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pH-Cycling Models to Evaluate the Effect of Low Fluoride Dentifrice on Enamel De- and Remineralization

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Since the currently available pH-cycling models do not differentiate the anti-caries potential of dentifrices with low fluoride (F) concentration, two models were developed and tested in the present. Bovine enamel blocks were subjected to the models and treated with F solutions containing from 70 to 280 µg F/mL in order to validate them in terms of dose-response effect. The models were also tested by evaluating the dentifrices Colgate Baby (500 µg F/g, as a low fluoride dentifrice), Tandy (1,100 µg F/g, as an active F-dentifrice) and Crest (1,100 µg F/g, as positive control). Enamel mineral loss or gain was assessed by surface and cross-sectional microhardness, and lesion depth was analyzed by polarized light microscopy. The pH-cycling models showed F dose-response effect either reducing enamel demineralization or enhancing remineralization. The low F dentifrice presented anti-caries potential, but it was not equivalent to the dentifrices containing 1,100 µg F/g. These data suggest that the models developed in this study were able to evaluate the anti-caries potential of low F dentifrice either on resistance to demineralization or on enhancement of remineralization.

Key Words: demineralization, remineralization, fluoride, dentifrice.

INTRODUCTION

In spite of the progress of *in situ* and *in vivo* research in cariology, laboratory tests are still widely used to evaluate dental caries, mainly the effect of fluoride (F) on inhibition of enamel-dentin demineralization and enhancement of remineralization. Among these protocols, there is a consensus that pH-cycling models may be used because they mimick caries development *in vivo* (1). Nevertheless, before using to estimate the anti-caries potential of F products, these models should be previously validated in terms of dose-response (2).

Therefore, the major focus of researchers has been the development of *in vitro* models that meet the suggested ADA guidelines associated with topical evaluations of fluoride (F) dentifrice (2). According to these guidelines (3), a pH-cycling model should show F dose-response effect but, unfortunately, most publications referring to these models are abstracts rather than full-text papers (2). Furthermore, according to ADA guidelines a pH-cycling model should show dose-response effect differentiating dentifrices containing 0, 250 and 1,100 µg F/g. Therefore, it is not mandatory that these models are able to differentiate the anti-caries effect of a low F dentifrice containing 500-550 µg F/g from the conventional 1,000-1,100 µg F/g, and the committee only recommended (3) that a 500 ppm F treatment group could be included to obtain a "mini-dose-response".

As far as low F dentifrice is concerned, it has been suggested as an alternative to reduce the risks of dental fluorosis (4) because, although caries decline in developed and developing countries (5) is explained by

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widespread F-dentifrice use, it has also been considered to be a risk factor for fluorosis (6). However, in addition to the fact that the anti-caries efficiency of low F dentifrice is not clearly established (7), the *in vitro* models available to evaluate the anti-caries potential of F dentifrice are unable to differentiate the effect of low F dentifrice compared to the conventional 1,000-1,100 µg F/g range (8-9). Moreover, regarding the substrates used in these investigations, although bovine enamel has several advantages over human enamel (10), it has not been thoroughly characterized in a pH-cycling study (11). Therefore, the goal of this study was to develop, validate and test pH-cycling models to evaluate the anti-caries potential of low F dentifrices either to inhibit demineralization or to enhance remineralization, using bovine enamel as substrate.

MATERIAL AND METHODS

Experimental Design

Four independent studies were carried out. For two of them, fluoride solutions (0, 70, 140 and 280 µg F/mL) were used to validate the models to evaluate F effect on inhibition of enamel demineralization (named demineralizing pH-cycling model) and to enhance remineralization (named remineralizing pH-cycling model). The other two studies used the validated models testing 4 dentifrices (3 fluoridated commercial dentifrices and 1 non-fluoridated control formulation) in their ability to interfere with enamel de- and remineralization.

The experimental units were bovine enamel blocks. Independent comparisons among treatments of either fluoride solutions or dentifrices were done, considering the response variables: percentage of surface microhardness loss (%SML), integrated mineral loss (ΔZ) and lesion depth (LD), for the demineralizing model; and percentage of surface microhardness recovery (%SMR), percentage of integrated mineral recovery (%ΔZ) and LD for the remineralizing model. Additionally, analyses of the F dose-response effect were performed with the fluoride solutions, in both models.

