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ORIGINAL ARTICLE

In situ evaluation of the remineralizing capacity of pit and fissure sealants containing amorphous calcium phosphate and/or fluoride

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Abstract

Objective. This in situ study evaluated the remineralizing potential of pit and fissure sealants containing amorphous calcium phosphate (ACP) and/or fluoride in artificially induced carious lesions on smooth enamel surfaces. ***Material and methods.*** Ten volunteers who wore acrylic palatal devices were enrolled in this 5-day double-blind study and assigned to one of the following five groups: (I) demineralized enamel slab+Fluroshield (sealant with fluoride); (II) demineralized enamel slab+Aegis (sealant with ACP); (III) demineralized enamel slab+experimental sealant with fluoride (ESF); (IV) demineralized enamel slab +experimental sealant with fluoride/ACP (ACP-F); and (V) demineralized enamel slab (control). After the experimental period, the percentage of surface microhardness recovery (%SMHR) and the integrated loss of subsurface hardness (ΔKHN) were evaluated. The concentrations of fluoride, calcium and phosphorus in enamel were also determined. ***Results.*** The sealants containing ACP and/or fluoride presented a higher remineralizing capacity (%SMHR and ΔKHN) than that of the control group. Aegis provided either more efficient or similar remineralization than the other sealants. The association between ACP and fluoride did not show a greater efficacy in the remineralization. F, Ca and P concentrations in enamel varied according to the group. ***Conclusion.*** The pit and fissure sealants containing ACP were able to promote remineralization of artificially induced carious lesions on smooth enamel surfaces.

Key Words: *Calcium phosphates, fluorides, pit and fissure sealants, tooth remineralization*

Introduction

Prevention measures, when applied properly, show significant results in reducing dental caries. However, as the prevalence of occlusal caries is still high [1,2], pit and fissure sealants are recommended and commonly utilized since their introduction into dentistry.

Among the agents that have the potential to remineralize carious lesions or minimize caries development, the following may be mentioned: calcium and phosphate ions from saliva or other sources (dentifrices, chewing gums, beverages, remineralizing solutions and restorative materials) and fluoride from topical or systemic sources. The calcium phosphate in saliva is the natural defense against mineral tooth loss. As an additional method of caries control, the fluoride present in the oral environment plays an important role in the dental structure, reducing demineralization and potentiating remineralization. However, the effect of the fluoride ion may be limited by the availability of calcium and phosphate in the mouth (plaque/saliva) [3,4].

Amorphous calcium phosphate (ACP) has been identified as a possible precursor in the biological formation of hydroxyapatite. Dental applications based on the unique characteristics of ACP have been proposed, as well as the improvement of its properties and associations with similar products that have anti-demineralizing/remineralizing potential [5–12].

If a supplementary concentration of calcium and phosphate ions could be supplied without making fluoride insoluble, its efficiency would be increased, as observed in previous studies with dentifrices

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[13,14]. In this sense, the properties of calcium, phosphate and/or fluoride ions should be assessed in dental materials, such as pit and fissure sealants, in order to determine the additional benefit in prevention of dental caries and their effectiveness in enamel remineralization. The purpose of this study was to evaluate in situ the remineralizing potential of pit and fissure sealants containing ACP and/or fluoride in artificially induced carious lesions on smooth enamel surfaces.

Material and methods

Subject recruitment

Ten young adults (five males, five females) aged 20–29 years with normal non-stimulated salivary flow (≥0.2 ml/min) [15,16] were enrolled in this study. The study design was independently reviewed and approved by the Research Ethics Committee of the Dental School of Araçatuba (process number 2005-01860) and all participants signed an informed consent form. An intraoral examination confirmed the absence of carious lesions, periodontal disease or any other type of oral pathology. Smokers and individuals on medication or who presented with chronic systemic diseases were excluded from the study [17].

Specimen preparation

Enamel slabs ($4 \times 4 \times 2$ mm) were obtained from bovine incisor teeth that had been stored in 2% formaldehyde solution, pH 7.0, at room temperature for 30 days [18]. The enamel surface was polished and the slabs were cross-sectioned at 1 mm from the border, resulting in specimens with a volume of $4 \times 3 \times 2$ mm^3 (Figure 1A). Initial surface microhardness (SMH_1) was measured using a microhardness tester (Shimadzu Micro Hardness Tester HMV-2000; Shimadzu Corp., Kyoto, Japan) with a Knoop diamond indenter under a 25-g load for 10 s using image-analysis software (CAMS-WIN; NewAge Industries, Inc., Southampton, PA). Five indentations spaced 100 μm from each other were made on the slab surface at distances of 150, 300 and 600 μm from the sectioned enamel border, totaling 15 indentations (Figure 1B). Two hundred enamel slabs with an average SMH_1 of 320–360 KHN were selected for the study.

