pela dureza de superfície, foram usados em ambos. Para validar o modelo, blocos dentais (n=12/grupo) foram submetidos ao modelo de ciclagens de pH e tratados duas vezes ao dia com: água purificada (controle negativo) e soluções contendo 62,5; 125, 250 e 375 µg F/mL. Essas concentrações de F foram escolhidas para simular a diluição (1:3 g/g) que ocorre na cavidade oral quando dentifrícios contendo 250, 500, 1000 e 1500 µg F/g, respectivamente, são usados. O modelo durou 10 dias e os blocos permaneceram diariamente por 6 h em solução desmineralizante e 18 h em solução remineralizante a 37°C. Após a validação, o modelo foi testado com dentifrícios comerciais e blocos de esmalte (n=15/grupo) foram tratados com: dentifrício não fluoretado, 500 µg F/g, como dentifrício de baixa concentração de F, 1100 e 1450 µg F/g, como dentifrícios convencionais. A porcentagem de perda de superfície (%PDS) e área da lesão de cárie (ΔS) foi determinada. O efeito dose-resposta ao F foi analisado por regressão quadrática e o efeito do teste com dentifrícios pelo teste de Tukey. O modelo mostrou relação dose-resposta entre concentração de F e %PDS (R^2=0,7047; $p<0,01$) e ΔS (R^2=0,4465; $p<0,01$). Quando o modelo de ciclagens de pH foi usado para avaliar o potencial anticárie de dentifrícios fluoretados comerciais, a média (±DP) de %PDS e ΔS para o grupo tratado com 500 µg F/g foi 36,6±8,0 e 6298,5±1221,3, respectivamente, os quais foram significativamente maiores que os grupos tratados com dentifrícios de 1100 (25,2±8,7 e 4565,7±1122) e 1450 µg F/g (24,2±5,2 e 2339,1±879,7), respectivamente. Em conclusão, o modelo proposto validado é capaz de diferenciar o potencial anticárie de dentifrícios de baixa concentração de F (500 μg F/g) dos dentifrícios de concentração convencional (1000-1500 μg F/g).

Palavras-Chaves: Cárie dentária. Dentifrícios. Flúor. Esmalte decíduo.

SUMÁRIO

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Epígrafe

A mente que se abre a uma nova ideia jamais voltará ao seu tamanho original.

Albert Einsten

INTRODUÇÃO

A cárie dentária é uma doença biofilme e açúcar-dependente (Fejerskov, 2004), que ocorre devido a um desequilíbrio na perda e ganho de minerais entre as estruturas dentárias e o fluido do biofilme, sendo um processo dinâmico resultante de alterações ultraestruturais que, ao longo do tempo, se não forem controladas, podem levar à formação de uma lesão ou cavitação (Kidd, 2004). Para o controle da cárie dentária, as estratégias utilizadas devem se direcionar aos fatores etiológicos da doença e, embora haja evidência que o aconselhamento dietético colabora com a redução da cárie precoce em crianças (Feldens et al., 2007; Feldens et al., 2010), o sucesso do controle do biofilme dental como estratégia a nível populacional tem sido limitado (Nyvad, 2008), reforçando a utilização do fluoreto (F) como método mais indicado para controlar a cárie (Cury & Tenuta, 2008). Assim, embora o F não possa interferir com os fatores responsáveis pela doença, se estiver presente constantemente na cavidade bucal, poderá interferir no seu desenvolvimento e progressão (Tenuta & Cury, 2010).

Em acréscimo, o uso de dentifrícios fluoretados tem sido relatado como o grande responsável pelo declínio de cárie (Rølla et al., 1991) em países desenvolvidos (Bratthal et al., 1996) e em desenvolvimento (Cury et al., 2004). O efeito anticárie de dentifrícios fluoretados é devido ao aumento da concentração de F na cavidade bucal toda vez que os dentes são escovados com esses produtos. Assim, além da desorganização do biofilme pela escovação, o biofilme que não foi completamente removido é enriquecido com F, podendo interferir com posteriores eventos de desmineralização-remineralização (Tenuta et al., 2009). Dessa forma, diante da exposição desses residuais de biofilme a carboidratos e com a consequente queda de pH, o F presente no fluido do biofilme irá interferir com o processo de cárie, reduzindo a desmineralização. Por outro lado, quando o pH do biofilme voltar à normalidade, o F ainda presente no biofilme irá atuar na ativação da remineralização. Além disso, é conhecido na literatura que há uma maior disponibilidade de F no fluido do biofilme após o uso de dentifrícios com concentração convencional de F do que o uso de dentifrícios de baixa concentração de F (Tenuta et al., 2009).

No entanto, o potencial anticárie de dentifrícios fluoretados é influenciado por alguns fatores, como a frequência (Marinho et al., 2003) e o período de uso (Kusano et al., 2011), bem como a concentração de F (Walsh et al., 2010) e, com relação à concentração de F, tem sido demonstrado que dentifrícios com concentração convencional de F (1000-1500 ppm F) reduzem a incidência de cárie dentária tanto na dentição permanente (Walsh et al., 2010), quanto na dentição decídua (Santos et al., 2013). Considerando a atual relevância da concentração de F para exercer o potencial anticárie nos dentifrícios fluoretados, qualquer produto fluoretado antes de ser lançado no mercado deveria ser avaliado adequadamente.

