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Enamel Remineralization by Fluoride-Releasing Materials: Proposal of a pH-Cycling Model

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This study proposes a pH-cycling model for verifying the dose-response relationship in fluoride-releasing materials on remineralization *in vitro*. Sixty bovine enamel blocks were selected for the surface microhardness test (SMH_1). Artificial caries lesions were induced and surface microhardness test (SMH_2) was performed. Forty-eight specimens were prepared with Z 100, Fluroshield, Vitremer and Vitremer ¼ diluted - powder/liquid, and subjected to a pH-cycling model to promote remineralization. After pH-cycling, final surface microhardness (SMH_3) was assessed to calculate percent recovery of surface microhardness ($\%SMH_R$). Fluoride present in enamel (µg F/mm^3) and in the pH-cycling solutions (µg F) was measured. Cross-sectional microhardness was used to calculate mineral content (ΔZ). There was no significant difference between Z 100 and control groups on analysis performed on - $\%SMH_R$, ΔZ, µg F and µg F/mm^3 ($p>0.05$). Results showed a positive correlation between $\%SMH_R$ and µg F/mm^3 ($r=0.9770$; $p=0.004$), $\%SMH_R$ and µg F ($r=0.9939$; $p=0.0000001$), ΔZ and µg F/mm^3 ($r=0.9853$; $p=0.0002$), ΔZ and µg F ($r=0.9975$; $p=0.0000001$) and between µg F/mm^3 and µg F ($r=0.9819$; $p=0.001$). The pH-cycling model proposed was able to verify *in vitro* dose-response relationship of fluoride-releasing materials on remineralization.

Key Words: tooth remineralization, dental materials, fluoride.

INTRODUCTION

The role of *in vitro* models is to facilitate the generation of enough quantitative data to give confidence for investigators moving into in clinical trials (1). pH-cycling protocols have served as powerful means for providing mechanistic insights into the caries process and preventive measures, and applications of these models for these purposes continue today. The pH-cycling test involves artificial enamel lesions treated daily with oral care products and cycled in de- and remineralizing solutions to mimic oral pH-fluctuation patterns.

According to the Council on Dental Therapeutics (2), caries models are needed to determine the dose-response relationship of fluoride products in enamel remineralization. Caries models with human teeth are commonly used to measure the dose-response relationship and its influence on caries inhibition and enamel remineralization (3). There are few studies on enamel remineralization, and most of them used high cariogenic challenge models and assessed demineralization inhibition, rather than enamel remineralization involving caries lesions. Vieira et al. (4) described a pH-cycling model that determined the dose-response relationship between fluoride and remineralization, using solutions with different fluoride concentrations.

An *in vitro* model that demonstrates the potential of fluoride incorporated into the material and its response to the adjacent enamel is very important to evaluate the remineralization of caries lesions and/or reduction of secondary caries. This study proposes a pH-cycling model to verify the dose-response relationship of fluoride-releasing material on enamel remineralization.

MATERIAL AND METHODS

Preparation of Enamel Blocks

Enamel blocks (4 x 4 x 3 mm) were obtained from bovine incisor teeth and stored in 2% formaldehyde for

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30 days at room temperature (5). The enamel surface was polished and blocks were cross-sectioned at 1 mm from the edge of each block (Fig. 1A). The larger part (3 x 4 x 3 mm) of the block was used in the experiment, the smaller part discarded.

Surface Microhardness Analysis

Blocks were submitted to baseline surface microhardness (SMH_1) analysis, using a microhardness tester (Shimadzu Microhardness Tester HMV-2000; Shimadzu Corp., Kyoto, Japan), with a Knoop diamond, under a load of 25 g for 10 s (6). Five indentations spaced 100 µm away from each other were made at distances of 150, 300, 450 and 600 µm from each specimen edge, up to a total of 20 indentations (Fig. 1A) (7). Enamel blocks with an average SMH_1 between 350 and 380 KHN were included in the study.

Artificial Carious Lesions

Lateral surfaces of each specimen, except for the top enamel surface, were coated with acid-resistant varnish (area = 12 mm^2) and artificial caries lesions were created by immersing each enamel block in 24 mL of demineralizing solution (1.3 mmol/L Ca, 0.78 mmol/L P, 0.05 mol/L acetate buffer, 0.03 µg F/mL, pH 5.0) at 37^oC for 16 h (8). This method produces a subsurface enamel demineralization without surface erosion (9). Surface microhardness (SMH_2) was measured again and other 20 indentations created among the initial ones (Fig. 1A).

