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pH-cycling Model to Verify the Efficacy of Fluoride-releasing Materials in Enamel Demineralization

E Rodrigues • ACB Delbem
D Pedrini • MSR Oliveira

Clinical Relevance

Due to the commercial availability of many fluoride-releasing materials, it is necessary to develop methods to analyze their cariostatic action, mainly as a per dose of fluoride for effect of response.

SUMMARY

The current study proposes a pH-cycling model to verify the dose-response relationship of fluoride-releasing materials in their ability to reduce *in vitro* demineralization. Sixty bovine enamel blocks (4 x 3 x 3 mm) were selected, using baseline surface microhardness (SMH_1) evaluations at different distances from the enamel sectioned border (150, 300, 450 and 600 µm). Specimens (n=48) were prepared with Z100, Fluroshield and Vitremer at the standard powder/liquid ratio and at a ¼ diluted-powder/liquid ratio. The 12 remaining specimens were used as a control group. The specimens were submitted to a pH-cycling model with high cariogenic challenge. After pH-cycling, final surface microhardness (SMH_2) was assessed to calculate the percentage change of surface microhardness ($\%SMH_c$). Next, the fluoride present in enamel (µg F/mm³) and in pH-cycling solutions (µg F) was measured. Cross-sectional microhardness was done to calculate the mineral content (ΔZ). Data from $\%SMH_c$, ΔZ and µg F were analyzed by analysis of variance ($p<0.05$), while µg F/mm³ analysis was done using the Kruskal-Wallis test. The results showed a correlation between $\%SMH_c$ and µg F/mm³ ($r^2=0.4129$; $p<0.0001$), $\%SMH_c$ and µg F ($r^2=0.4932$; $p<0.0001$), ΔZ and µg F/mm³ ($r^2=0.4573$; $p<0.0001$), µg F/mm³ and µg F ($r^2=0.3029$; $p<0.0001$) and between ΔZ and µg F ($r^2=0.5276$; $p<0.0001$). The pH-cycling model allowed the *in vitro* verification of the dose-response relationship of fluoride-releasing materials in the demineralization of enamel.

Eliana Rodrigues, DDS, PhD, Department of Child and Social Dentistry, UNESP—São Paulo State University, Araçatuba Dental School, SP, Brazil

Alberto Carlos Botazzo Delbem, DDS, MS, PhD, professor, Department of Child and Social Dentistry, UNESP—São Paulo State University, Araçatuba Dental School, SP, Brazil

*Denise Pedrini, DDS, MS, PhD, professor, Department of Surgery and Integrated Clinic, UNESP—São Paulo State University, Araçatuba Dental School, SP, Brazil

Mary Silvia Ramos de Oliveira, DDS, UNESP—São Paulo State University, Araçatuba Dental School, SP, Brazil

*Reprint request: Rua José Bonifácio, 1193, Vila Mendonça—Araçatuba, SP, CEP: 16015-050, Brazil; e-mail: pedrini@foa.unesp.br

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INTRODUCTION

In vitro studies indicate that fluoride-containing materials are associated with a reduction in secondary caries

through anticariogenic action by the inhibition of tooth demineralization and an increase in the potential for tooth structure remineralization.[1-2]

Fluoride-releasing materials have been evaluated in conditions that do not simulate the oral environment, caries dynamic or the real concept of the action of the fluoride mechanism.[3] When discussing the models utilized for testing dental materials, the critical issues are related to dose-response. It is important to understand the F^- concentrations required to reduce and/or prevent lesions localized near the material (secondary caries) and the influence of the release rate vs the pattern of release[4] (any alteration in the model will affect the response of the material examined).

