EFECTO DE UN DENTÍFRICO EXPERIMENTAL CONTENIENDO MICROPARTÍCULAS DE BIOSILICATO® EN SUPERFICIE DE ESMALTE EROSIONADO IN VITRO: ESTUDIO COMPARATIVO.
THE EFFECT OF AN EXPERIMENTAL DENTIFRICE CONTAINING BIOSILICATE® MICRON-PARTICLES ON ERODED ENAMEL SURFACE IN VITRO: A COMPARATIVE STUDY.

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Resumen
Objetivos: Este estudio evaluó in vitro el efecto de un dentífrico experimental (DE) con micropartículas de una vitrocerámica bioactiva - Biosilicato® (Mp-Bios) en la fórmula, para tratar la superficie erosionada del esmalte. Material y métodos: sesenta muestras de esmalte dental bovino (EDB) (4x4x3mm) fueron separadas en grupos experimentales (n = 10 por grupo): G1-control (agua destilada), G2-DE con Mp-BIOS 7.5%; G3-DE sin Mp-BIOS; G4- DE con monofluorfosfato - 1500ppm; G5- DE con NaF- 500ppm, y G6- DE con 7,5% de micropartículas de Bioglass 45S5 y sometidas a espectroscopia (FTIR), microscopía electrónica de barredura (MEB) y análisis de microdureza superficial (MS) , antes y después de 7 días de desafíos erosivos (inmersión en ácido láctico, pH = 4,3, 1 vez al día, durante 1 hora). Después de cada desafío erosivo, las muestras fueron expuestas a suspensiones de los dentífricos (15 min), seguido por inmersión en saliva artificial entre los desafíos erosivos. Otras sesenta muestras de EDB fueron sometidas a pruebas de rugosidad antes y después de una prueba de cepillado con los dentífricos evaluados. Las variaciones porcentuales de MS y rugosidad se analizaron con las pruebas de ANOVA-Tukey ($\alpha = 0,05$). Resultados: Las menores variaciones de MS ocurrieron para G4, G2, G6, G3, G5 y G1, respectivamente. G2, G6 ($p < 0,5$) y G4 ($p < 0,01$) fueron más eficientes que G1 y G5 en el EDB reendurecido. Variación porcentual de rugosidad se observó sólo en G4, donde la superficie de las muestras se hicieron más lisas. La MEB mostró una superficie EDB alterado en G1, G3 y G5, y FTIR demostró que hidroxicarbonatoapatita se había formado en la superficie del EDB cuando el DE contenía partículas bioactivas (G2 y G6). Conclusiones: los resultados sugieren que la formulación de dentífrico con micropartículas de Biosilicato® podría ser una opción para tratar el esmalte erosionado.

PALABRAS CLAVES: esmalte, erosión, pasta dental, biomateriales, dentífrico.

Introduction

Nowadays, the integrity of the enamel, dentin and cement surfaces have been challenged by further aggressive situations such as, acidogenic diets, **gastroesophageal reflux disease(2), eating disorders(8) and** increased use of tooth-whitening products(18). Because the critical pH of dental enamel is approximately 5.5, any solution with a lower pH value may cause erosion, particularly if the attack is of long duration, and repeated over time. Saliva and salivary pellicle counteract the acid attacks but if the challenge is severe, a total destruction of tooth tissue follows. Because enamel acid erosion is a surface phenomenon, the protection and treatment of tooth surfaces with acid-resistant and/or remineralizers agents appears to be an interesting strategy. The effectiveness of fluoride as a therapy for erosion is still under debate(4,15). Therefore, the search for innovative agents, with a cost-benefit as good as or better than that reached by fluorides remains a promising aim.

In the dental field of developing new technologies to guarantee oral health, dentifrices represent an ancient product that has been re-invented throughout the years with innovative formulations(20). Because dentifrices (together with toothbrushes) represent the most easy-to-use and accessible products for oral self-care, they have become vehicles for a wide variety of therapeutic or preventive (antimicrobial and remineralizing) agents, such as fluorides(15), triclosan(3), chlorhexidine(17), medicinal plants(1) , chlorine compounds(13), CPP-ACP(12) and bioglasses(5).