Dental Material Preparation and Enamel Block Adaptation

Using a metal matrix (3 x 2 x 1 mm), twelve samples were prepared for each group involving the following materials: composite resin Z 100 (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 Vitremer (3M/ESPE); resin-modified glass ionomer cement Vitremer (diluted at a powder/liquid ratio of 1:4); and 12 untreated blocks (control). Materials were prepared and applied following manufacturers' instructions, except for the diluted material (Vitremer), and then light activated for 40 s (Fig. 1B). Following preparation, materials were attached to the enamel blocks with utility wax (Kota Ind. and Com. Ltda, São Paulo, SP, Brazil) (Fig. 1C). The specimens were again laterally coated with acid-resistant varnish, except for the top surface of the enamel (12 mm^2) and material (3 mm^2) (7).

pH-Cycling Test

The effect of fluoride on the remineralization of the artificial caries lesions was evaluated. The cycle alternated between the demineralizing (DE) solution (2.0 mmol/L Ca and P, 0.075 mol/L acetate buffer, 0.03 µg F/mL, pH 4.7; 0.75 mL/mm^2) and the remineralizing (RE) solution (1.5 mmol/L Ca, 0.9 mmol/L P, 0.15 mol/L KCl, 0.02 mol/L cacodylate buffer, 0.04 µg F/mL, pH 7.0; 0.25 mL/mm^2) during 6 days, described as follows: 8 a.m. - all specimens were immersed in the RE solution; 12:00 - specimens were washed with deionized water and immersed in the DE solution; 2 p.m. - specimens were washed and immersed in the same RE solution used at 8 a.m.; 4 p.m. - specimens were washed and immersed in a new RE solution, in which they were kept until 8 a.m. the next day, when the RE solution was replaced again as a new cycle started (Fig. 1C).

After pH cycling (6 days), the material attached to each enamel block was removed and discarded. The blocks were then submitted to final surface microhardness (SMH_3) test as another 20 indentations spaced 100 µm apart from each other were created (Fig. 1C). The percentage recovery of the surface microhardness ($\%SMH_R=[(SMH_3 - SMH_2)/(SMH_1 - SMH_2)]x100$) was calculated.

Cross-Sectional Microhardness Analysis

All blocks were longitudinally sectioned into two halves. To measure cross-sectional microhardness (CSMH), one half of each block was embedded in acrylic resin (Buehler Transoptic Powder, Lake Bluff, IL, USA) and the binding surface between block and the material discarded was then polished to allow for the cross-sectional microhardness test at a load of 25 g for 10 s. Thirty-two indentations (4 rows of 8 indentations each) were created at distances 10, 30, 50, 70, 90, 110, 220 and 330 µm away from the edge of the block. Rows were spaced 150 µm apart (Fig. 1E) (7). CSMH values were considered significant at distances up to 90 µm, at all of which the mineral content returned to 95% of the sound enamel level. Mineral content (ΔZ) was then measured by calculating the integrated area of the dental enamel before (Z_1) and after (Z_2) pH cycling [$\Delta Z=(Z_2 - Z_1)$].

Analysis of Fluoride in Enamel and pH-Cycling Solutions

The other half of each specimen was sectioned again into two halves to obtain 2 x 2 x 3 mm blocks, all of which were attached to dental mandrels using ethyl cyanoacrylate adhesive (Super Bonder, Loctite, Itapevi, SP, Brazil). The mandrels were coupled to a dental low-speed handpiece (Dabi-Atlante, Ribeirão Preto, SP, Brazil), which was attached to a modified microscope (Fig. 1D) (10). Fifty micrometers of enamel were then obtained from each block using a crystal polystyrene tube (J-10, Injeplast, São Paulo, SP, Brazil) containing a self-adhesive polishing disc (600-grid; 13-mm diameter) (Silicon-Carbide, Buehler) (11). Each block was washed with 0.4 mL of deionized water, which was kept in the tube and another 0.4 mL of 1 mol/L HCl was added. The tubes were then shaken for 30 min and 0.8 mL of 0.5 mol/L NaOH was added (10). Enamel fluoride uptake was measured by using an ion-selective electrode (Orion 9609-BN; Orion Research, Inc., Beverly, MA, USA) and a digital ion analyzer (Orion 720 A; Orion Research, Inc.), calibrated with fluoride concentrations ranging from 0.05 up to 0.8 μg/mL and TISAB III ("Total Ionic Strength Adjustment Buffer", Orion Research, Inc). For the fluoride analysis concerning the pH-cycling solutions, calibration was carried out with concentrations ranging from 0.025 to 0.4 μg F/mL (TISAB III/solution, 1:10 ratio, pH 5.0) (Fig. 1F). Results regarding de- and remineralizing solutions were summed up (6 days of pH-cycling) and expressed as μg F.