Os modelos in vitro de ciclagens de pH são amplamente usados para estimar o potencial anticárie de dentifrícios fluoretados (Featherstone et al., 1986; ten Cate et al., 1990; ten Cate et al., 2006; Queiroz et al., 2008; Featherstone et al., 2011; Tenuta & Cury, 2013). Esses modelos foram inicialmente propostos por ten Cate & Duijster (1982), que avaliaram a perda e ganho de minerais do esmalte dental em condições semelhantes ao que ocorre intra-oralmente, após períodos de exposição à solução desmineralizante e remineralizante. O modelo de ciclagens de pH de ten Cate & Duijster (1982) foi modificado posteriormente por vários experimentos para testar produtos fluoretados, até estabelecer resultados similares aos resultados in vivo relatados por Featherstone et al. (1986).

A grande vantagem dos estudos envolvendo o desenvolvimento de lesões de cárie artificiais é a possibilidade de testar os fatores de forma controlada, com métodos de análise bastante sensíveis (White, 1995). Adicionalmente, permitem a exposição dos substratos dentais a períodos alternados de desmineralização e remineralização, simulando as variações dinâmicas que ocorrem no pH e na saturação mineral associadas com o processo de cárie natural (White, 1995). Dessa forma, mimetizam o desenvolvimento e progressão da cárie in vivo, de modo que a duração do período de desmineralização simula a soma de vários períodos de quedas de pH que ocorrem no biofilme dental presente na superfície do esmalte, após o metabolismo bacteriano de carboidratos (ten Cate et al., 1988). Assim, a função dos modelos in vitro de ciclagens de pH é facilitar a geração de dados quantitativos suficientes para fornecer ao investigador confiança na elaboração de ensaios clínicos (Cummins, 1995).

Em adição, os modelos in vitro de ciclagens de pH são capazes de mostrar efeito dose-resposta a diferentes concentrações de F na inibição da desmineralização dental (Argenta et al., 2003). Segundo Proskin et al. (1992), estudos com dentifrícios para demonstrar a eficácia dos produtos deveriam apresentar a validade da metodologia. No entanto, poucos modelos foram validados em termos de dose-resposta ao F e, os modelos que foram validados usaram dentes bovinos como substratos dental (Vieira et al., 2005; Queiroz et al., 2008) para avaliar o efeito de dentifrício de baixa concentração de F na redução da desmineralização ou aumento da remineralização.

O esmalte dos dentes bovinos é mais poroso e apresenta maior progressão da lesão artificial que o esmalte dental humano (Featherstone & Mellberg, 1981). Em acréscimo, em relação ao esmalte dental humano, a progressão da cárie dentária no esmalte de dente decíduo é mais rápida quando comparada com o esmalte de dente permanente (Sonju Clasen et al., 1997), pois o esmalte dental decíduo é mais solúvel que o permanente (Wang et al., 2006). Além disso, o efeito do F na redução da desmineralização do esmalte humano pode ser diferente em termos de dose-resposta do que o efeito encontrado em esmalte dental bovino (Vieira et al., 2005; Queiroz et al., 2008). Adicionalmente, existe modelo validado para avaliar o efeito de dentifrícios fluoretados na desmineralização do esmalte dental humano permanente (Argenta et al., 2003), mas não para dentes decíduos.

Considerando que não existe um modelo de ciclagens de pH validado em termos de dose-resposta ao F na redução da desmineralização em esmalte de dente decíduo, esse estudo foi realizado. Adicionalmente, usando o modelo validado, dentifrícios comerciais fluoretados com diferentes concentrações de F foram testados.

CAPÍTULO 1

Validation of a pH-cycling model to evaluate the effect of fluoridated toothpastes on enamel demineralization of human primary teeth

ABSTRACT

The effect of fluoridated toothpaste on caries prevention in primary dentition is concentration-dependent. However, there is no pH-cycling model validated to assess the dose-response to fluoride (F) in human primary enamel. This study validated a pH-cycling model in terms of F dose-response to estimate the toothpaste efficacy on enamel demineralization. Human primary enamel slabs, selected by surface microhardness, were submitted to the pH-cycling model, which lasted 10 days, remaining daily for 6 h in demineralizing solution and 18 h in remineralizing solution. Twice/day, they (n=12/group) were treated with: water (negative control) or solutions containing 62.5; 125; 250 or 375 µg F/mL. After validation, the model was tested with commercial toothpastes and enamel slabs (n=15/group) were treated with: no F toothpaste, 500 µg F/g, as low F toothpaste, 1100 and 1450 µg F/g, as standard toothpastes. The percentage of surface microhardness loss (%SMHL) and carious lesion area (ΔS) were determined. F dose-response effect was analyzed by quadratic regression and the toothpaste effect by Tukey's test. The model showed dose-response relationship between F concentration and %SMHL (R^2=0.7047; $p<0.01$) and ΔS (R^2=0.4465; $p<0.01$). The mean (±SD) of %SMHL and ΔS for the group treated with 500 µg F/g was 36.6±8.0 and 6298.5±1221.3, respectively, which were significantly higher than those treated with 1100 (25.2±8.7 and 4565.7±1122) and 1450 µg F/g toothpastes (24.2±5.2 and 2339.1±879.7), respectively. In conclusion, this validated model is able to differentiate the anticaries potential of toothpaste with low F concentration (500 µg F/g) from those with standard concentrations (1000-1500 µg F/g).

