

A modified pH-cycling model to evaluate fluoride effect on enamel demineralization

Modelo de ciclagens de pH para avaliar o efeito do fluoreto na desmineralização do esmalte

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ABSTRACT: Since *in vitro* pH-cycling models are widely used to study dental caries, they should allow evaluations of fluoride effect on early stages of caries development. Therefore, acid etching on enamel surface must be avoided, enabling surface microhardness (SMH) analysis. In the present study, the pH-cycling model originally described by Featherstone et al.⁹ (1986) was modified to preserve the enamel surface and to produce early carious lesions that could be evaluated using SMH and cross-sectional microhardness (CSMH) measurements. In order to validate this modified model, a dose-response evaluation with fluoride was made. Human enamel blocks with known SMH were submitted to such regimen with the following treatments: distilled deionized water (DDW; control) and solutions containing 70, 140 and 280 ppm F. Data from %SMH change and ΔZ (mineral loss) showed a statistically significant negative correlation between F concentration in treatment solutions and mineral loss. In conclusion, the modified pH-cycling model allowed the evaluation of changes on the outermost enamel layer during caries development, and a dose-response effect of fluoride reducing enamel demineralization was observed.

DESCRIPTORS: Dental enamel; Demineralization; Fluorine; Fluorides.

RESUMO: Considerando que os modelos de ciclagens de pH são amplamente usados para estudar a cárie dental, eles deveriam possibilitar a avaliação do efeito do fluoreto nos estágios iniciais do desenvolvimento da cárie. Desse modo, o ataque ácido erosivo na superfície do esmalte deve ser evitado, possibilitando a análise da microdureza de superfície (MDS). No presente estudo, o modelo de ciclagens de pH descrito por Featherstone et al.⁹ (1986) foi modificado para preservar a superfície do esmalte e para produzir lesões iniciais que pudessem ser avaliadas usando MDS e microdureza do esmalte seccionado longitudinalmente. Para validar esse modelo modificado, uma avaliação dose-resposta a fluoreto foi feita. Blocos de esmalte dental humano de MDS conhecida foram submetidos a tal regime com os seguintes tratamentos: água deionizada (controle) e soluções contendo 70, 140 e 280 ppm F. Os dados de variação de MDS e ΔZ (perda mineral) mostraram correlações negativas e estatisticamente significativas entre as concentrações de F dos tratamentos e a perda de mineral pelo esmalte. Em conclusão, foi possível avaliar as variações de mineral ocorridas nas camadas mais superficiais do esmalte durante o desenvolvimento de cárie, e um efeito dose-resposta do F reduzindo a desmineralização do esmalte foi observado.

DESCRIPTORES: Esmalte dentário; Desmineralização; Flúor; Fluoretos.

INTRODUCTION

Irrespective of the progress in *in situ* and *in vivo* experimentation about caries research, *in vitro* tests are still widely used. A Medline/Biosis search of the last five years revealed over 300 citations documenting *in vitro* tests associated with caries. Several models were developed, and the present knowledge about the mechanisms of dental caries development, fluoride (F) effect and preventive measures on caries is due mainly to *in vitro* studies.

Among these models, those mimicking *in vitro* caries processes (pH-cycling), as suggested by ten

Cate, Duijsters¹⁶ (1982), have been used for different purposes¹⁸; the most important application has been the evaluation of fluoride effect on caries. Different pH-cycling models were developed to evaluate the fluoride effect in either reducing enamel demineralization⁹ or enhancing remineralization¹⁷. These approaches have been used with tremendous success in cariology.

However, *in vitro* testing protocols show limitations, and one of them is to be unable to study the effect of fluoride on all stages of the caries process, mainly the early stage. Thus, in the presence of acids an incipient dissolution of the sound enamel

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surface occurs, which is followed by the precipitation of less soluble minerals like fluoride in the surface layer¹³. Subsequently, some acids, which remained within the enamel pores, diffuse into inner enamel regions, resulting in the dissolution of the underlying enamel. Mineral components then back diffuse into the surface layer and precipitate in this region. At the same time, a portion of the dissolved ions diffuses out of the enamel surface into the environment. The surface layer appears unaltered because it is continuously regenerated. Thus, the caries process is initiated by a slight dissolution of the enamel surface¹³. Considering the importance of the surface layer in caries progression, the evaluation of changes in this region is relevant and surface microhardness (SMH) measurement is a suitable technique for this purpose.