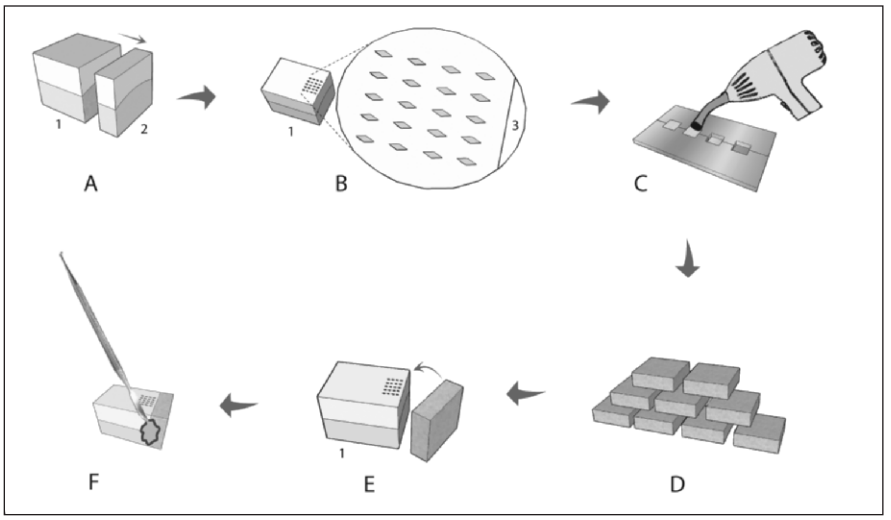

Figure 1. Schematic presentation. *A–Section of the block (1. block 3 x 4 mm used in the research; 2. Piece of the block 1 x 4 mm discarded), B–Four rows of five indentations each—150, 300, 450 and 600 µm from the enamel sectioned border (3), C–Polymerization of sample, D–Samples, E—Samples adapted onto the enamel blocks (1), F—Samples fixed with wax.*

Several *in vitro* studies have shown fluoride release from materials placed in bovine enamel.[5-7] Bovine teeth are commonly used, because these teeth are easy to obtain (slaughterhouse), have a greater area of usable enamel and present a decreased variability in composition when compared to human enamel.[8] Bovine teeth are easy to use and promote a uniform methodology, especially with assessment methods that are simple, such as surface microhardness and enamel uptake of fluoride.[9]

The laboratory models that analyze various applications of fluoride should simulate the balance between demineralization and remineralization using pH-cycling (alternated cycles). Remineralization occurs not only during periods of neutral pH, when minerals precipitate from oral fluids into enamel defects, but also during caries development.[1] It is important to reproduce the *in vitro* conditions of high cariogenic challenge in order to analyze the capacity of fluoride-releasing materials that might interfere with this dynamic process. The pH-cycling model developed by Vieira and others[10] determined a dose-response relationship for fluoride solutions in different concentrations. However, this model was not utilized for fluoride-releasing materials. This is important, because the literature shows several pH-cycling models, but few that demonstrate a dose-response relationship. Thus, the aim of the current study was to use a pH-cycling model proposed in the literature,[10] adapting it to allow for verification of the dose-response relationship of fluoride-releasing materials in reducing *in vitro* demineralization.

METHODS AND MATERIALS

Preparation and Selection of Enamel Blocks

Enamel blocks (4 x 4 x 3 mm) obtained from bovine incisors were stored in a 2% formaldehyde solution for 30 days at room temperature.[11-12] The enamel surface of the blocks was ground flat with water-cooled carborundum discs (600, 800 and 1,200 grit Carbimet Paper Discs, 30-5108-320, Buehler, Lake Bluff, IL, USA) and polished with a felt (Polishing Cloth Buehler 40-7618, Buehler) that was dampened with a diamond spray (1 µm; Extec I Water Based Diamond Permanent Polishing suspension, Enfield, CT, USA), resulting in the removal of about 120 µm of the enamel. Due to the quantity of enamel removed during polishing procedures and curvature of the block, only the central portion of the specimens exhibited a flat surface. Therefore, a 1 mm section of the enamel block (Figure 1A) was prepared to perform the surface microhardness testing for the 150 µm of the enamel border (Figure 1B). Next, baseline surface microhardness (SMH_1) analysis was performed on the enamel blocks using a microhardness tester (Shimadzu Micro Hardness Tester HMV-2000, Shimadzu Corp, Kyoto, Japan) with a Knoop diamond under a 25g load for 10 seconds.[13] Five indentations spaced 100 µm from each other were made at distances of 150, 300, 450 and 600 µm from the enamel sectioned border, for a total of 20 indentations (Figure 1B). Enamel blocks with an average SMH_1 between 350 and 380 KHN were selected for this study.