Demineralizing Solution Preparation

Firstly, 0.05 M acetate buffer pH 5.0, 50% saturated with respect to enamel solubility was prepared. In this solution, it was detected 1.28 ± 0.058 mmol/L of Ca, 0.74 ± 0.005 mmol/L of inorganic phosphorus (P_i) and 0.023 ± 0.006 µg F/mL. From these results, 0.05 mol/L acetate buffer, pH 5.0 and containing 1.28 mmol/L Ca, 0.74 mmol/L P_i and 0.03 µg F/mL was prepared from the salts $Ca(NO_3)_2.4H_2O$, KH_2PO_4 and NaF, respectively. This demineralizing solution was used in both pH-cycling models (de- and remineralizing) and also to induce caries-like lesions on enamel blocks subjected to the pH-cycling caries reversal model.

Enamel Block Preparation

Flattened and polished bovine enamel blocks (4x4x3 mm) were prepared (12). An adhesive strip (2.0 x 4.0 mm) was attached to the enamel and the remaining surfaces of the block were coated with an acid-resistant varnish, so that an area of 8.0 mm^2 was exposed to the treatments. Baseline enamel surface microhardness was determined (13) and 197 enamel blocks with hardness of 353.4 ± 12.2 kg/mm^2 were selected for this study.

Demineralizing pH-Cycling Model

Model Validation - Dose-Response Test: Fifty-two blocks were randomly assigned to 4 groups (n=13) and submitted to one of the following treatments: distilled deionized water (DDW, as negative control) and solutions containing 70, 140 and 280 µg F/mL. These F (NaF) concentrations were chosen to simulate the dilution (1:3 w/w) that occurs in the oral cavity when dentifrices containing 275, 550 and 1100 µg F/g, respectively, are used (14). The pH-cycling regimen took 8 days, and the blocks were kept at 37°C for 4 h in the demineralizing solution and 20 h in the remineralizing solution. Twice a day (before and after immersion in the demineralizing solution), the blocks were washed with DDW and subjected to one of the groups of treatments for 5 min under agitation. After treatments, the blocks were washed and individually kept in the demineralizing solution. The remineralizing solution used contained 1.5 mmol/L Ca, 0.9 mmol/L P, 150 mmol/L KCl, 0.05 µg F/mL in 0.1 mol/L Tris buffer, pH 7.0. The proportion of demineralizing and remineralizing solutions *per* area of block was 6.25 mL/mm^2 and 3.12 mL/mm^2, respectively. On the 4th day, the de- and remineralizing solutions were replaced by fresh ones. After the 8th cycle, the blocks remained in the remineralizing solution for additional 24 h until the analyses (13).

F-Dentifrice Evaluation. The effect of F dentifrice on inhibiting demineralization was tested using the same conditions described before. Forty blocks were randomly assigned to 4 groups (n=10) that were subjected to one of the following treatments: non-fluoridated dentifrice (negative control); Colgate Baby (500 µg F/g, as a low fluoride dentifrice, Colgate-Palmolive, São Bernardo do Campo, SP, Brazil); Tandy (1,100 µg F/g, as an active F-dentifrice, Colgate-Palmolive); and Crest Cavity Protection Regular (1,100 µg F/g, as a positive control, considered a "gold standard", Procter & Gamble, Cincinnati, OH, USA). The blocks were treated twice a day for 5 min with dentifrice/water slurries (1:3 w/w). All products were silica-based dentifrices.

Remineralizing pH-Cycling Model

Caries-Like Lesion Formation. A preliminary study to induce caries-like lesions on bovine enamel blocks was conducted. Forty blocks were immersed individually in the previously described demineralizing solution (2 mL/mm^2 of enamel area) from 8 to 64 h and mineral loss was evaluated. The time of 32 h was chosen to induce caries-like lesion on enamel because the enamel blocks presented measurable caries-like subsurface lesions without surface erosion, allowing the evaluation of mineral loss or gain by determining surface microhardness. Next, 105 enamel blocks of known surface microhardness (SMH) (sound enamel) were subjected to the demineralizing solution for 32 h, the SMH was again determined (demineralized enamel) and these blocks with caries-like lesions were used for the further analyses.