However, the use of SMH to evaluate the effect of fluoride on early caries lesions is not possible because the pH-cycling models that have been developed up to the present moment promote an acid etching on the enamel surface. When this occurs, the changes on the surface cannot be properly evaluated. According to Zero¹⁹ (1995), SMH measurement is a highly sensitive and reproducible method used to evaluate *in situ* studies. Using SMH it is possible to study early stages of enamel-dentine demineralization^{1,4,20,21} or enamel remineralization^{20,22}. Furthermore, this technique is desirable since it also allows hardness determination in the same substrate before and after the treatments³, reducing the experimental error. In addition, White¹⁷ (1987) found a high correlation ($r^2 = 0.94$; $p < 0.01$) between the remineralization of early carious lesions measured by SMH and micro-radiography. Thus, a pH-cycling model that promotes early enamel carious lesion, which may be evaluated by SMH measurement and cross-sectional microhardness (CSMH) measurement at a very close distance from the surface must be proposed.

Thus, an *in vitro* modified pH-cycling model, allowing the use of SMH and CSMH to evaluate the initiation and progression of the caries process, and the study of fluoride effect on enamel demineralization, was developed.

MATERIAL AND METHODS

Experimental design

Enamel blocks (4 x 4 x 2 mm) were prepared from impacted human third molars stored in 2% formaldehyde solution, pH 7.0, for at least a month^{4,17}. The enamel surface was polished to re-

move the outer 50 μm ¹¹. Baseline SMH determination was accomplished using a Future-Tech FM-ARS microhardness tester (Future-Tech Corp., Tokyo, Japan) with a Knoop diamond under 25-g load for 5 s. Five indentations were made at the center of the enamel surface. Enamel blocks with baseline SMH between 360 and 420 KHN (Knoop hardness) were selected for the study. The objectives of baseline SMH determination were to select the blocks and to calculate the SMH change that occurred after pH-cycling.

Thirty-two enamel specimens, with all the surfaces protected with an acid-resistant varnish except for a circular central area of 3.14 mm², were randomized into 4 treatment groups: distilled and deionized water (DDW; control) and solutions containing 70, 140 and 280 ppm F. These fluoride concentrations correspond to the dilution (1:3) of dentifrices containing 275, 550 and 1100 ppm F in the oral cavity⁶. All fluoridated solutions were prepared with NaF and DDW.

pH-cycling regimen

The pH-cycling model originally described by Featherstone et al.⁹ (1986) was modified by several experiments (data not shown) until establishment of a set of conditions preserving the enamel surface and producing early carious lesions that could be evaluated using SMH and CSMH measurements. Eight blocks from each group were submitted for 5 days to a pH-cycling regimen. The blocks were kept individually in a demineralizing solution (2.0 mM calcium, 2.0 mM phosphate, 0.030 ppm F, in 75 mM acetate buffer, pH 4.3) for 3 h (20 ml per block), and in a remineralizing solution (1.5 mM calcium, 0.9 mM phosphate, 150 mM of KCl, 0.050 ppm F in 20 mM cacodylic buffer, pH 7.4) for 20 h (10 ml per block) each day. After each cycle, the blocks were returned to the same solutions. This cycle was repeated daily for 5 days and the enamel blocks remained in the remineralizing solution for 2 days until the analyses. Solution treatment was performed twice a day (before and after the demineralizing period) by immersion of the blocks for 5 min in 10 ml of the solution per block under agitation. The experiment was carried out at 37°C. De- and remineralizing solutions contained thymol to avoid fungal growth.

Microhardness determination

After pH-cycling, SMH and CSMH were determined in all enamel blocks. For both determinations, a 25-g load was used for 5 s. For SMH measure-

ment, two rows of five adjacent indentations spaced by 100 µm were made on both sides of the five baseline indentations. The mean values of the five baseline indentations and the ten measurements after treatments were then averaged within a treatment group and the percentage change of surface microhardness (SMC) was calculated. A 25-g load was used, because in a pilot study it was observed that the 50-g load provoked cracking on the surface of the blocks from the control group. It was also found that the value of KHN at the surface of sound enamel was not statistically different using the 25-g load or the 50-g. Thus, with the lower load it would be possible to find differences between treatments without going beyond the enamel resistance during the penetration of the indenter.