Keywords: Dental caries. Dentifrices. Fluoride. Primary enamel.

INTRODUCTION

It has been well described in the literature the mechanism of action of fluoride (F) on the control of dental caries, reducing demineralization and activating remineralization [ten Cate, 1999]. In addition, a decline of dental caries prevalence and incidence has been observed both in developed countries from the 80' [Bratthall et al., 1996] and developing countries from the 90' [Cury et al., 2004]. This phenomenon is mainly attributed to the use of fluoridated toothpaste [Rolla et al., 1991], which is considered the most rational way to use F, due to the association of biofilm disruption with F concentration increase in the oral cavity [Tenuta and Cury, 2013].

The anticaries potential of fluoridated toothpastes is influenced by some factors, such as the frequency [Marinho et al., 2003] and the period of use [Kusano et al., 2011], as well as the F concentration [Walsh et al., 2010]. With regard to F concentration, there is clear evidence that toothpastes should contain at least 1000 µg F/g to control dental caries either in permanent [Walsh et al., 2010] or primary teeth [Santos et al., 2013]. Therefore, considering the relevance of F concentration for the anticaries efficacy of toothpastes, any fluoridated formulation before launching onto the market should be properly evaluated.

In vitro pH-cycling models are widely used to estimate the anticaries potential of fluoridated toothpastes [ten Cate et al., 1990; Featherstone et al., 2011; Tenuta and Cury, 2013]. These models simulate and mimic the caries process, but very few of them were validated in terms of dose-response effect to F and it has been suggested that the models must be previously validated and present a dose-response effect to F [Proskin et al., 1992]. Besides, the available validated models used bovine teeth [Vieira et al., 2005; Queiroz et al., 2008] to evaluate the effect of low F toothpaste on the reduction of enamel demineralization or enhancement of remineralization.

Bovine enamel is more porous than human enamel, showing a higher progression of artificial carious lesion [Featherstone and Mellberg, 1981].

Furthermore, in relation to human enamel, the progression of dental caries in primary teeth is faster when compared to permanent teeth [Sonju Clasen et al., 1997], considering that primary enamel is more soluble than the permanent one. Therefore, the effect of fluoride on the reduction of demineralization of human enamel can be different in terms of dose-response than that found for bovine enamel [Vieira et al., 2005; Queiroz et al., 2008]. In addition, there is a validated model to evaluate the effect of fluoride toothpaste on enamel demineralization of human permanent teeth [Argenta et al., 2003], but not for primary enamel.

As there is no validated pH-cycling model presenting dose-response effect to F in human primary enamel on the reduction of demineralization, this study was conducted. Also, using this validated model, the anticaries potential of commercial fluoridated toothpastes with different concentrations was evaluated.

MATERIALS AND METHODS

Ethical Considerations

This study was approved by the Research and Ethics Committee of Piracicaba Dental School - University of Campinas (Protocol n° 060/2012). The use of primary human teeth was ethically conducted according to the Brazilian guidelines (Resolution n° 196 of the National Health Council, Health Ministry, Brasilia, DF, 10/03/1996).

Experimental Design

Two independent studies were carried out. For both studies, the experimental units were enamel slabs (3 x 3 x 2 mm) obtained from sound human primary incisor and molar teeth and selected by surface microhardness (SMH). The first study validated the pH-cycling model, using F solutions to evaluate the dose-response effect to F on enamel demineralization. The slabs were submitted to a pH-cycling model and randomized among 5 treatment groups (n=12): purified water (negative control), solutions with 62.5, 125, 250 and 375 µg F/mL. These F concentrations were chosen to simulate the dilution (1:3 w/w) that occurs in the oral cavity [Duke and Forward, 1982] when toothpastes containing 250, 500, 1000 and 1500 µg F/g, respectively, are used. The pH-cycling model developed lasted 10 days and the slabs remained individually in a demineralizing solution for 6 h and in a remineralizing solution for 18 h each day. The experiment was carried out at 37°C and the volume of demineralizing and remineralizing solution per area of enamel surface was 6.25 and 3.12 mL/mm^2, respectively; these solutions were changed on the 6^{th} day. Twice a day, before and after immersion in the demineralizing solution, the slabs were washed with purified water and submitted to the treatment groups described above under agitation on a shaker for 5 min at room temperature. In the second study, the validated model was used to test the anticaries potential of commercial F toothpastes. The slabs were randomized among 4 treatment groups (n=15): toothpaste with no F concentration (negative control), toothpaste with low F concentration (500 µg F/g, Colgate Baby®), toothpaste with 1100 µg F/g (Tandy®) and toothpaste with 1450 µg F/g (Colgate Total®). In both studies, the volume of F solution or toothpaste suspension was 10 mL per slab. The percentage of surface microhardness loss (%SMHL) and carious lesion area (ΔS) were analyzed in dental slabs at the end of each study. F concentration in enamel was also analyzed in the slabs of the second study.