Statistical Analysis

Computer software (GMC, version 2002) was used for statistical analysis at 5% significance level. Surface microhardness recovery (%SMH_R) and mineral content (ΔZ) data had normal (Kolmogorov-Smirnov) and homogeneous (Cochran) distribution and were subjected to ANOVA and Tukey's test. Data regarding to enamel (μg/mm^3)/solution (μg) fluoride concentrations had non-normal distribution and were analyzed by the Kruskal-Wallis and Miller's tests. %SMH_R, ΔZ, μg F and μg F/mm^3 data were subjected to regression analysis and adjusted according to their tendency.

RESULTS

Table 1 shows the percentage of surface microhardness recovery (%SMH_R) according to indentations and groups. No statistically significant difference ($p>0.05$) was observed for %SMH_R regarding the distances. Significant differences ($p<0.05$) were found among the fluoride-releasing materials, concerning indentations at distances of 300 and 450 μm.

The regression test showed a positive correlation between %SMH_R and μg F/mm^3 (r=0.9770; p=0.004 - Fig. 2), %SMH_R and μg F (r=0.9939; p=0.0000001 - Fig. 3), as well as between μg F and μg F/mm^3 (r=0.9819; p=0.0010 - Fig. 4). In relation to the fluoride-releasing materials, %SMH_R values concerning the enamel fluoride uptake (Fig. 2) were similar to those regarding the de- and remineralizing solutions (Fig. 3). The fluoride concentrations in both enamel and pH-cycling solutions were found to increase proportionally (Fig. 4).

A positive correlation was observed between ΔZ and μg F/mm^3 (r=0.9853; p=0.0002) (Fig. 5) and ΔZ and

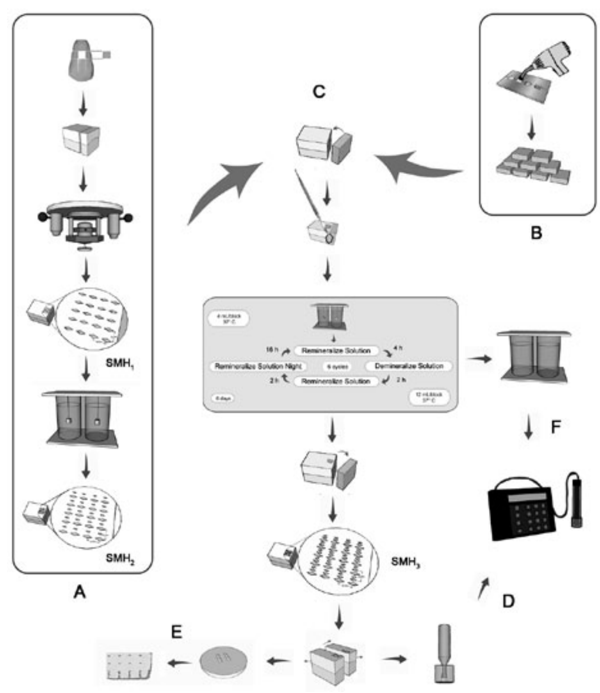

Figure 1. Schematic diagram of the methodology. (A) Preparation of the enamel blocks, baseline surface microhardness analysis (SMH_1), production of artificial carious lesions and surface microhardness analysis (SMH_2); (B) Dental material preparation; (C) Enamel block adaptation (material/enamel block), pH-cycling model, material was dismounted and discarded, final surface microhardness analysis (SMH_3) and blocks bisected longitudinally; (D) Microabrasion (analysis of fluoride in enamel); (E) Cross-sectional microhardness (CSMH) and (F) Analysis of fluoride in pH-cycling solutions.