A new bioactive material that is a >99.5% crystallized (P_2O_5–Na_2O–CaO–SiO_2 glass-ceramic powder-Biosilicate®), which was developed by a multidisciplinary research group(25), is being proposed as a suitable remineralizing agent in an experimental fluoride-free dentifrice formulation. Given the successful history of the use of biomaterials in bone regeneration(7), biomaterials, such as bioactive glasses and glass-ceramics, have been proposed for enamel and dentin regeneration. The similarity between bone, dentin, and enamel led to the hypothesis that bioactive glasses and glass-ceramics could be applicable to regenerate eroded dental surfaces via the *in-situ* deposition of hydroxy carbonate apatite (HCA). Additionally, the antimicrobial properties of bioactive materials could be an additional advantage in improving oral health(26, 27).

The first experiments with Biosilicate® showed that this novel material increases osteogenesis in cell culture(10), and i*n vivo* tests indicated its good performance in bone regeneration(16). An *in vitro* study showed the effects of the micron-sized (1-20 μm) particles of Biosilicate® in human dentin(21). Observations from this study indicated that an HCA-bonded layer was deposited on the dentin surface and in the dentinal tubules. As a consequence, a clinical study was carried out to investigate Biosilicate particles as a desensitizing agent(22). The results from the 6-month clinical study showed a very significant decrease in dentin hypersensitivity pain in patients treated with Biosilicate® particles mixed with distilled water. Also, an in vitro comparative study showed that micron-sized particles from Biosilicate were efficient in occluding dentinal tubules in dentin discs submitted to carbamide peroxide at 16%(14).

Following these positive results with Biosilicate® particles, the present investigation was designed to evaluate an experimental fluoride-free dentifrice containing Biosilicate® micron-sized particles. Importantly, the crystalline character of Biosilicate® offers an advantage over all other types of bioglasses because crystallization significantly changes the fracture characteristics of glass, yielding less sharp and less abrasive particles(23), which could then be safely brushed against teeth and gingiva.

The research hypothesis was that the micron-sized particles of the developed bioactive glass-ceramic could be an option as a therapeutic agent in a fluoride-free dentifrice formulation. To test this hypothesis, we evaluated comparatively two parameters: i) the effect of this experimental dentifrice on bovine enamel surface challenged by an acid solution and ii) the abrasive effect of the experimental dentifrice on bovine enamel.

Materials and Methods

Preparation of the bovine enamel specimens

One hundred and twenty (120) freshly extracted intact bovine incisors (with no cracks or erosion) stored in physiological saline solution at room temperature had the crowns separated from the roots using dental hand pieces. The facial sides of the crown were gently cut out with a diamond saw under water cooling to provide quadrangular BDE blocks that were successively ground (Polishing machine, Struers, Denmark) on wet silicon carbide paper with grain sizes ranging from 300 to 2000. The thickness and size of the flat BDE specimens (4x4x3 mm, n=120) were checked with a micrometer (Mitutoyo, Tokyo, Japan). All the specimens were sonicated and stored in 1.5 ml safe-lock tubes (Eppendorf Brazil, São Paulo, Brazil) with artificial saliva for one week until the start of the experiment.

Study design

The BDE specimens randomly received an identification number ranging from 1 to 120. The specimens 1 to 60 (Group A, n=60) were used to test the effect of the products on enamel surface, and specimens 61 to 120 (Group B, n=60) were used to test the abrasiveness of the toothpastes on enamel surface. The specimens were randomly allocated into six groups (A=G1, G2, G3, G4, G5, G6 and B= G1, G2, G3, G4, G5, G6; n=10 per group). To provide a comparative evaluation, the experimental dentifrice was compared to controls, to other commercial brand fluoride dentifrices, and to other experimental dentifrices with similar therapeutic agents. The products used in the comparative evaluation are described in Table 1

Table 1. Products tested in the study: experimental groups, therapeutics agents and manufacturers.