Sample Preparation and Enamel Block Adaptation

Twelve samples were prepared for each group using the following materials and a metal matrix (3 x 2 x 1 mm): resin composite—Z100 (3M ESPE, St Paul, MN, USA), pit and fissure sealant—Fluroshield (Dentsply Ind and Com, Rio de Janeiro, RJ, Brazil), resin-modified glass ionomer cement mixed at the standard pow-

der/liquid ratio, Vitremer (3M ESPE) and a diluted resin-modified glass ionomer cement—Vitremer ¼ diluted—mixed at ¼ the powder/liquid ratio, with the remaining 12 samples left untreated to serve as a control group. The materials were prepared and placed following the manufacturer's instructions, with the exception of Vitremer ¼ diluted, which had its powder/liquid ratio altered as described. After sample preparation, the materials were randomly attached to the sectioned surfaces of the enamel blocks and fixed with assistance wax (Kota Ind and Com Ltda, São Paulo, SP, Brazil) (Figures 1C, D, E and F). The specimens were then coated with an acid-resistant varnish, except for 4 mm of the enamel block around the sample and the sample surfaces.

pH-cycling

The effect of fluoride in interfering with the dynamic caries process was evaluated. The caries process occurs when the cariogenic challenge is greater during demineralization than during the remineralization process, a model based on Vieira and others.[10] All specimens were immersed in demineralizing solution for six hours—DE (2.0 mmol/L Ca and P, 0.075 mol/L acetate buffer, 0.04 ppm F, 2.2 mL/mm^2 of enamel surface, pH 4.7) and remineralizing solution for 18 hours—RE (1.5 mmol/L Ca, 0.9 mmol/L P, 0.15 mol/L KCl, 0.02 mol/L cacodylate buffer, 0.05 ppm F, 1.1 mL/mm^2 of enamel surface, pH 7.0) for five days at 37°C. The specimens were then submerged in the remineralizing solution for an additional two days prior to surface microhardness analysis.

Microhardness Analysis

The surface microhardness (SMH) and cross-sectional microhardness (CSMH) analyses were performed using a Shimadzu HMV-2000 microhardness tester and a Knoop diamond under a 25g load for 10 seconds. After pH-cycling, the final surface microhardness (SMH_2) was measured. Five indentations, spaced 100 µm from each other and at distances of 150, 300, 450 and 600 µm from the enamel sectioned border, were made (Figure 2B). The percentage change of surface microhardness [$\%SMH_c = 100(SMH_2 - SMH_1)/SMH_1$)] was calculated. After SMH analysis, all blocks were longitudinally sectioned through the center of the exposed enamel (Figure 2C). To measure CSMH, half of each block was embedded in acrylic resin (Buehler Transoptic Powder, Lake Bluff, IL, USA) and the cut surfaces were exposed and polished (Figure 2D). Next, the cross-sectional microhardness test was performed utilizing the same load for SMH testing. Testing was performed on each block, with 32 indentations distributed in four rows of eight indentations at distances of 10, 30, 50, 70, 90, 110, 220 and 330 µm (Figure 2E). The first row began 150 µm from the enamel-sectioned border that remained in contact with the material, with the other three rows performed 300, 450 and 600 µm from the first. CSMH values, up to the 90 µm depth, were converted to mineral content (volume % mineral). The integrated area of sound (Z_1) and pH-cycled (Z_2) enamel was calculated, followed by calculating the mineral content [$\Delta Z=(Z_2 - Z_1)$]. The depth of the lesion was measured as the distance from the surface where the mineral content was 95% of the sound enamel level.

Analysis of Fluoride in Enamel and pH-cycling Solutions

The remaining half of each specimen was sectioned to obtain 2 x 2 x 3 mm blocks of enamel (Figure 2F). The blocks were then attached to mandrels using an ethyl cyanoacrylate adhesive (Super Bonder, Loctite, Itapevi, SP, Brazil) coupled to a handpiece (Dabi-Atlante, Ribeirão Preto, SP, Brazil) that was fixed to the top of a modified microscope (Figure 2G). Fifty micrometers of enamel were removed in crystal polystyrene tubes (J-10, Injeplast, São Paulo, SP, Brazil) containing self-adhesive polishing discs (13-mm diameter) (400 grades of Carbimet Paper Discs, Buehler), based on Weatherell and others.[14] The block surfaces were then washed with 0.4 mL of deionized water inside the tubes and 0.4 mL HCl 1 mol/L was added to the tubes. The tubes were agitated for 30 minutes, then 0.8 mL NaOH 0.5 mol/L was added.[15] Fluoride measurements were performed with an ion-selective electrode (Orion 9609-BN, Orion Research, Inc, Beverly, MA, USA) and a digital ion analyzer