μg F (r=0.9975; p=0.0000001) (Fig. 6) as lower values of mineral loss were attributed to larger values of fluoride found in the enamel and pH-cycling solutions.

Figure 7 shows significant differences ($p<0.05$) among fluoride-releasing materials regarding ΔZ, considering indentations at distances of 150 and 300 μm.

DISCUSSION

ten Cate and Duijsters (12) introduced a dynamic model concept with alternating periods of de- and remineralization. Chemical analysis during pH-cycling provides kinetic data on mineral uptake and loss and how this is affected by therapeutic agents. Various models for pH cycling have been proposed and *in vitro* studies (4,5,10,13) have been designed to investigate the development and/or remineralization of dental caries.

In this study, a pH-cycling model was used to determine the dose-response (fluoride/remineralization) relationship using bovine teeth (4), as well as to analyze the ability of fluoride-releasing materials to remineralize dental enamel. When compared to bovine teeth, human teeth have a highly variable composition and a curvature that makes it difficult to obtain flat surface enamel and to verify the dose-response relationship between fluoride and dental enamel remineralization (14). Caries lesion development rates concerning bovine permanent teeth are found to be similar to those of human primary teeth (13,15). Although bovine enamel may differ from human enamel in some aspects, it is widely used to investigate anticariogenic and remineralizing agents *in vitro* involving subsurface lesions (15). A methodology was created to measure the enamel surface microhardness in order to verify differences among the tested materials.

Distances at 150, 300, 450 and 600 μm, determined by the surface microhardness test, allowed verifying the dose-response relationship considering all fluoride-releasing materials, showing higher remineralizing efficacy in areas closer to the restoration margins (16). Significant difference was observed among the fluoride-releasing materials concerning $\%SMH_R$; however, no significant difference was found between distances in each group, suggesting that distances at 300 and 450 μm might be more reliable for the $\%SMH_R$ analysis because they are much less likely to be affected by cracks and other alterations that could occur during specimen preparation.

In some *in vitro* or *in situ* studies (16-18), conventional cavities were made on dental enamel to test different materials. In the present study, no cavities were made to allow for more than two surface microhardness tests. It is well known that placing the material in the cavity could result in material overflowing, covering initial and post-demineralization indentations and, consequently, hindering subsequent microhardness tests.

Cross-sectional microhardness tests allow for evaluating body lesions in depth (16,19). In the present study, significant differences were found among the fluoride-releasing materials; a higher mineral gain was observed for distances at 150 and 300 μm, probably due to the fact that they lied closer to the material. The surface and cross-sectional microhardness tests showed better results for the distance at 300 μm concerning fluoride-releasing materials.

The potential for remineralization of enamel in the *in vitro* model was demonstrated using fluoride-releasing materials - Vitremer and Fluroshield. With regard to fluoride release and uptake, the results of this study were consistent with findings in the literature (18).

Table 1. Percentage recovery of surface microhardness ($\%SMH_R$) (mean ± SD, n=12) according to the distances and the groups.

Distance (μm)	Control	Z 100	Fluroshield	Vitremer	Vitremer ¼
150	11.7 ± 6.2 a,e*	10.2 ± 7.6 a	20.9 ± 6.3 b,e,f	39.7 ± 7.3 c,d	46.1 ± 7.6 d,h
300	13.0 ± 5.5 a,e	10.5 ± 8.6 a	21.9 ± 7.1 b,f	37.7 ± 6.0 c,h	48.8 ± 6.8 d
450	14.1 ± 6.7 a,e	11.4 ± 6.9 a,e	22.6 ± 8.7 b,f	33.0 ± 6.8 c,g	47.6 ± 5.6 d,h
600	14.6 ± 5.9 a,e	11.8 ± 7.1 a,e	23.0 ± 8.9 b,f,g	30.3 ± 7.6 c,f	43.2 ± 7.2 d,h
Total	13.3 ± 1.3 A+	11.0 ± 1.4 A	22.1 ± 0.9 B	35.2 ± 4.3 C	46.4 ± 2.4 D

Means followed by different letters are significantly different (Tukey; $p<0.05$). *lower case letters: comparison of $\%SMH_R$ between groups and distances. +uppercase letters: comparison of total values of $\%SMH_R$ among groups.