Group	Therapeutic agent	Manufacturer
G1	Control: distilled water	----------
G2	Experimental dentifrice formulation containing 7.5% Biosilicate® particles	Biosilicate®: Vitrovita, São Carlos, SP, Brazil. Dentifrice: HELP Laboratory: Research and Development (Ribeirão Preto, SP, Brazil)
G3	Experimental dentifrice formulation	Dentifrice: HELP Laboratory: Research and Development (Ribeirão Preto, SP, Brazil)
G4	Dentifrice Sorisso® (1500 ppm de MFP), reactive calcium carbonate	Colgate do Brazil, São Paulo, SP, Brazil
G5	Dentifrice Colgate Baby® (500 ppm NaF)	Colgate do Brazil, São Paulo, SP, Brazil
G6	Experimental dentifrice formulation containing 7.5% of bioglass type 45S5	Bioglass type 45S5: Vitrovita, São Carlos, SP, Brazil. Dentifrice: HELP Laboratory: Research and Development (Ribeirão Preto, SP, Brazil)

The variables measured were micro-hardness and roughness of the bovine enamel surface before and after applying the dentifrices in two different conditions. These conditions were 1) cycles of erosive challenges (CEC) on enamel surface, which involved cycling the specimens in artificial saliva, acid conditions, the dentifrice slurry and artificial saliva again and 2) a simulation of the tooth brushing process to evaluate the abrasiveness of the dentifrices.

Scanning electron microscopy (SEM) and Fourier transform infra-red spectroscopy (FTIR)

One specimen from each group (A: G1, G2, G3, G4, G5, G6) was randomly chosen and submitted to SEM and FTIR analyses before and after the cycle of erosive challenges on bovine enamel. Also, the micron-sized particles of Biosilicate® and bioglass type 45S5 were analyzed at a SEM to observe morphological aspects.

Surface micro-hardness measurements

Specimens in each group (Group A: G1, G2, G3, G4, G5, G6) had their surface micro-

hardness measured using a micro-hardness tester (HMV-2000, Shimadzu, Kyoto, Japan) with a load of 25 g for 5 s at three different points on the bovine enamel surface before and after the seven-day cycles of erosive challenges.

Roughness measurements

Specimens in each group (Group B: G1, G2, G3, G4, G5, G6) had their surface roughness (Ra) measured before and after the tooth brushing test with a profilometer (SJ-201-P, Mitutoyo, Kawasaki, Japan) with a cut-off of 0.08 mm.

Dentifrices

Dentifrice formulations were prepared to incorporate Biosilicate® (G2) or bioglass 45S5 (G6), both at 7.5% (wt %), with the following components: carboxymethylcellulose, methyl *p*-sodium hydroxybenzoate, sodium saccharin, menthol oil, propylene glycol, glycerol, sorbitol, flavor, hydrated silica, thickening silica, and sodium lauryl sulfate. The strict control group (G3) contained the same components except the bioactive materials. A very popular commercial dentifrice (1500 ppm of fluoride) was employed in G4, and a low fluoride (500 ppm) dentifrice was used in G5.*Enamel surface events: cycles of erosive challenges*

Specimens (Group A: G1, G2, G3, G4, G5, G6) were submitted to seven cycles of erosive challenge (one per day, for 7 days) at room temperature. Initially, the specimens were stored in individual tubes with artificial saliva for 12 h. Thereafter, each specimen was transferred to an individual tube with acid solution (5mL, lactic-acid with pH 4.3) and kept for 1 hour. Next, the specimens were washed with distilled water for one minute and inserted in another individual tube with the remineralizing solution (4mL, dentifrice suspensions prepared with distilled water in a 1: 3 proportion (w/w)) for 15 minutes. Sequentially, the specimens were pulled out of the tubes, washed again with distilled water for 1 minute and inserted in the individual tubes with fresh artificial saliva (5mL) where they were kept for 12 h to complete one cycle. The artificial saliva, acid solution, and remineralizing solution were changed at each cycle. The effect of the dentifrices was determined by evaluating the micro-hardness of the specimens before and after the cycles.