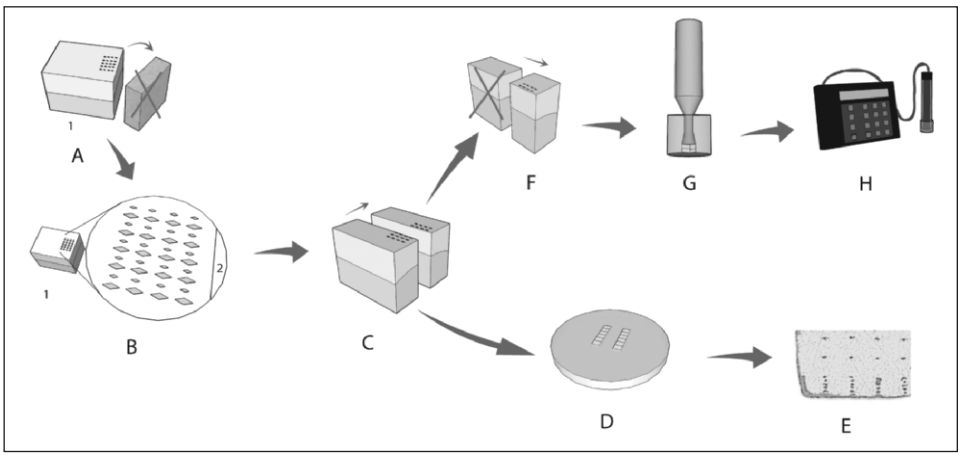

Figure 2. *Diagrammatic representation. A—Removing of the sample, B—Five indentations, spaced 100 µm from each other–150, 300, 450 and 600 µm from the enamel sectioned border (2), C—block longitudinally sectioned, D—Half of each block embedded in acrylic resin, E—Cross-sectional microhardness (4 rows of 8 indentations each), F—Half of each block sectioned again, G—Removing of the enamel in crystal polystyrene tubes, H—Fluoride measurement.*

(Orion 720 A, Orion Research, Inc) calibrated with standards containing from 0.05 µg up to 0.8 µg F/mL in TISAB III ("Total Ionic Strength Adjustment Buffer," Orion Research, Inc) (Figure 2H). The results were expressed in µg F/mm³. For analysis of fluoride in the pH-cycling solutions, TISAB III at a 1:10 ratio (TISAB/solution) and a pH of 5.0 were used. Calibration was performed with standards containing from 0.0625 to 1.0 µg F/mL in TISAB III. The results of the DE and RE solutions were added. The results were expressed in µg F.

Statistical Analysis

For the statistical analysis, GMC version 2002 software was used at a significance limit of 5%. First, the normality and homogeneity of the samples were tested using the Kolmogorov-Smirnov and Cochran tests, respectively. Data from $\%SMH_c$, ΔZ and µg F testing presented a normal and homogeneous distribution and were submitted to ANOVA, followed by the Tukey's test. The values of µg F/mm³ in enamel were heterogeneous and submitted to the Kruskal-Wallis test, followed by the Miller's test. The $\%SMH_c$, ΔZ, µg F and µg F/mm³ data were submitted to regression analysis and adjusted according to tendency.

RESULTS

Table 1 demonstrates that there were no significant differences between $\%SMH_c$ and distances in the control and Z100 groups. Among the fluoride-releasing materials at distances of 300 and 450 µm, there were statistically significant differences for $\%SMH_c$. For the total values mean, significant differences were also found for $\%SMH_c$ among fluoride-releasing materials ($p<0.05$).

Figure 3 demonstrates the correlation between $\%SMH_c$ and µg F/mm³ ($r^2=0.4129$; $p<0.0001$), while, Figure 4 shows the correlation between $\%SMH_c$ and µg F ($r^2=0.4932$; $p<0.0001$). For the fluoride-releasing materials, greater values of fluoride were found in the enamel (µg F/mm³) (Figure 3) and in the pH-cycling solutions (µg F) (Figure 4) at lower values of $\%SMH_c$, and these materials were all statistically different among themselves ($p<0.05$).