Preparation of Tooth Specimens

After at least 30 days stored in 2% formaldehyde solution pH 7.0 [White, 1987], sound molar and incisor primary teeth were visually examined with a magnifying glass (magnification 10 X) to exclude those with fractures, carious lesions and hypoplasia. Enamel slabs (3 x 3 x 2 mm) were cut in the third middle of the crowns. The dentin was flattened and the central area of the enamel surface was polished flat. The baseline SMH of dental slabs was determined using a Future-Tech FM-ARS microhardness tester with a Knoop diamond under 50-gram load for 5 s. Three indentations spaced 100 µm apart from each other were made at the center of the enamel surface and enamel slabs with baseline SMH of 331.0±18.8 kg/mm² were selected. A circular piece of contact paper with 2 mm of diameter was placed over the polished central surface of enamel slabs, which were then totally coated with acid-resistant varnish (nail polish, RISQUÉ®, Brazil, SP). After drying, the contact paper was removed and a surface area of 3.14 mm² was exposed to the treatments.

pH-cycling model development

pH-cycling solutions

The demineralizing solution, 50% saturated with respect to primary enamel, used in the pH-cycling model was defined in order to produce a subsurperficial carious lesion. To prepare the solution (n=3), enamel powder (particles of 74 - 105 µm) was obtained from crowns of primary teeth and added to 0.05 M acetate buffer pH 5.0 (0.5 g/L), which were kept under agitation for 96 h at 37°C to create a 100% saturated solution [Queiroz et al., 2008]. The filtered solution was diluted with an equal volume of the same buffer obtaining a 50% saturated solution with respect to the solubility of primary dental enamel. This solution is able to induce a typical enamel subsurface demineralization without erosion [Moreno and Zahradnik, 1974]. F, phosphorus (P) and calcium (Ca) concentrations were determined in these solutions by ion selective electrode, colorimetrical analysis and atomic absorption, respectively. An average of 0.038 µg F/mL, 0.81 mM P and 1.71 mM Ca was found in the solutions. From these results, a demineralizing solution containing 0.05 M acetate buffer 50% saturated of F, P and Ca with respect to primary enamel was prepared, using the reagents 100 ppm F standard, KH_2PO_4 and $CaCl_2$, respectively. This solution was used in both studies.

The remineralizing solution (1.5 mM Ca; 0.9 mM P; 150 mM KCl; 0.05 µg F/g in 20 mM cacodylic buffer pH 7.4) was used to mimick the supersaturation found in saliva to apatitic minerals [ten Cate and Duijster, 1982; Argenta et al., 2003]. This solution was also used in both studies.

Dose-response effect to fluoride

Sixty slabs were randomly distributed into 5 groups (n=12) and submitted to one of the following treatments: purified water (negative control) and solutions with 62.5, 125, 250 and 375 µg F/mL. These concentrations of F were chosen because they simulate the dilution (1:3 w/w) that occurs in the oral cavity [Duke and Forward, 1982] when toothpastes containing 250, 500, 1000 and 1500 µg F/g, respectively, are used. Fluoridated solutions were prepared with NaF and F concentrations were checked in all solutions through analysis with ion selective electrode before use.

The pH-cycling regimen lasted 10 days and the slabs were kept at 37°C for 6 h in the demineralizing solution and the rest of the daily cycle in the remineralizing solution. Before immersion in the demineralizing solution, slabs were treated with the respective groups under agitation on a shaker (TE-140, TECNAL, SP, BRAZIL) for 5 min at room temperature. After the treatment, the slabs were washed with purified water, and then individually kept in the demineralizing solution. After 6 h, the slabs were washed and again treated with the respective groups, completing the daily cycle in the remineralizing solution. The proportion of demineralizing and remineralizing solution volume per area of exposed enamel surface was 6.25 and 3.12 mL/mm^2, respectively. On the 6th day, the demineralizing and remineralizing solutions were replaced by fresh solutions and after the 10th cycle the slabs remained in the remineralizing solution for 24 h for posterior analysis.

Test of the pH-cycling model with F toothpastes

The effect of commercial F toothpastes on the inhibition of enamel demineralization of primary teeth was tested using the same pH-cycling model described before. Sixty slabs were randomly distributed into 4 groups (n=15) and submitted to one of the following treatments: non-fluoridated toothpaste (negative control), toothpaste with 500 µg F/g (Colgate Baby®), toothpaste with 1100 µg F/g (Tandy®) and toothpaste with 1450 µg F/g (Colgate Total®). The F and non-fluoride toothpaste were purchased from Colgate Ind. Com. Ltda., São Bernardo do Campo, SP, Brazil. The slabs were treated twice a day, before and after the immersion in the demineralizing solution, for 5 min with toothpaste/water slurries (1:3 w/w), under agitation on a shaker (TE-140, TECNAL, SP, BRAZIL) and at room temperature. All the toothpastes were NaF/silica-based and F concentrations were checked in all toothpastes through analysis with ion selective electrode before use.

Microhardness Analysis

After the pH-cycling, SMH of the enamel slabs was measured again. The mean values of three indentations spaced 100 μm from each other and from the baseline ones was averaged and the percentage of surface microhardness loss [%SMHL = 100 (SMH after pH-cycling – baseline SMH)/baseline SMH] was calculated. After SMH analysis for the first study, all slabs were longitudinally sectioned near to the center of the exposed enamel for cross-sectional microhardness (CSMH) analysis. For the second study, 12 slabs from each group were used for CSMH determination and 3 slabs from each group were analyzed for total F in enamel.