Specimens (Group B: G1, G2, G3, G4, G5, G6) were brushed using an automatic brushing machine with identical and new toothbrushes, with a load of 200 g and 350 strokes per minute for 1 minute, using a dentifrice slurry with a 1:2 dentifrice to distilled water ratio. The abrasiveness of each dentifrice was evaluated by the enamel roughness variation determined by profilometry of the specimens before and after tooth brushing.

Statistics

The data for the SM and roughness were collected before and after the events on the BDE surface (*cycles of erosive challenges* and tooth brushing tests) for each specimen. The percent variations of SM and roughness for each specimen were analyzed with an analysis of variance followed by the Tukey's test using the GraphPad 5.00 software for Windows.

Results

Representative SEM images from the baseline BDE are shown in Figure 1; they show evidence of a flat and smooth surface.

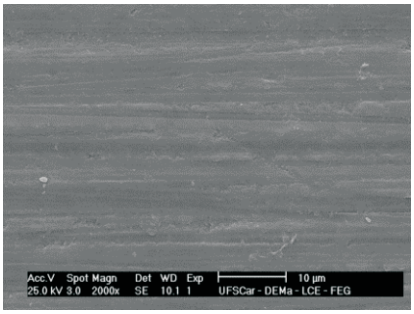

Figure 1. SEM image from a bovine enamel specimen before the Des-Re process.

Figure 2 shows particles of bioglass type 45S5 (1.0 to 4.5 µm) and particles of Biosilicate® (1.0 to 1.5 µm). The morphological difference between the particles obtained from the crystallized vitroceramic (B) and those from a partially crystallized one (A) is shown in Figure 2, where the edges present in

the bioglass type 45S5 are not seen in the Biosilicate® particles.

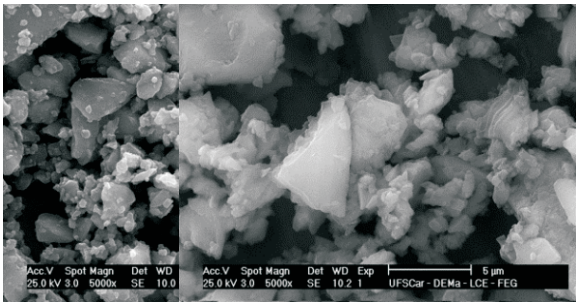

Figure 2. (A) Bioglass type 45S5 particles and (B) Biosilicate® particles.

Figure 3 shows representative SEM images from specimens of DBE submitted to the *cycles of erosive challenges*. SEM revealed morphological differences brought about by the various products applied. Using Figure 1 as a baseline SEM image, the SEM images in Figure 3 show that in both the control group (G1) and the strict control group (G3), the enamel surfaces were affected by the acid solution, and no regeneration occurred with the application of distilled water or a dentifrice without a therapeutic agent. The SEM images of the specimens treated with the experimental dentifrice (G2), commercial brand dentifrice (G4), and experimental dentifrice containing bioglass type 45S5 (G6) showed no visual changes on the BDE surface, suggesting that these products played a role in the remineralization / regeneration of the enamel during the *cycles of erosive challenges*. Surprisingly, the specimens treated with the commercial brand fluoride dentifrice (G5) looked similar to the control specimens.