After SMH analysis, all blocks were longitudinally sectioned through the center of the exposed enamel. To measure CSMH, the two halves of each block were embedded in acrylic resin and the cut surfaces were exposed and polished. Three rows of 7 indentations each were made, one in the central region of the dental enamel exposed and the other two 100 µm below and above this. The indentations were made at 10 µm up to 130 µm from the outer enamel surface. The mean values at all 3 measuring points at each distance from the surface were then averaged. CSMH values were converted to mineral content (volume % mineral) using the relation: $mineral\ content = 4.3(\sqrt{KHN}) + 11.3^{13}$. The set of data representing each artificial carious lesion (in each enamel block) was curve-fitted. The area under the lesion tracing was calculated by means of the trapezoidal rule (in units of volume percent mineral x µm), and subtracted from the normal enamel value to give the parameter ΔZ⁸. Data of volume % mineral at each distance from the enamel surface were also presented.

Polarized microscopy analysis

After CSMH analysis, the embedded enamel half blocks were sectioned in order to obtain a longitudinal section of 100 µm (± 10). These sections were mounted for examination under a polarizing light microscope at 20 X magnification (DMLSP, Leica, Wetzlar, Germany) after imbibition in deionized water. Digital images were taken with a specific software (Image-Pro Plus, Media Cybernetics, Silver Spring, USA).

Statistical analysis

Analysis of variance (ANOVA) and Student's paired *t*-test were used to compare surface microhardness before and after the treatments. To show the dose-response effect of fluoride the data of %SMC and ΔZ were analyzed by ANOVA and regression. The difference between treatments with regard to baseline SMC and volume % mineral at each distance from enamel surface were evaluated by the Tukey test. For all statistical analysis, SAS (Software Analyses System) was used and the significance limit was set at 5%.

RESULTS

Table 1 shows that baseline SMH was not significantly different among the groups. A significant decrease in SMH was observed after pH-cycling for all treatment groups. A dose-response effect was found between fluoride concentrations in the treatment solutions and %SMC ($R^2 = -0.9048$; $p < 0.0001$, linear correlation), and ΔZ ($R^2 = -0.9858$; $p = 0.0287$, polynomial correlation).

Graph 1 shows that all groups were statistically efficient in reducing the volume % mineral loss in comparison with the control. It was also shown that a caries lesion of approximately 50-60 µm depth was produced.

TABLE 1 - Surface microhardness of enamel blocks (SMH) before and after pH-cycling, percentage of enamel surface microhardness change (%SMC) and mineral loss (ΔZ) according to the treatments (mean ± sd; n = 8).

Treatments	SMH before	SMH after	%SMC*	ΔZ**
DDW	379.4 ± 20.6a,A	42.0 ± 28.0B	-89.1 ± 6.7	1,493.1 ± 289.9
70 ppm F	383.7 ± 32.6a,A	138.5 ± 36.6B	-63.3 ± 10.8	660.5 ± 167.2
140 ppm F	388.2 ± 31.9a,A	133.3 ± 14.0B	-65.5 ± 3.3	445.1 ± 259.1
280 ppm F	372.6 ± 28.6a,A	234.0 ± 41.2B	-36.8 ± 12.0	248.4 ± 151.8

DDW: distilled and deionized water.

Means followed by distinct letters are statistically different ($p < 0.05$). Capital letters show difference between before and after pH-cycling and lower case letters show differences between the treatments. * $R^2 = -0.9048$; $p < 0.0001$; ** $R^2 = -0.9858$; $p = 0.0287$.