Model Validation - Dose-Response Test. Sixty-five enamel blocks, presenting caries-like lesions, were randomly assigned to 5 groups (n=13). Four groups were submitted to the same conditions already described for the pH-cycling demineralizing model and the 5th extra-group was not submitted to any treatment and was kept for analysis of the baseline caries-like lesion. The pH-cycling regimen took 8 days and this number of cycles was determined by pilot study since after 11 days in the remineralizing solution, there is maximum rehardening of enamel surface, irrespective of F concentration used to enhance remineralization. The blocks were kept for 2 h in the demineralizing solution and for 22 h in remineralizing solution at 37ºC. Three times a day (9:00, 14:00 and 17:00 h), the blocks were washed with DDW and submitted for 1 min to one of the treatments under agitation. After the treatments, the blocks were washed again. On the 4th day, the de- and remineralizing solutions were replaced by fresh ones. After another 4-day cycle, the enamel remineralization was evaluated.

F-Dentifrice Evaluation. Forty enamel blocks, presenting caries-like lesions, were randomly assigned to 4 groups (n=10). They were subjected to the pH-cycling caries reversal model described before, and submitted to the dentifrices as already described for the pH-cycling demineralizing model.

Microhardness Analysis

After the demineralizing pH-cycling and treatments, enamel SMH was again determined (12) and the %SML was calculated. Subsequently, all the blocks were longitudinally sectioned through the center and the cross-sectional microhardness was measured on the inner surface of one of the halves, to the determination of the area of mineral loss (ΔZ) (12). After the remineralizing pH-cycling and treatments, SMH was again determined and percentage of SMH recovery (%SMR) was calculated. Afterwards, all enamel blocks were longitudinally sectioned and prepared as described above. In one half of the specimens, the integrated mineral recovery value for each experimental group was calculated (ΔZ_2), compared to that (ΔZ_1) of the extra-group of enamel blocks presenting caries-like lesions and the percentage of integrated mineral recovery was calculated ($\%\Delta Z = \Delta Z_1 - \Delta Z_2 \times 100/ \Delta Z_1$).The microhardness tester (Future-Tech FM Corp., Tokyo, Japan), coupled to FM-ARS software, was used for these analyses and a Knoop indenter was used with a 25-g load for 5 s (13).

Polarized Light Microscopy Analysis

Longitudinal sections of 100 ± 10 µm were obtained from the remaining half of each block. Sections were embedded in DDW, mounted on glass-slides and the artificial caries lesion depth was analyzed in a polarized light microscope (DM LSP, Leica, Wetzlar GmbH, Germany), as previously detailed (15).

Statistical Analysis

The assumptions of equality of variances and normal distribution of errors were respectively checked

with the Hartley and Shapiro-Wilks tests for all response variables. If necessary, data were transformed according to the Box-Cox method. The differences between the F-dentifrices or F-solutions treatments were evaluated by ANOVA and Tukey's test (α=0.05) for all variables. To test the dose-response effect of F-solutions, %SML, ΔZ, %SMR, %ΔZ and LD data were analyzed by ANOVA and regression analysis (α=0.05). The analyses were performed with the SAS System 8.01 software (SAS Institute Inc., Cary, NC, USA).