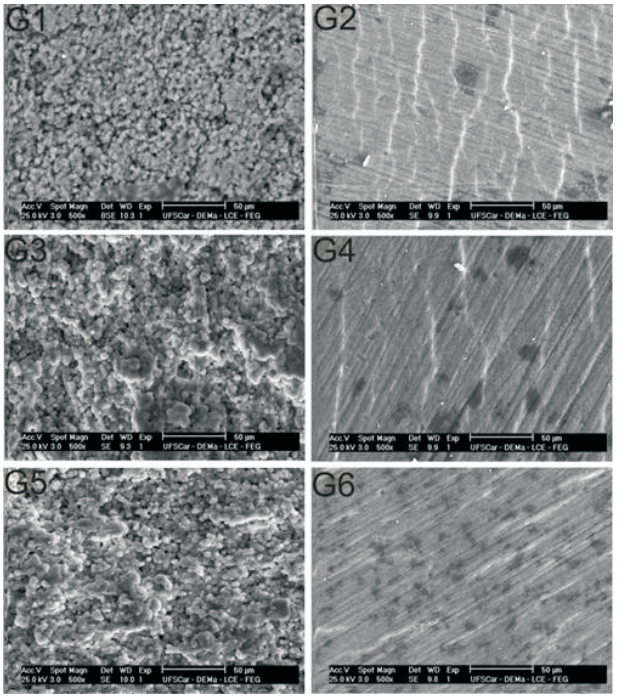

Figure 3. SEM images of the surface of the specimens after the Des-Re process. In the upper left corner, letter and number identify the group to which the image belongs.

Figure 4 shows representative FTIR results from the DBE surfaces. There were two different patterns of peaks; the first showed the groups G1, G3, G4, and G5 with no surface changes of the peaks that remained at the same position; and the second showed the groups G2 (Biosilicate) and G6 (bioglass) exhibiting significant changes. A small shift between 1062 cm^{-1} and 1050 cm^{-1} (G2 and G6) was observed, and the peak at 1100 cm^{-1} almost disappeared. This change was very important as it indicated the formation of HCA on the enamel surface triggered by the bioactive particles. The peaks observed for BDEs treated with dentifrices containing the bioactive material were coincident with peaks observed with Biosilicate® or bioglass type 45S5 after 24 hours in SBF **("a" spectrum**); this is strongly suggested by the peak at 564 cm^{-1}. After the *cycles of erosive challenge*, the **"b"** spectrum was the representative image for the bovine enamel treated with the experimental dentifrices containing the bioactive glass-ceramics (G2 and G6). The peaks G2 and G6

were quite similar; therefore, only one (b) line was plotted to represent them. The main spectral peaks for the molecular vibrations of Biosilicate® and 45S5 were observed at 460, 536, 930 nm and at 1124, 602, 574 nm for bovine enamel. From these spectra, it was suggested that Biosilicate® formed a thin layer of HCA on the bovine enamel surface. The double peaks at 602 and 574 nm were the most important P-O crystal vibrational bend modes associated with HCA and they could be clearly observed on the BDE from G1, G3, G4, and G5 (**"c" and "d" spectrum**) in enamel surfaces treated or not treated with fluoride dentifrices (G1 and G3 versus G4 and G5).

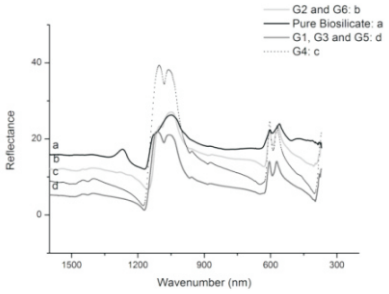

Figure 4. Representative FTIR analyses of the BDE surfaces before the Des-Re process.

Figure 5 shows the variation of the surface micro-hardness (SM) of the specimens exposed to the *cycles of erosive challenges*. The smallest changes in SM were observed in increasing order for G4, G2, G6, G3, G5, and G1, indicating that the commercial brand fluoride-dentifrice (G4) was the most efficient in maintaining the SM of the BDE exposed to acid conditions, followed by the dentifrices in which a glass (G6) or glass-ceramic biomaterial (G2) was used as therapeutic agent. A statistically significant difference in SM was found between the control group (G1) and G2, and between G4 and G6. The product used in G2 was statistically significantly more efficient than its strict control (G3) or G5. As the specimens in G5 demonstrated the highest SM variation (with variation defined as decrease in SM), statistically significant differences were observed between G5 and G2, between G5 and G6, and between G5 and G4. The SEM images showed more disturbed enamel surfaces for G1, G3, and G5, which was consistent with greater decreases in SM.