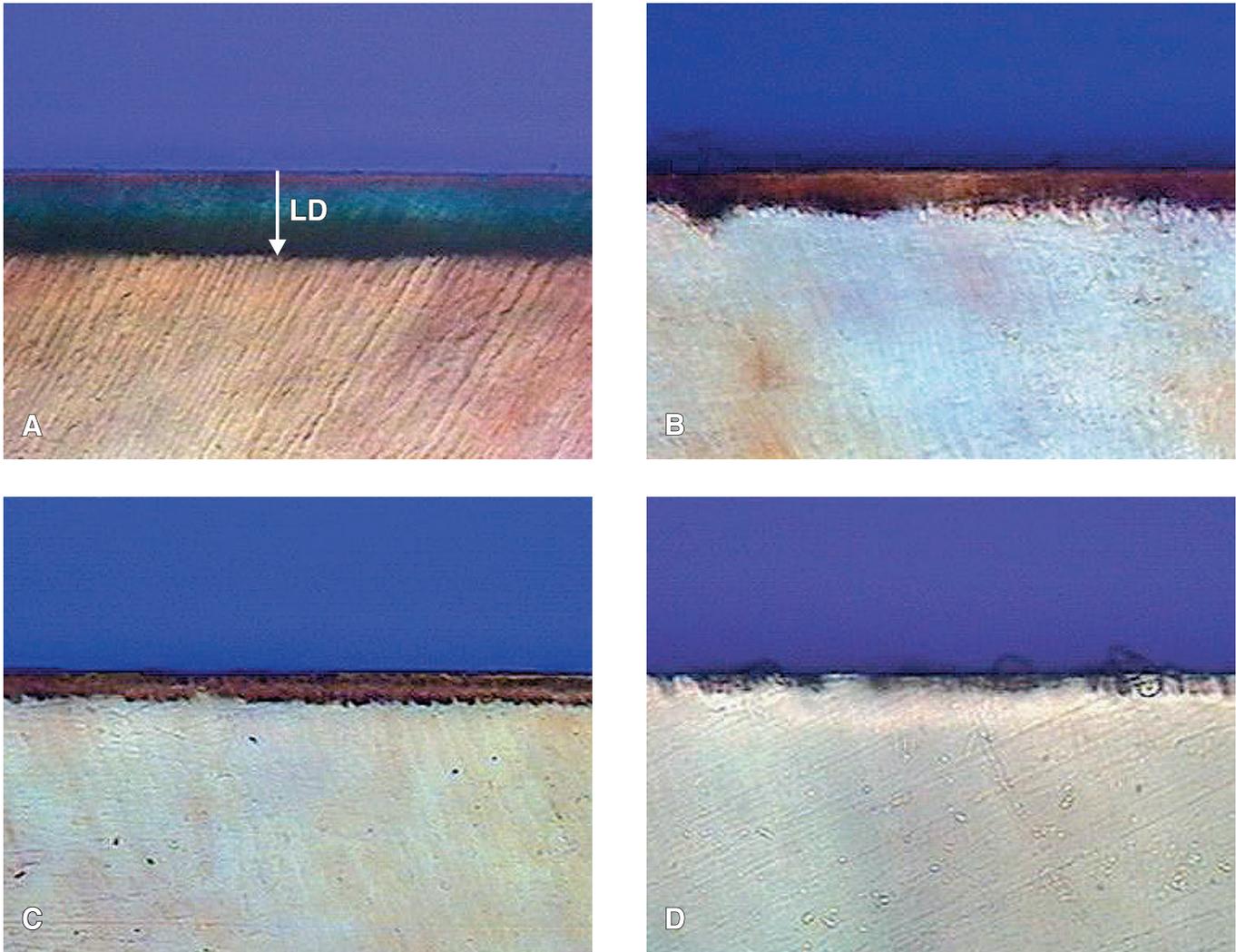
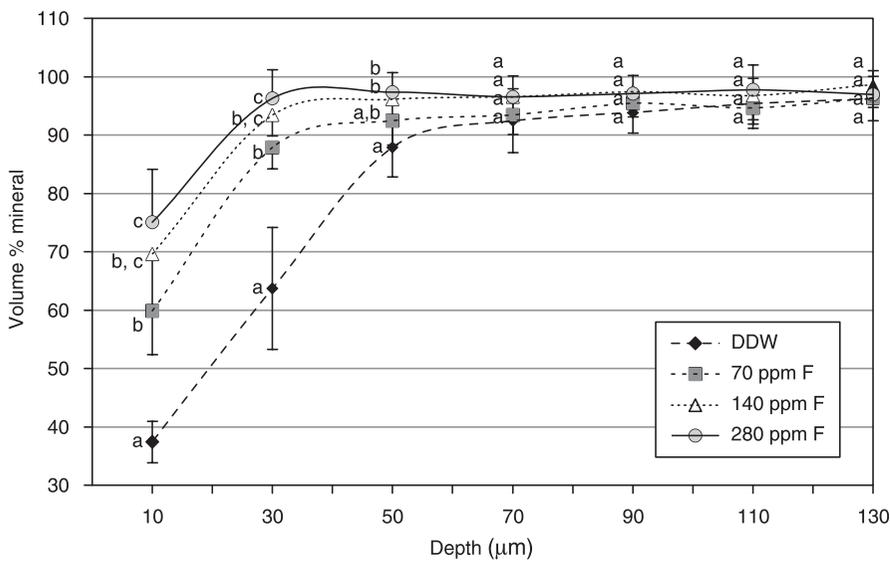