To perform CSMH, half of each slab was embedded in acrylic resin so that the cut surface was exposed and polished. The analysis was conducted using Knoop diamond under a 25-gram load for 5 s. Three rows of 13 indentations each were made at 10 up to 60 μm (10 μm between each other) and from 60 to 200 μm (20 μm between each other) depths from the outer surface of enamel slabs. The mean values at all 3 measuring points at each distance from the surface were then averaged and expressed as Knoop hardness number (kg/mm^2), since there is a discrepancy in the literature regarding conversion of hardness to mineral concentration [Featherstone et al., 1983; Kielbassa et al., 1999]. The area of hardness loss was calculated by numerical integration using a trapezoidal rule by the difference between the area under the curve (kg/mm^2 x μm) of the sound enamel minus the area of the demineralized one [Ana et al., 2012].

Fluoride concentration in enamel

After the pH-cycling study with the commercial toothpastes, three slabs of each group were submitted to extraction by acid etching. Two enamel layers were sequentially removed by immersion of each slab in 0.4 mL of 0.5 M HCl for 15 and 30 s under agitation. An equal volume of TISAB II, pH 5.0, modified with 20 g NaOH/L, was added to each solution containing the dissolved enamel layer [Paes Leme et al., 2003]. F measurements were performed using an ion-selective electrode (Orion 96-09) and an ion analyzer (Orion EA-940, Orion Research Inc., Boston, USA), previously calibrated with standard F solutions ranging from 0.031 to 4 μg F/mL. The amount of F found in each layer of enamel removed was summed and the results expressed in μg F/cm^2.

Statistical analyses

To verify data normality, the Kolmogorov-Smirnov test was used and a logarithmic transformation was done for the data of regression analysis of ΔS. The %SMHL and ΔS were analyzed by one-way analysis of variance (ANOVA), followed by Tukey test ($p<0.05$). To evaluate the dose-response effect of fluoride, the data were analyzed by ANOVA and the regression rate coefficient was determined. For all analyses, 5% was considered the limit of significance and the analyses were performed with the statistical software R 3.0.1 (www.r-project.org).

RESULTS

The treatment groups of the first study, with the fluoridated solutions, did not differ among each other with regard to baseline SMH of the enamel slabs ($p>0.05$): negative control (331.7 ± 14.5), 62 µg F/mL (331.2 ± 13.3), 125 µg F/mL (330.8 ± 17.7), 250 µg F/mL (330.3 ± 14.5) and 375 µg F/mL (331.1 ± 14.5). Highly significant dose-response effects were found to the response variables (Fig. 1 and 2) throughout the increase of F concentrations in the solutions, when data were analyzed by regression: %SMHL (R^2 = 0.7047; $p<0.01$) and ΔS (R^2 = 0.4465; $p<0.01$).

With respect to the evaluation of the toothpastes using the pH-cycling model, baseline SMH was not significantly different among the groups: negative control (330.5 ± 19.1), 500 µg F/g (329.9 ± 20.0), 1100 µg F/g (330.8 ± 19.6) and 1450 µg F/g (330.3 ± 19.8). All fluoridated toothpastes significantly reduced the %SMHL and ΔS in relation to the negative control group (Table 1). The slabs treated with toothpaste with low F concentration (500 µg F/g) had greater %SMHL and area of carious lesion (ΔS) than the slabs treated with standard toothpastes with 1100 µg F/g and 1450 µg F/g. With relation to the toothpastes with standard F concentration, there was no difference between the groups with toothpastes of 1100 and 1450 µg F/g when %SMHL was analyzed ($p>0.05$), even though toothpaste with 1450 µg F/g showed greater anticaries effect than 1100 µg F/g in relation to ΔS ($p<0.05$). Data of F in enamel (Table 1) showed a statistically significant increase as a function of F concentration in toothpastes.