There was a significant correlation between µg F and µg F/mm³ ($r^2=0.3029$; $p<0.0001$ (Figure 5). With the mineral content calculation up to 90 µm of depth, sig-

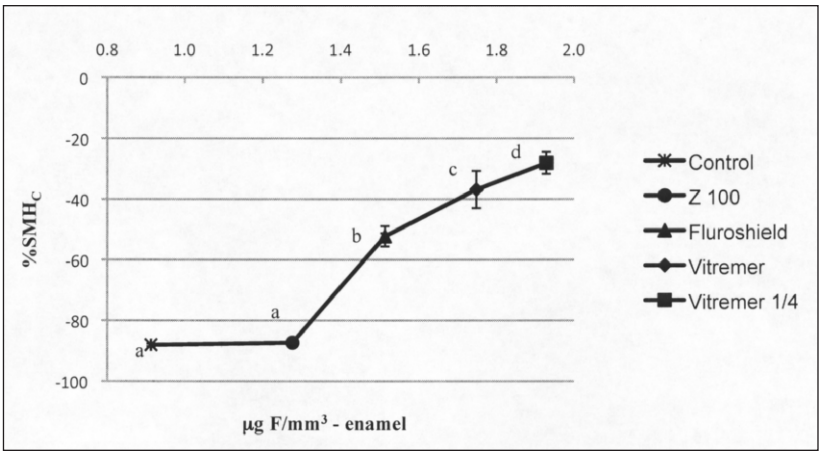

Figure 3. *Percentage change of surface microhardness ($\%SMH_C$) (mean ± se, n=12) per fluoride present in enamel (µg F/mm³), according to groups. Means followed by distinct letters are significantly different (p<0.05).*

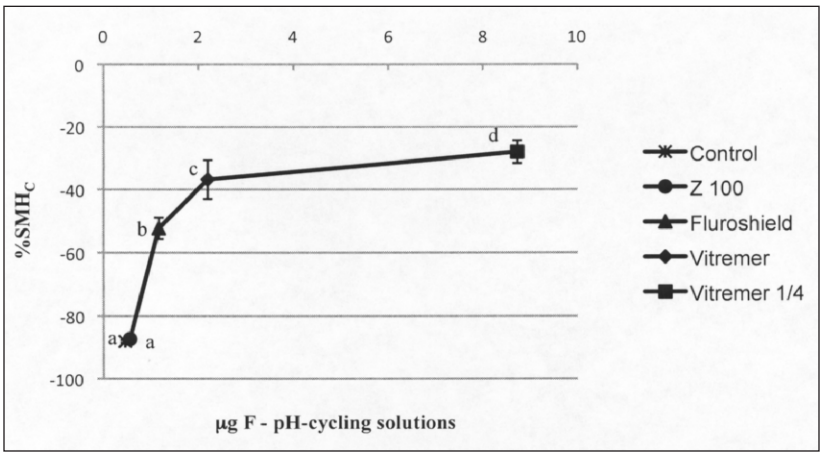

Figure 4. *Percentage change of surface microhardness ($\%SMH_C$) (mean ± se, n=12) per fluoride present in pH-cycling solutions (µg F), according to groups. Means followed by distinct letters are significantly different (p<0.05).*

Table 1: *Percentage Change of Surface Microhardness ($\%SMH_C$) (mean ± sd, n=12) According to Distance and Group*

Distance	Group				
(µm)	Control	Z 100	Fluroshield	Vitremer	Vitremer ¼
150	-89.1 ±7.8a*	-86.4 ±8.4a	-43.4 ±10.7b,d	-23.3 ±6.1e	-20.6 ±5.3e
300	-88.1 ±10.7a	-86.9 ±6.9a	-50.7 ±10.0b,c	-31.9 ±8.9d	-25.4 ±8.6e
450	-87.9 ±10.0a	-89.0 ±5.5a	-56.8 ±10.9c	-39.9 ±9.1d	-28.6 ±9.1e
600	-87.0 ±12.4a	-86.8 ±6.4a	-58.1 ±11.4c	-52.2 ±13.7b,c	-37.6 ±8.0d
Total	-88.0 ±10.1A+	-87.3 ±6.7A	-52.2 ±11.9B	-36.8 ±14.4C	-28.1 ±9.9D

*Means followed by distinct letters are significantly different (5%). *lower case letters: comparison of $\%SMH_C$ between groups and distances. +capital letters: comparison of total values of $\%SMH_C$ among groups.*