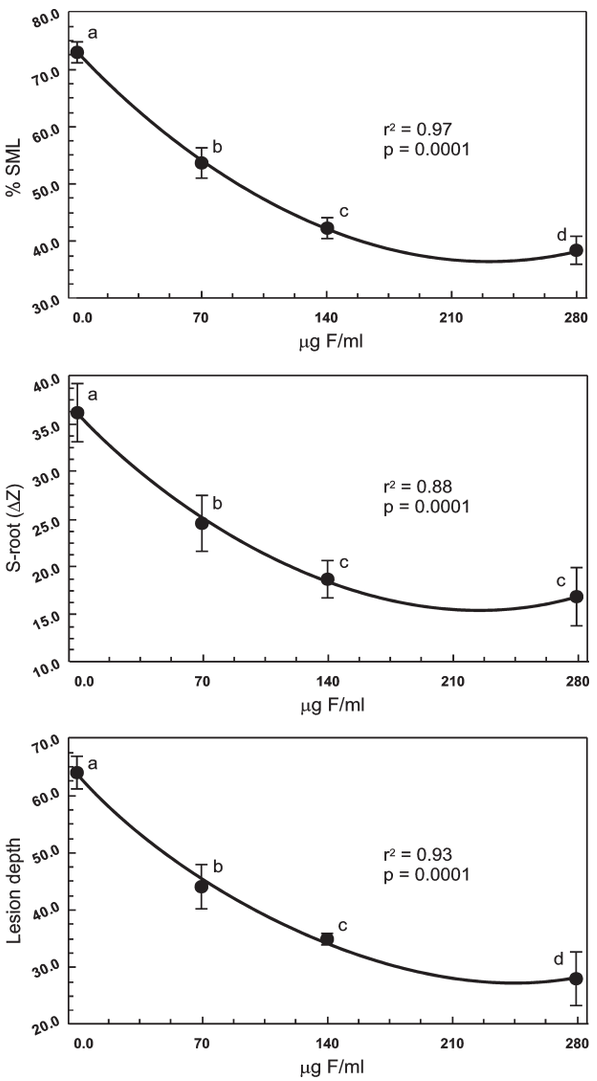

Figure 1. Effect of fluoride concentration (μg F/mL) on reduction of enamel demineralization evaluated by surface microhardness (top), area of mineral loss (center) and lesion depth (bottom).

RESULTS

Demineralizing pH-Cycling Model

ΔZ data of both fluoride solution and fluoride dentifrice experiments were transformed according to the root-square function. Highly significant dose-response effects were found to the response variables (Fig. 1) throughout the increase of fluoride concentrations in the solutions, when data were adjusted to quadratic fits: %SML (R^2 = 0.97; p = 0.0001), ΔZ (R^2 = 0.88; p = 0.0001) and LD (R^2 = 0.93; p = 0.0001). Table 1 shows that all F-dentifrices significantly reduced enamel demineralization, either evaluated by %SML, ΔZ or LD, compared to the negative control group ($p<0.05$). However, the dentifrices containing 1,100 μg F/g were more efficacious in reducing enamel demineralization than the low F dentifrice ($p<0.05$), but the active F-dentifrice and the positive control did not differ significantly ($p>0.05$).

Remineralizing pH-Cycling Model

%SMR data of the fluoride solutions experiment

Table 1. Results (original values) according to the treatments with fluoride solutions (n = 13) or dentifrices (n = 10) for the pH-cycling demineralizing model. (Means ± SD)[a].

Treatment groups with F-solutions (μg F/mL)	Variables		
	%SML[b] (kg/mm²)	ΔZ[c] (vol. % min x μm)	LD[d] (μm)
0	72.9 ± 0.9	1314.5 ± 228.9	64.0 ± 1.8
70	53.7 ± 1.7	615.4 ± 242.6	44.1 ± 5.9
140	42.2 ± 1.6	356.4 ± 72.7	35.0 ± 1.0
280	38.4 ± 1.1	292.5 ± 97.3	28.1 ± 6.5
Dentifrices			
Negative control	74.8 ± 8.2	1569.5 ± 215.1	84.2 ± 2.8
Low fluoride	47.7 ± 10.1	789.1 ± 122.1	50.6 ± 4.6
Active dentifrice	33.8 ± 4.3	399.8 ± 65.7	30.1 ± 1.4
Positive control	35.4 ± 6.5	376.4 ± 125.1	32.9 ± 1.8

[a]Fluoride solutions and dentifrices whose means are connected by brackets do not differ statistically ($p<0.05$); [b]Percentage of surface microhardness loss; [c]Integrated mineral loss; [d]Lesion depth.

and ΔZ data of the fluoride dentifrice experiment, were transformed according to the root-square and log_{10} functions, respectively. Highly significant dose-response effects were found to the response variables (Fig. 2) throughout the increase of the fluoride concentrations in the solutions, when data were adjusted to cubical: %SMR ($R^2 = 0.86$; p = 0.0001); and quadratic fits: %ΔZ ($R^2 = 0.87$; p = 0.0001) and LD ($R^2 = 0.80$; p = 0.0001). Table 2 shows that all F-dentifrices presented higher %SMR, or %ΔZ, and lower LD compared to the negative control group (p<0.05). The low F dentifrice showed lower efficacy in enhancing remineralization than the dentifrices containing 1,100 µg F/g (p<0.05), which did not differ statistically (p>0.05).