The resin-modified glass ionomer (Vitremer) released significantly more fluoride than the sealant (Fluroshield) and this more than composite resin.

The desire to evaluate a material not commonly used in studies contributed to the choice of Vitremer - diluted at a powder/liquid ratio of 1:4. When compared with other fluoride-releasing materials, diluted Vitremer obtained the best results of remineralization of enamel lesions. This performance may be due to differences in the powder/liquid ratio; a lower ration results in increased solubility, and hence greater fluoride release (20).

It would be wise to consider the percentage

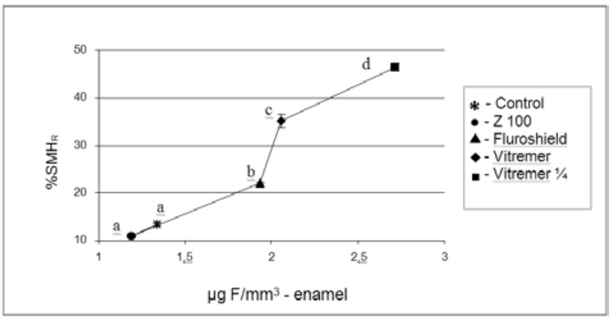

Figure 2. Percentage recovery of surface microhardness (%SMH_R) (mean ± SE, n=12) *per* fluoride present in enamel (µg F/mm^3), according to groups. Means followed by different letters are significantly different (Miller test; p<0.05).

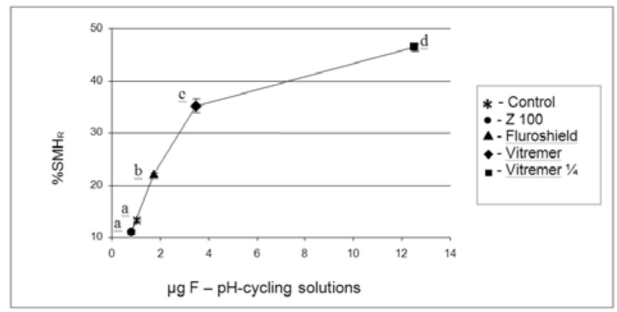

Figure 3. Percentage recovery of surface microhardness (%SMH_R) (mean ± SE, n=12) *per* fluoride present in pH-cycling solutions (µg F), according to groups. Means followed by different letters are significantly different (Miller test; p<0.05).

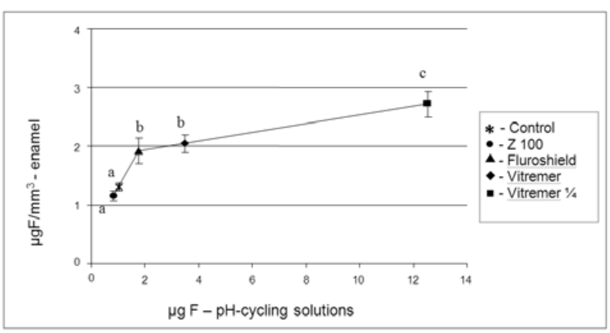

Figure 4. Fluoride present in enamel (µg F/mm^3) (mean ± SE, n=12) *per* fluoride present in pH-cycling solutions (µg F), according to groups. Means followed by distinct letters are significantly different (Miller test; p<0.05).

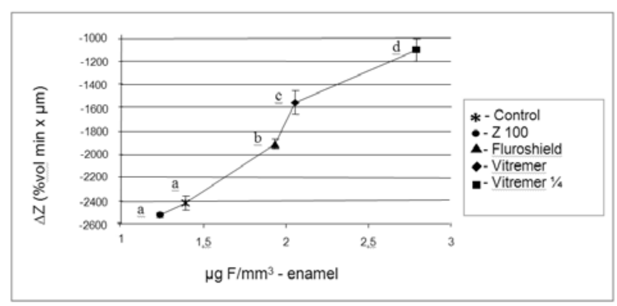

Figure 5. Mineral content (ΔZ) (mean ± SE, n=12) *per* fluoride present in enamel (µg F/mm^3), according to groups. Means followed by distinct letters are significantly different (Miller test; p<0.05).