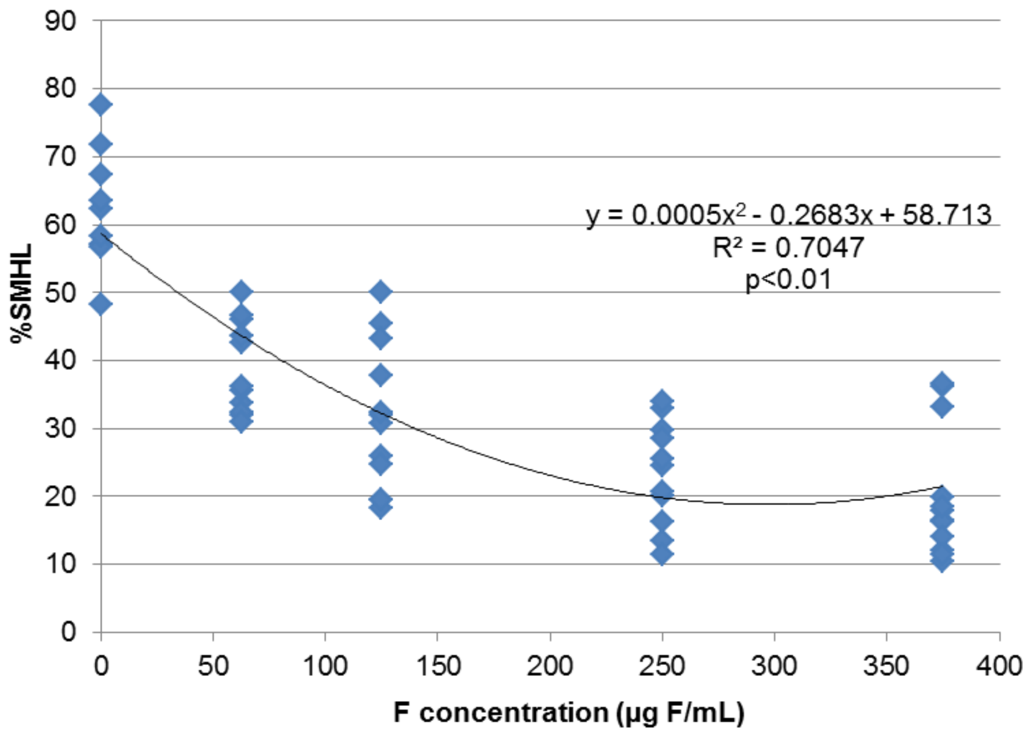

Fig 1. Percentage of surface microhardness loss (%SMHL) of primary enamel slabs in relationship to F concentration in solutions (µg F/mL) (n=12/group; except for groups negative control and 62.5 µg F/mL, which were 9 and 11, respectively).

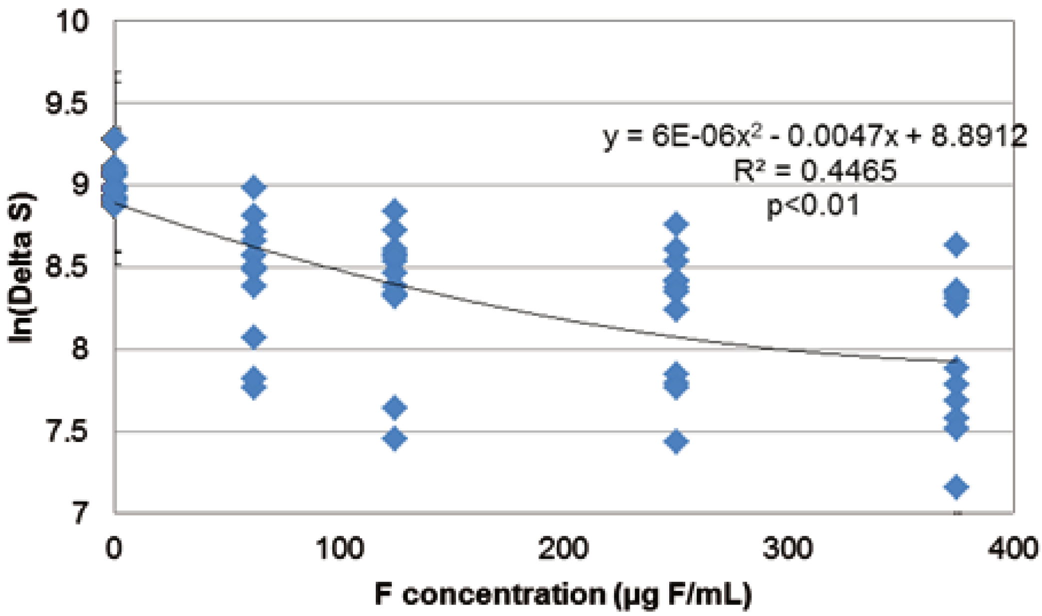

Fig. 2 Area of carious lesion of enamel (Kg/mm².µm) in relationship to F concentration in solutions (µg F/mL) (n=12/group).