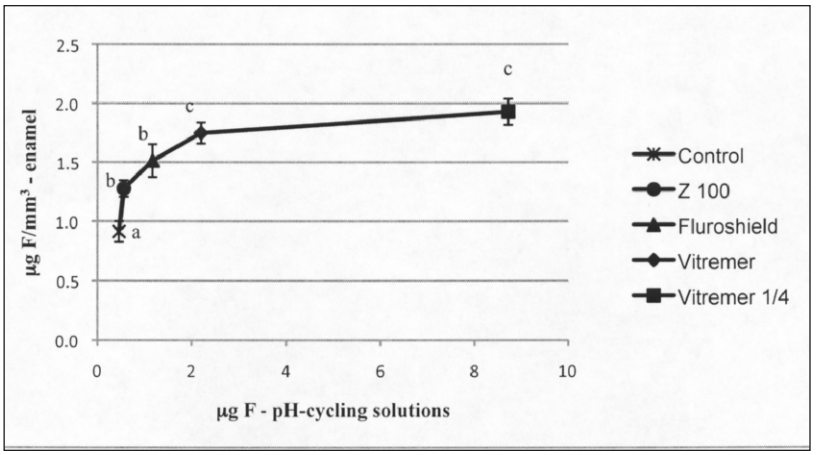

Figure 5. *Fluoride present in enamel (µg F/mm³) (mean ± se, n=12) per fluoride present in pH-cycling solutions (µg F), according to groups. Means followed by distinct letters are significantly different (*p*<0.05).*

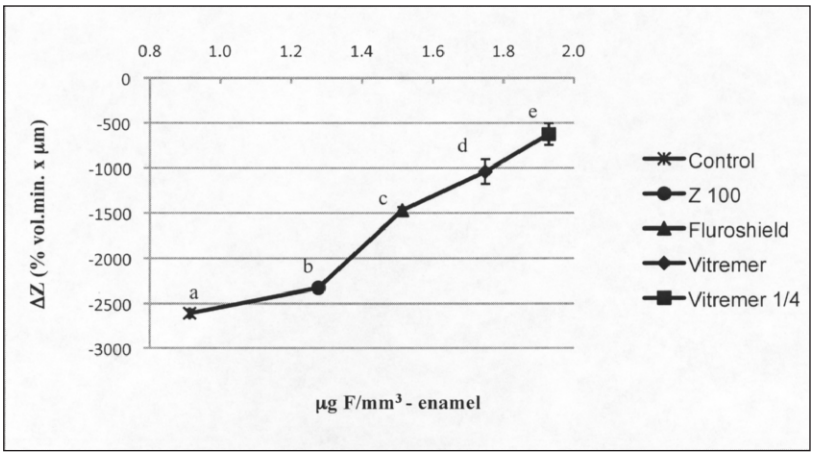

Figure 6. *Mineral content (ΔZ) (mean ± se, n=12) per fluoride present in enamel (µg F/mm³), according to groups. Means followed by distinct letters are significantly different (*p*<0.05).*

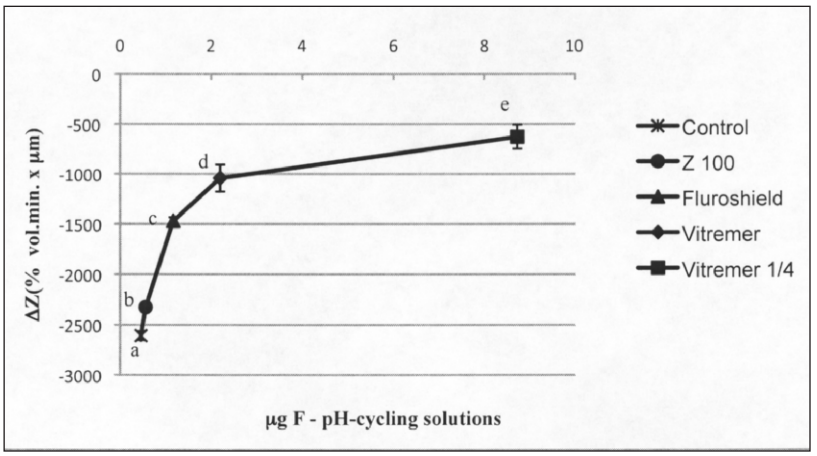

Figure 7. *Mineral content (ΔZ) (mean ± se, n=12) per fluoride present in pH-cycling solutions (µg F), according to groups. Means followed by distinct letters are significantly different (*p*<0.05).*