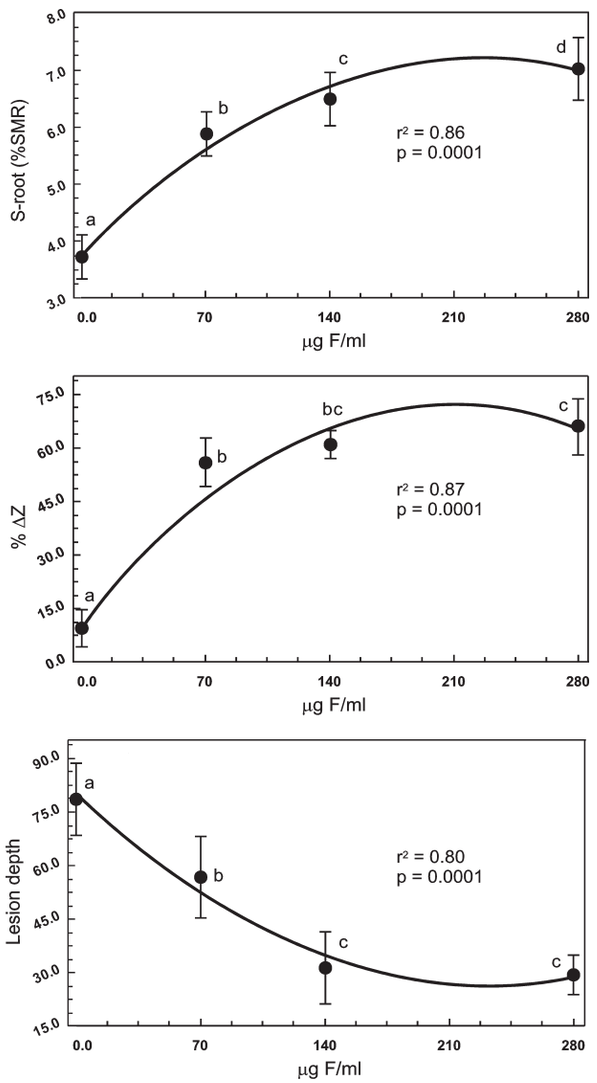

Figure 2. Effect of fluoride concentration (µg F/mL) on enhancement of enamel remineralization evaluated by surface microhardness (top), area of mineral recovery (center) and lesion depth (bottom).

DISCUSSION

The pH-cycling models developed were designed to evaluate *in vitro* the anti-caries potential of dentifrice with low fluoride (F) concentration, either to reduce enamel demineralization or to enhance remineralization, compared to conventional dentifrice presenting 1,000-1,100 µg F/g. To validate them, F solutions of known concentrations similar those found during tooth brushing with F-dentifrice (14) were first used to evaluate the dose-response effect of F on enamel. Next, using the developed pH-cycling models, a commercially available low F-dentifrice was evaluated against a positive control dentifrice ("gold standard") and an active 1,100 µg F/g Brazilian dentifrice (15,16).

The findings showed that both pH-cycling models developed in this study presented F dose-response effect for all analyzed variables (Tables 1 and 2, and

Table 2. Results (original values) according to the treatments with fluoride solutions (n = 13) or dentifrices (n = 10) for the pH-cycling remineralizing model. (Means ± SD)[a].