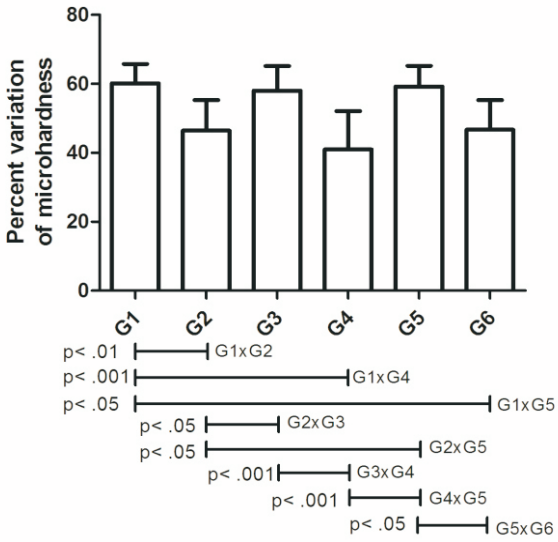

Figure 5. Variation in the specimens' micro-hardness exposed to Des-Re process.

For surface roughness variation (Figure 6), a statistically significant difference was found only when G4 was compared with G5. Actually, for G4 specimens, the surface became smoother after the toothbrush test.

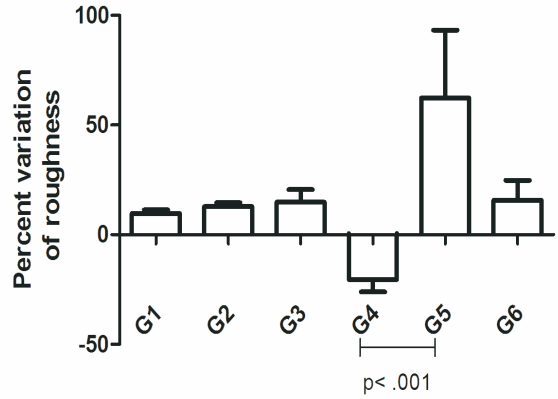

Figure 6. Variation in the specimens' roughness after the tooth brushing test.

Discussion

Currently, there is no standard protocol for erosion experiments in general, and in particular, for testing agents to prevent or to treat erosion of the enamel and dentin[4]. The experimental design of this study was therefore elaborated to test the effect of therapeutic

agents on enamel surface submited to *cycles of erosive challenges*.

From the literature there are evidences that fluoride agents are a good option to lead with eroded enamel, although no consensus regarding the best approach had been established yet. Moretto et al., 2010(9) evaluated *in vitro* the effect of dentifrices with different fluoride concentrations as well as of a low-fluoridated dentifrice supplemented with trimetaphosphate (TMP) on enamel erosion. The alterations of the enamel were quantified using the Knoop hardness test. The results suggested that the 500 microg F/g plus 3% TMP and 5,000 microg F/g dentifrices had a greater protective effect when compared with the 1,100 microg F/g dentifrice. Considering the side-effect of the fluoride products, non-fluoride therapeutic agents, are also investigated to treat dental eroded surfaces. A study evaluated the effect of CCP-ACP on bovine enamel eroded by cola drink. The results showed the enamel became hardener after four applications of CCP-ACP paste[19].