FIGURE 1 - Polarized light microscopy of the caries lesions from the groups control (A) and treated with F at different concentrations: 70 (B), 140 (C) and 280 (D) ppm. LD = caries lesion depth.



GRAPH 1 - Enamel volume percentage mineral according to the treatments and the distance (µm) from the surface. Different letters show statistical significance ($p < 0.05$) among treatments at each distance from the enamel surface, and bars denote standard deviations.

Figure 1 shows typical subsuperficial caries lesions found after the pH-cycling, with preservation of the surface zone. It also shows qualitatively the effect of treatments with F, reducing the depth of the lesions.

DISCUSSION

For the present pH-cycling model, modified from that described by Featherstone et al.⁹ (1986), some alterations were made. Fluoride was added at low concentrations to the demineralizing and remineralizing solutions. Fluoride in low concentration can be present in the solutions as a contaminant of the reagents used^{5,15}. However, the reagents used in the present study did not contain fluoride, which was added to the solutions used and their concentrations were checked with an ion specific electrode. The low fluoride concentrations used (0.05 and 0.03 ppm F) did not interfere in the response of the treatments, as can be observed in Table 1, and were relevant for enamel surface preservation (Figure 1, control). The effect of fluoride at low concentrations is well known¹⁴. Moreover, these concentrations could simulate the remaining fluoride in oral environment after toothbrushing with fluoridated dentifrices and even that found in saliva due to the regular use of fluoridated water. In addition, the pH of the remineralizing solution was raised, and at 7.4 we observed the best conditions for preserving the enamel surface.

In the present study, the demineralization period lasted for 3 hours per day and the total duration of the regimen was 7 days. According to ten Cate, Duijsters¹⁶ (1982), this demineralization period simulates a low cariogenic challenge. However, a 60% volume mineral loss was observed in the DDW group at 10 μ m from the enamel surface (Graph 1). In addition, the lesion depth is similar to that found *in vitro* by Featherstone et al.⁹ (1986), whose study showed a correlation with clinical data of caries development. Therefore, after a one-week treatment, it was possible to evaluate SMH and CSMH at very close distances from the surface. This fact supports the effectiveness of this modified model in simulating early carious lesions and in studying the initiation and progression of the caries process. It should be emphasized that keeping the enamel blocks in the remineralizing solution for additional 2 days after the 5 days of pH-cycling is relevant to preserve the enamel surface layer allowing the SMH determination.

In order to validate this modified model and to mimic the use of fluoridated dentifrices, a dose-

response evaluation was made. Data from %SMC and ΔZ (Table 1) showed a statistically significant negative correlation between the concentration of fluoride in treatment solutions and mineral loss. This suggests that the pH-cycling model proposed is adequate for studying *in vitro* fluoride treatments in early enamel lesions.

It is clearly established that the major interactions of hard tissue substrate with the oral environment occur at the surface layer of the sample. This effect is also observed with fluoride, which reacts with dental tissues at the most superficial layers¹². Thus, the evaluation of early surface changes in dental enamel seems to be sensitive for measuring the fluoride efficacy and may present the greatest predictive value with respect to anti-caries effectiveness¹⁹. Additionally, the pH-cycling models developed up to the present are limited to the study of the changes in the subsurface region and cannot accurately evaluate changes occurring in the outer 25 μ m of surface enamel^{2,7}. Thus, data suggest that the modified pH-cycling model is suitable for evaluating these changes near the enamel surface.

Another modification made was the use of 25-g load instead of 50-g load, which has been currently used to evaluate SMH¹⁹. In the present study, a 25-g load was tested and greatly increased the sensitivity of the method allowing the determination of SMH even when the cariogenic challenge was severe.

In summary, the modified pH-cycling model allowed the evaluation of changes on the outermost enamel layer during caries development and, in addition, a dose-response effect of fluoride reducing enamel demineralization was observed.

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