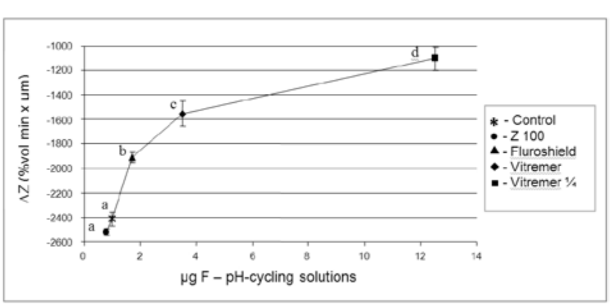

Figure 6. Mineral content (ΔZ) (mean ± SE, n=12) *per* fluoride present in pH-cycling solutions (µg F), according to groups. Means followed by different letters are significantly different (Miller test; p<0.05).

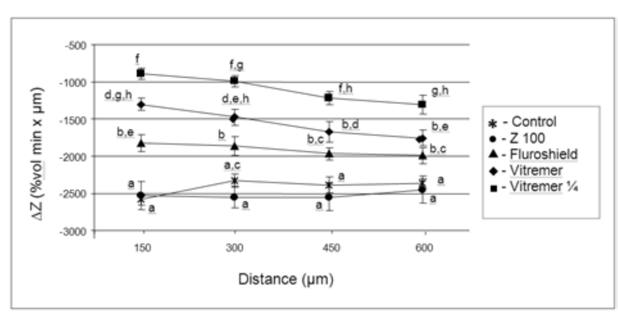

Figure 7. Mineral content (ΔZ) (mean ± SE, n=12) *per* distance of the material (µm). Means followed by different letters are significantly different (Tukey's test; p<0.05).

of mineral loss in the artificial caries lesions and the enamel remineralization values obtained in the control to measure the dose-response (fluoride/remineralization) relationship. To validate a remineralization model, the percentage of alteration in surface microhardness in bovine enamel should be close to 80% right after artificial caries lesions are made and enamel remineralization between 10 and 20% should be obtained in the control. If mineral loss is higher than 80%, remineralization might not occur; if it is lower than 80%, it would be impossible to find differences among materials. The pH-cycling solutions used in the present study reproduced adequate conditions to induce fluoride release, favoring enamel remineralization.

Few studies in the literature evaluated fluoride-releasing materials to induce remineralization; most studies assessed the ability of these materials to inhibit demineralization. The *in vitro* model described in the present study should be further used to investigate fluoride-releasing materials and their efficacy in remineralizing dental enamel.

In conclusion, the proposed pH-cycling model showed a satisfactory dose-response (fluoride/remineralization) relationship, as the fluoride-releasing materials were proven effective in remineralizing dental enamel.

RESUMO

Este trabalho propôs um modelo de ciclagem de pH verificando a relação dose-resposta de materiais que liberam flúor na remineralização *in vitro*. Foram selecionados 60 blocos de esmalte bovino pelo teste de microdureza de superfície (SMH_1). Realizou-se indução de cárie e microdureza de superfície pós-cárie (SMH_2). Corpos-de-prova (n=48) dos grupos Z 100, Fluroshield, Vitremer e Vitremer diluído ¼ foram fabricados e submetidos à ciclagem de pH para promover a remineralização. Após, avaliou-se a microdureza de superfície final (SMH_3) para cálculo da porcentagem de recuperação da microdureza de superfície ($\%SMH_R$). Determinou-se o flúor presente no esmalte (μg F/mm^3) e nas soluções de ciclagem (μg F). O teste de microdureza em secção longitudinal foi realizado para cálculo do conteúdo mineral (ΔZ). Entre os grupos controle e Z100 não houve diferença significativa nas análises realizadas - $\%SMH_R$, ΔZ, μg F e μg F/mm^3 (p>0,05). Houve correlação positiva entre a $\%SMH_R$ e μg F/mm^3 (r=0,9770; p=0,004), $\%SMH_R$ e μg F (r=0,9939; p=0,0000001), ΔZ e μg F/mm^3 (r=0,9853; p=0,0002), ΔZ e μg F (r=0,9975; p=0,0000001) e também entre μg F/mm^3 e μg F (r=0,9819; p=0,001). O modelo de ciclagem de pH proposto foi adequado para verificar relação dose-resposta *in vitro* de materiais que liberam flúor na remineralização.

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