In this study, the SM and SEM was the main outcome of interest for testing the bioglass (type 45S5) and its crystalline counterpart as proposed agents to treat eroded enamel surface. There was no statistically significant difference between the SMs at baseline, indicating an efficient random distribution of the specimens. The bovine enamel in the control group (G1) showed the highest variation in SM as expected because distilled water had no effect in regenerating / rehardening the enamel surface exposed to acid conditions. Experimental dentifrices containing the bioactive materials of glass-ceramic (G2) and glass (G6) and the commercial brand fluoride-dentifrice (G4) were able to guarantee significantly lower SM variation than distilled water. The strict control dentifrice (G3) did not show the same effect that was observed for G2 and G6, indicating that the proposed therapeutic agents of Biosilicate® and bioglass type 45S5 were responsible for the lower variation in SM; or for re-hardening the enamel. There were no statistically significant differences between the experimental dentifrices (G2 and G6) compared to the commercial brand fluoride dentifrice containing 1,500 ppm of monofluorphosphate of sodium and calcium carbonate as remineralizing agents (G4), suggesting that the experimental and fluoride dentifrice had the same performance. However, when these experimental dentifrices (G2 and G6) were compared to the commercial brand fluoride dentifrice containing only 500 ppm of the fluoride compound (G5), a statistically significant difference was found, indicating that the fluoride-free experimental dentifrices (G2 and G6) were much more efficient than the low fluoride dentifrice (G5). Indeed, the literature confirms the benefits of using fluoride toothpaste in preventing caries in children and adolescents when compared to placebo, but the effects were only significant for fluoride concentrations of 1000 ppm and above(24). The commercial brand fluoride dentifrice (G4) showed the best results for SM variation. The dentifrice used in the group G4 was one of the most commonly used in Brazil, and it has two active remineralizing agents of MFP and reactive calcium carbonate. Also, there was no statistically significant difference between the G2 and G6 groups indicating that Biosilicate® and bioglass type 45S5 had the same effect on SM.

The representative SEM images showed regular BDE surfaces for groups G2, G4, and G6, and it showed visual surface changes for G1, G3, and G5. The morphological variation in the enamel surfaces matched the variations in SM, and the higher SM variations occurred in the specimens from G1, G3, and G5, suggesting that when distilled water (G1), an experimental dentifrice without a therapeutic agent (G3), and a dentifrice with only 500 ppm of NaF (G5) were used, the enamel surface was disturbed.

For the roughness variation, a significant difference in variation was found only when we compared the commercial brand fluoride dentifrice (G4) with the commercial brand low fluoride dentifrice (G5). The former made the surface of the bovine enamel smoother than before, whereas the latter

showed the greatest increase in roughness. The percent composition of the ingredients in the formulations of the dentifrices G4 and G5 were not available from the manufacturers. It is possible that the dentifrice with the lowest amount of fluoride also has a higher level of abrasives, and the commercial brand dentifrice with the two remineralizing agents (MFP and calcium carbonate) had a low amount of abrasives. Hara et al. 2008, (6), described the interplay between fluoride and abrasives on mineral surfaces, showing that fluorides reduced the surface loss in enamel at all abrasive levels. Muray and Shaw 1980 (11) suggested that the lower fluoride content the higher the abrasives content should be to guarantee adequate oral health status.

Given the results of all the experiments and considering the limitations of this *in vitro* study, our research hypothesis was confirmed, and therefore, the micron-sized particles of Biosilicate® are an interesting option (in fluoride-free dentifrice formulations) as a therapeutic agent for treating eroded enamel. Additional questions to be answered in subsequent future investigations are as follows: i) Given that the bioactive vitro-ceramic triggered the HCA layer formation on the enamel, what is the thickness of this layer and how strong is its resistance to acid challenges?; ii) How does the acidity influence the reactions occurring on the bioactive particle surfaces?; iii) What would be the interplay between the microorganisms in the mouth, particularly *streptococcus mutans*, and the bioactive glasses?; iv) What is the optimal dose of the bioactive material into a dentifrice formulation?; and v) What would be the interplay between fluoride and bioactive vitro-ceramics in dentifrice formulations?

Conclusions

The results from this *in vitro* study suggest that Biosilicate® micron-particles could be used as a remineralizing agent in a fluoride-free dentifrice formulation.

Acknowledgments

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