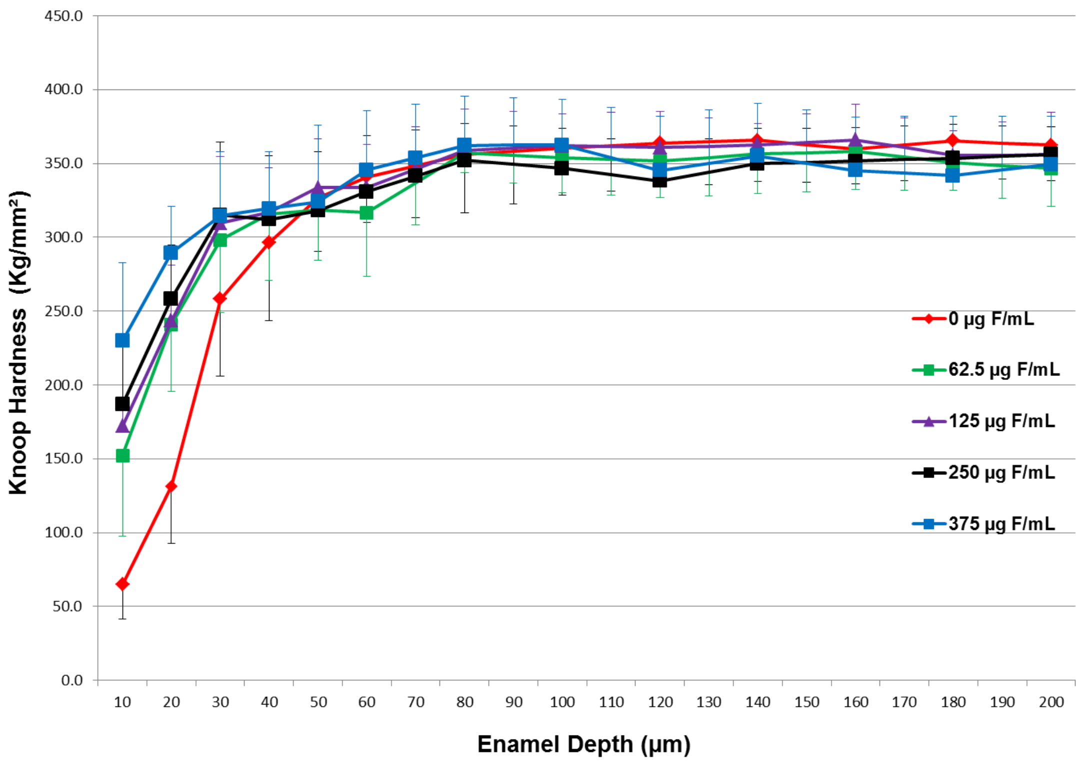

Figure 3. Enamel hardness (kg/mm2) according to the treatments and the distance (µm) from the surface (n=12/group). Bars denote standard deviations.

Table 1. Percentage of surface microhardness loss (%SMHL), area of carious lesion (ΔS) and F in enamel, according to the toothpaste used for treatment of the enamel slabs of human primary teeth in the validated pH-cycling model (Mean ± Standard deviation).

Toothpastes (µg F/g)	**%SMHL (Kg/mm²)**	**ΔS (Kg/mm².µm) (n=12)**	**F in enamel (µg F/cm²) (n=3)**
0 (Negative Control)	64.8 ± 8.6 [a] (n=10)	7,831.5 ± 991.1 [a]	0.6 ± 0.2 [a]
500 (Colgate Baby)	36.6 ± 8.0 [b] (n=11)	6,298.5 ± 1,221.3 [b]	2.5 ± 0.3 [b]
1100 (Tandy)	25.2 ± 8.7 [c] (n=13)	4,565.7 ± 1,122.0 [c]	5.2 ± 0.6 [c]
1450 (Colgate Total)	24.2 ± 5.2 [c] (n=14)	2,339.1 ± 879.7 [d]	10.4 ± 0.7 [d]

%SMHL = percentage of surface microhardness loss; ΔS = area of carious lesion. Lower case letters show statistical differences among the treatments ($p<0.05$).

DISCUSSION

The efficacy of F toothpastes has been estimated using in vitro models, which may be good indicators of the anticaries efficacy of a toothpaste formulation, supporting the conduction of a clinical trial [Tenuta and Cury, 2013]. An important prerequisite of these models, according to ADA (American Dental Association), is that they present a dose-response effect to F [Proskin et al., 1992] and this might be tested using solutions with increasing F concentrations prior to the test with the toothpaste formulations. The failure to fulfill this requirement will end up in biased models and the comparison of toothpaste efficacies will be compromised by the lack of response to F doses [Tenuta and Cury, 2013].

The pH-cycling model developed in this study was designed to evaluate the anticaries potential of toothpaste with low F concentration on enamel demineralization of human primary teeth compared to standard toothpastes containing 1100-1450 µg F/g. To validate it, fluoridated solutions, considered the ideal vehicle for the quantitative information on the optimum dose of F [Damato et al., 1990] and with known concentrations similar to those found during tooth brushing with F toothpastes [Duke and Forward, 1982], were first used to evaluate the dose-response effect to F. Next, using the developed and validated pH-cycling model, commercially available low F toothpaste (containing 500 µg F/g) and standard toothpastes with 1100 and 1450 µg F/g were tested.

The development of this model is relevant because in the literature there is no in vitro pH-cycling model validated to evaluate the effect of F toothpaste on the reduction of enamel demineralization of human primary teeth. Human primary teeth have a greater tendency to demineralization and mineral dissolution when compared with permanent teeth, due to the higher porosity they present [Wang et al., 2006]. In addition, the progression of dental caries in human primary teeth is faster mainly due to a higher carbonate content found in this substrate, which increases its solubility [Cutress, 1972]. The findings in this study showed a dose-response effect of the proposed model to F for both %SMHL and ΔS, with a statistically significant correlation between the concentration of F in the solutions tested and both variables, as can be observed in Figures 1 and 2, respectively. This supports that this pH-cycling model is adequate for studying F effect on human primary enamel, either evaluating the carious lesion formation by SMH or caries progression by CMSH.

To accomplish this, a series of experiments was conducted to establish a set of conditions, which allowed the anticaries effect of F on the subsurface lesions able to be analyzed by SMH and CMSH. The time of 60 h of immersion of the slabs in the demineralizing solution was chosen, because in a previously pilot study conducted, the daily acid challenge of 6 h in a total of 14 days allowed a carious lesion formation possible to demonstrate F effect and to be analyzed by surface microhardness. This time is in accordance to the study of Featherstone et al. [1986] that simulated a clinical condition and is a reasonable period for subjects who snack frequently [Featherstone et al., 1986].