nificant correlation was observed between ΔZ and µg F/mm³ (r^2=0.4573; p<0.0001). In others words, the greater amount of fluoride present in enamel indicated a reduced mineral loss (Figure 6). A significant correlation was found between ΔZ and µg F (r^2=0.5276; p<0.0001). When there was a greater amount of fluoride in the pH-cycling solutions, there was less mineral loss (Figure 7). For materials that released a greater quantity of fluoride (Vitremer and Vitremer ¼ diluted), there was a reduction in ΔZ with greater distance. There was a significant difference in ΔZ at distances of 150 µm and 600 µm for these materials when analyzed individually with each material. Statistically similar ΔZ results were observed for the control (without material) and Z100 (without fluoride) groups (Figure 8).

DISCUSSION

The establishment of a sensitive methodology to assess the dose-response relationship for fluoride-releasing materials is of interest to the dental community. The initial studies that evaluated the *in vitro* effects of fluoride on the demineralization and remineralization of enamel were performed with static models.[16] ten Cate and Duijsters[17] introduced the concept of a dynamic model, with alternating periods of demineralization and remineralization. This *in vitro* model was extremely useful for determining the effects of topical fluoride, achieving results similar to studies performed *in vivo*.[16] Models that differ from pH-cycling have been presented, with the suggestion that those *in vitro* studies had an increased importance for studies on the progression or remineralization of dental caries.

The pH-cycling model, developed to determine the dose-response relationship of fluoride products (solutions and dentifrices), uses bovine teeth,[10] based on the advantages of using bovine teeth in place of human teeth.[8] These advantages include the fact that bovine enamel responds quicker to a cariogenic challenge or remineralizing conditions than human enamel,[18] enabling its use in *in vitro* studies. Factors influencing *in vitro* lesions produced in bovine enamel, such as time of demineralization, pH of solution, time of remineralization, composition of the demineralized and remineralized solutions and duration of pH-cycling, were altered to establish the methodology allowing the assessment of the dose-response relationship of fluoride products.[10]

In the current study, other changes were necessary, in addition to the alterations defined by Vieira and others,[10] to establish a protocol for surface microhardness analysis (SMH). Knoop microhardness testing was performed at distances of 150, 300, 450 and 600 µm inside the enamel-sectioned border to define the distance where there was a difference between fluoride-releasing materials that was not related to outside factors. For example, measurements of 150 µm were more susceptible to trine and other alterations during the process of block isolation, making differential evaluation difficult. Therefore, in Table 1, the distances of 300 µm and 450 µm were great enough that differences could be observed between fluoride-releasing materials with a lower incidence of outside factors, allowing for an improved precision of analysis. Several *in vitro* studies[7,19-24] demonstrated an increased inhibition of demineralization from the enamel area adjacent to the material. However, when trying to compare materials that release much fluoride, it was difficult to determine the differences between fluoride-releasing materials at a distance of 150 µm. A similar issue is observed when using a greater distance of material (600 µm) where there is a decrease in the effect of fluoride between the various materials, which is hard to differentiate. Thus, the distances of 300 µm and 450 µm are more favorable for discerning the differences between the materials.

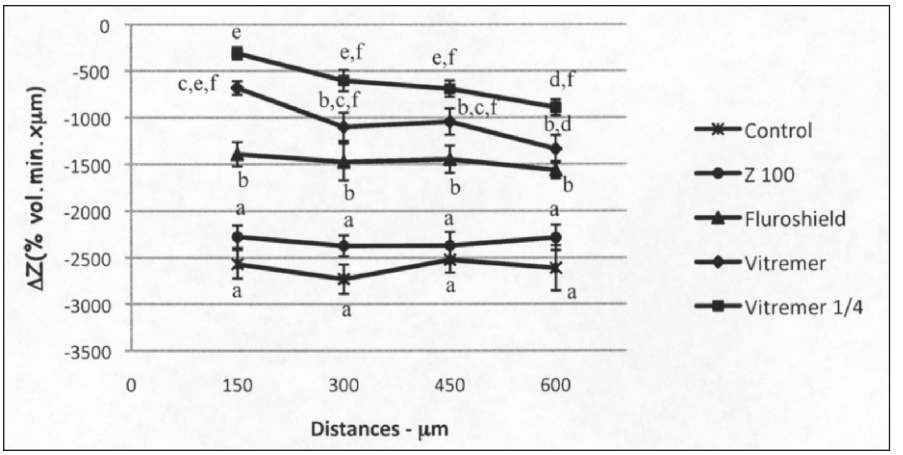

Figure 8. *Mineral content (ΔZ) (mean ± se, n=12) per distance of the material (µm). Means followed by distinct letters are significantly different (p<0.05).*