Treatment groups with F-solutions (µg F/mL)	Variables		
	%SMR[b] (kg/mm²)	%ΔZ[c] (vol. % min x µm)	LD[d] (µm)
0	13.9 ± 2.7	9.6 ± 5.5	78.4 ± 5.2
70	34.5 ± 4.7	56.0 ± 6.7	56.9 ± 1.4
140	42.1 ± 6.2	61.0 ± 4.0	31.4 ± 2.7
280	49.1 ± 7.0	65.8 ± 8.8	29.5 ± 6.9
Dentifrices			
Negative control	9.1 ± 3.2	6.2 ± 1.4	64.7 ± 5.2
Low fluoride	26.2 ± 2.2	33.2 ± 11.0	46.1 ± 3.6
Active dentifrice	38.8 ± 4.0	56.2 ± 11.1	18.9 ± 6.5
Positive control	40.9 ± 3.2	52.3 ± 5.6	20.7 ± 9.4

[a]Fluoride solutions and dentifrices whose means are connected by brackets do not differ statistically (p<0.05); [b]Percentage of surface microhardness recovery; [c]Percentage of integrated mineral recovery; [d]Lesion depth.

Figs. 1 and 2). This suggests that these pH-cycling models are adequate for studying *in vitro* fluoride effect on bovine enamel, either evaluating early caries by surface microhardness change or caries progression by cross-sectional microhardness or polarizing microscopy. The data are relevant because one of the barriers the use of bovine enamel in *in vitro* studies is the fact that its surface is susceptible to erosion (11). However, even human enamel is susceptible to erosion when subjected *in vitro* to pH-cycling, and modification of the widely used models is required to evaluate early caries (13).

It should be emphasized that although our research group has developed other pH-cycling models (13,17) using human or bovine enamel as substrate, they have not been used to evaluate F products. The models developed in the present study, in addition to being of novel design, extended the previous results (17) for bovine enamel because they were tested using commercially available F-dentifrices, one of them considered as a "gold standard".

The Brazilian fluoride dentifrice Tandy, which contains 1,100 µg F/g, was equivalent to the positive control dentifrice Crest, reducing demineralization and enhancing remineralization of enamel (Tables 1-2). These findings have clinical relevance because this dentifrice is consumed by 20% of the young Brazilian population (18) and F-dentifrices have made an important contribution to caries decline in Brazil (5). Furthermore, this *in vitro* study confirms *in situ* findings about the anti-caries efficacy of this formulation (15,16,19).

However, although the tested low F dentifrice was effective in reducing enamel demineralization and enhancing remineralization in comparison to the non-fluoridated negative control dentifrice, it did not have an equivalent performance to that of the positive control or the active dentifrice containing 1,100 µg F/g. Thus, although the low F dentifrice may be safer than the conventional one in terms of dental fluorosis risks, its use should be recommended according to the children's caries activity (20).

In conclusion, the findings of the present study suggest that the developed *in vitro* models using bovine enamel as substrate, in addition to presenting F dose-response effect, were also able to evaluate the anti-caries potential of a low F dentifrice, either to inhibit enamel demineralization or to enhance enamel remineralization, compared to the conventional dentifrice containing 1,100 µg F/g. Furthermore, these models could be used to evaluate the anti-caries potential of fluoride mouthrinse (225 µg F/mL).

RESUMO

Tendo em vista que os modelos atuais de ciclagens de pH não diferenciam o potencial anti-cárie de dentifrícios com baixa concentração de fluoreto (F), dois modelos foram desenvolvidos e testados. Blocos de esmalte bovino foram submetidos aos modelos e tratados com soluções de concentrações crescentes de F (70 a 280 µg F/mL) para validar os modelos em termos de dose-resposta. A seguir, os modelos foram testados avaliando o potencial anti-cárie dos dentifrícios Colgate Baby (500 µg F/g, dentifrício de baixa concentração), Tandy (1.100 µg F/g, como controle ativo) e Crest (1.100 µg F/g, como controle positivo). Perda ou ganho de mineral pelo esmalte foi avaliada por microdureza e profundidade de lesão de cárie foi avaliada por microscopia de luz polarizada. Os modelos de ciclagens de pH desenvolvidos mostraram efeito do F dose-resposta quer seja na redução da desmineralização como na remineralização do esmalte. O dentifrício de baixa concentração de F mostrou ter potencial anti-cárie, o qual não foi equivalente aos dentifrícios contendo 1.100 µg F/g. Os resultados sugerem que os modelos desenvolvidos são capazes de avaliar o potencial anti-cárie de dentifrício de concentração reduzida de F, quer seja na sua capacidade de aumentar a resistência do esmalte a desmineralização como na ativação da remineralização.

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