Considering the importance of the surface layer in caries studies, the evaluation of mineral changes in this region is relevant and SMH measurement is a suitable technique for this purpose. In the present study, the measurement of SMH was possible due to the standardization of the demineralizing solution (1.71 mM calcium; 0.81 mM phosphate; 0.038 ppm F; acetate buffer 0.05 M; pH 5.0), 50% saturated with respect to enamel of human primary teeth, allowing a typical enamel subsurface demineralization without erosion [Moreno and Zahradinik, 1974]. Furthermore, the presence of low F concentration in the demineralizing (0.038 ppm F) and remineralizing (0.05 ppm F) solutions was relevant for enamel surface preservation (Fig 3, negative control group), because in the presence of F, surface etching is eliminated and subsurface demineralization promoted [White, 1987]. These concentrations did not interfere with the responses to the treatments and additionally could simulate part of the remaining F in the oral environment after toothbrushing with F toothpastes [Vieira et al., 2005].

Although the pH-cycling solutions aimed to preserve the enamel surface, some slabs presented mineral loss on the surface enough to interfere with the measurement of SMH as can be seen in the number of dental slabs analyzed in each group in Figure 1 and Table 1. This fact may be related to the own variability existing among the enamel slabs obtained from primary teeth. Human teeth show a variable composition and are difficult to obtain [Zero, 1995]. A limitation of this study was the difficulty of collecting primary teeth, and consequently, we used teeth erupted in different periods and may be exposed to different acid challenges and F exposure in the oral cavity. However, even considering that bovine teeth are readily available, the use of human teeth in researches must be considered the most appropriate substrate with relation to clinical relevance [Zero, 1995]. Furthermore, the aim of this study was the development of a model specifically for primary teeth.

The period of immersion of the slabs in the demineralizing solution in the present study allowed the formation of a caries lesion with approximately 70 µm in depth, as can be seen in Figure 4 and this depth was similar to that found in the study of Featherstone et al. [1986]. Although the total period of 84 h of immersion of the slabs in the demineralizing solution in the study of Featherstone et al. [1986] was higher than in our model (60 h), the key conclusion is that the depth of demineralization, which occurred in this pH-cycling study in 10 days, was closely similar to that observed in vivo [Featherstone et al., 1986]. Additionally, regarding the daily treatments with F, while 5 min may be considered a long period of brushing, it is a good estimate of the effective retention time for elevated F levels in the mouth [Featherstone et al., 1986].

When the pH-cycling model was used to evaluate the anticaries potential of commercial toothpastes, the findings clearly show that the F toothpastes present anticaries potential, because they were able not only to incorporate F in enamel (Table 1), but also to interfere with the phenomena of caries development, reducing enamel demineralization, as we can see in the results of %SMHL and ΔS

(Table 1). However, although the low F toothpaste was effective in reducing enamel demineralization in comparison to the non-fluoridated toothpaste (Table 1), it did not show the same anticaries efficacy as standard toothpastes. Despite the limitations of an in vitro study, our results are consistent with a systematic review recently published in the literature [Santos et al., 2013], showing that the anticaries potential of toothpaste with low F concentration would be compromised, since it differs from standard F toothpaste. Thus, the recommendation that young children should use low F toothpastes may jeopardize the anticaries benefits of this F method. Our results emphasize the clinical importance of the model developed, which can be used to test new products and formulations launched onto the market.

In summary, the validated model proposed in this study is able to differentiate the anticaries potential of toothpaste with low F concentration (500 µg F/g) from those with standard concentrations (1000-1500 µg F/g).

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CONCLUSÃO

Os dados sugerem que o modelo de ciclagens de pH desenvolvido nesse estudo para esmalte de dentes decíduos mostrou efeito dose-resposta ao F e foi capaz de avaliar o potencial anticárie de dentifrícios fluoretados diferenciando dentifrícios de baixa concentração de F (500 μg F/g) de dentifrício de concentração convencional (1000-1500 μg F/g).

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ANEXO 1

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

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Validação de modelo de ciclagens de pH para estimar o potencial de produtos fluoretados na redução da desmineralização do esmalte decíduo"**, protocolo nº 060/2012, dos pesquisadores Marilia Mattar de Amoêdo Campos Velo, Cínthia Pereira Machado Tabchoury e Jaime Aparecido Cury, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 01/08/2012.

The Ethics Committee in Research of the Piracicaba Dental School - University of Campinas, certify that the project **"Validation of pH-cycling model to estimate the potential of fluoridated products on the reduction of deciduous enamel demineralization"**, register number 060/2012, of Marilia Mattar de Amoêdo Campos Velo, Cínthia Pereira Machado Tabchoury and Jaime Aparecido Cury, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee on Aug 01, 2012.

Lívia M. A. Tenuta

Profa. Dra. Lívia Maria Andaló Tenuta
Secretária
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

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

ANEXO 2

Dear Mrs. Velo:

A manuscript titled Validation of a pH-cycling model to evaluate the effect of fluoridated toothpastes on enamel demineralization of human primary teeth (201406010) has been submitted by Mrs. Marilia Velo to Caries Research.

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