Some studies performed *in vitro* or *in situ* prepared conventional cavities in enamel for tests involving dental materials.[6,19,25-27] Instead of placing the material in a cavity in the current study, the material was attached to the enamel blocks to allow surface microhardness testing adjacent to the material. Placing the material in a cavity leads to covering of the baseline impressions. This would not allow final surface microhardness to be implemented.

The surface microhardness test was used as a simple, quick method of analysis, which also allowed for performance of other tests. This test evaluated the alterations of microhardness in each group to verify the dose-response relationship in a pH-cycling model. However, it is necessary that the control group presents a reduction in surface microhardness of approximately 80%.[10,28] This parameter allows the accuracy of the model utilized; whereas, if higher or lower mineral loss occurred, the inhibition of demineralization and the results of the study would not be significant. The current study presented a mean change in surface microhardness of 88% in the control group. For the cross-sectional microhardness test, the highest fluoride concentration was present in the pH-cycling solutions and was incorporated into dental enamel, while presenting the lowest mineral loss (ΔZ). Therefore, for the current pH-cycling model, the surface and cross-sectional microhardness tests were suitable to assess the fluoride-releasing materials.

The release of fluoride from restorative materials and the consequential alterations in fluoride content of the enamel were assessed by Norman and others,[29] showing that the materials did not always release more fluoride for incorporation in enamel. A correlation was observed between the fluoride present in enamel and that released into the pH-cycling solutions. The utilization of a pH-cycling model that simulates a cariogenic challenge promotes the determination of a dose-response relationship similar to what is found in a clinical setting. The incorporation of fluoride into enamel is not uniform, so that the distribution of fluoride into enamel does not remain limited to just the area next to the material (where the indentations of surface microhardness testing are performed). However, for comparison purposes, the microabrasion of enamel next to the material with an area of wear approximating the 2 x 2 mm/block must be accomplished. Thus, standardizing the size of the area of wear is important for reducing differences among blocks of a same group.

There was a significant correlation between µg F and µg F/mm^3, %SMH$_C$, µg F/mm^3 and ΔZ and µg F/mm^3. This fact can be linked to the appearance of a higher level of fluoride in enamel that had been removed by microabrasion (50 µm), corresponding to an area of greater mineral loss. While the analysis of fluoride content in enamel cannot be accepted as a critical indicator of the mechanism of action for fluoride, it is an excellent measure and demonstrates that keeping the fluoride uptake in enamel lesions was an important part of the testing profile.[30]

The result of the quantitative analysis of fluoride contained in the pH-cycling solutions for this study was expected (significant difference in values among fluoride-releasing materials). The result was expected, because of the composition of the pH-cycling solutions (DE/RE) that simulated the clinical application involving the surface interactions, ionic strength, environmental pH and degree of saturation, all of which contributed to the release of fluoride ions.[3] The evaluation of this pH-cycling model tested the dose-response relationship through surface microhardness analysis, cross-sectional microhardness analysis, and fluoride uptake in enamel and pH-cycling solutions. When the model was used to compare the effectiveness of dental materials, the surface microhardness test was found to be more suitable with regard to equipment, ease of application and inexpensive cost.

The effectiveness of fluoride-releasing materials in reducing the demineralization process of enamel must be tested *in vitro*, preceding *in situ* and *in vivo* studies. For *in vitro* studies, the pH-cycling models must reproduce the natural caries process and allow for verification of the dose-response relationship as the fluoride concentration varies.[30-31] Utilization of the pH-cycling model, including the implemented alterations in the current study, allowed the verification of a dose-response relationship using cross-sectional surface microhardness and by determining the fluoride present in enamel and pH-cycling solution.

CONCLUSIONS

The pH-cycling model proposed in this study allowed for the determination of a dose-response relationship of fluoride-releasing materials in the demineralization of enamel.

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