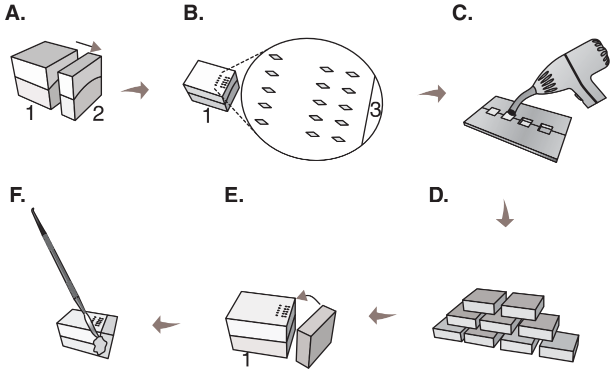

Figure 1. Schematic presentation. (A) Section of the block (1 = block $3 \times 4 \times 2$ mm used in the study; 2 = piece of the block $1 \times 4 \times 2$ mm that was discarded). (B) Five rows of three indentations each, 150, 300 and 600 μm from the enamel-sectioned border (3). (C) Polymerization of sample. (D) Samples. (E) Samples adapted onto the enamel blocks (1). (F) Samples fixed with wax.

Demineralization

Artificial carious lesions were produced by immersing each enamel slab (area = 12 mm^2) in 24 ml of a demineralizing solution [19] (1.3 mmol/l Ca, 0.78 mmol/l P, 0.05 mol/l acetate buffer, 0.0315 μg/ml F, pH 5.0) at 37°C for 16 h. Post-demineralization surface microhardness (SMH_2) was measured by making five indentations between the previous ones (SMH_1) at the same distances from the border in contact with the material (150, 300 and 600 μm).

Depth lesion analysis after demineralization

Approximately 600 μm-thick sections were obtained from the other demineralized blocks ($n = 10$) using a diamond saw (11-4243 series 15 HC; Diamond Buehler). The sections were ground and polished to a thickness of 100 μm in a polishing machine (Phoenix Beta-Vector; Buehler, Lake Bluff, IL), mounted on slides with distilled/deionized water and covered with a cover glass, the edges of which were sealed with ethylene resin. The sections were examined by polarized light microscopy (AxioPhot DSM-940 A; Zeiss, Oberkochen, Germany) at a magnification of ×400. Three areas in the central regions of the slices were analyzed by recording the presence and thickness of the superficial enamel layer and the depth of the lesion using Axiovision Software Rel. 4.3.

Preparation of sealant specimens

Forty specimens were prepared for each tested sealant [Fluroshield (Dentsply International Inc, Milford, DE; with fluoride); Aegis (Bosworth, Skokie, IL; with ACP); experimental sealant containing fluoride (ESF) (Bosworth); and experimental sealant containing ACP and fluoride (ACP-F) (Bosworth)] using a metallic matrix ($4 \times 2 \times 1$ mm^3). Polymerization was performed for 40 s at the top and bottom surfaces of the specimen (Figure 1C) using a halogen light-curing unit (VIP; Bisco, Schaumburg, IL) with a light intensity of 500 mW/cm^2 [20]. Following preparation, each sealant specimen was fixed with wax (Kota Ind. and Com. Ltda, São Paulo, Brazil) to the border of the enamel slabs that had been cross-sectioned (Figures 1D, 1E and 1F).

Intraoral procedures

Acrylic intraoral removable palatal devices were constructed with four cavities [21]: two in the region of the second premolar (one right, one left) and two in the region of the first molar (one right, one left). Each demineralized enamel slab/sealant specimen set or demineralized enamel slab (control) was fixed with wax (Kota Ind. and Com. Ltda) in one of the cavities of the palatal device. Ten days before the beginning of the experimental period, the volunteers received instructions on oral hygiene, a non-fluoride dentifrice [16] and a toothbrush. A 5-day double-blind cross-over study was designed with five groups, as follows: (I) demineralized enamel slab+Fluroshield; (II) demineralized enamel slab+Aegis; (III) demineralized enamel slab+ESF; (IV) demineralized enamel slab+ACP-F; and (V) demineralized enamel slab (control). The palatal devices were worn continuously during the experimental period, being removed for cleaning (15-s brushing) [22] and at meals [15,22]. A waiting time frame of 7 days was allowed between the groups [16,21], during which the volunteers kept using the non-fluoride dentifrice. A sample of the water drunk by the volunteers (10 ml) was collected on the second and fourth days of each experimental period in order to determine the fluoride concentration.

Microhardness measurements

The enamel slabs were removed from the acrylic palatal devices and the final surface microhardness (SMH_3) was measured by making five indentations between the previous ones (SMH_1 and SMH_2) at the same distances from the border in contact with the material (150, 300 and 600 μm). The percentage SMH change (%SMH) was calculated as follows: $\%SMH = 100 \times (SMH_3 - SMH_2)/(SMH_1 - SMH_2)$. After SMH_3 analysis, all slabs were longitudinally sectioned through the center of the enamel. To measure the cross-sectional microhardness (CSMH), half of each enamel slab ($2 \times 3 \times 2\ mm^3$) was embedded in acrylic resin (Buehler Transoptic Powder) and the cut surfaces were exposed and polished. CSMH testing was performed with the same load used for SMH_1 testing. Testing was performed on each enamel slab, with 24 indentations distributed in three rows of eight indentations each at depths of 10, 30, 50, 70, 90, 110, 220 and 330 μm. The first row began 150 μm from the border that remained in contact with the material, the other two rows being located 300 and 600 μm from the border. The mean values at all three measuring points at each distance from the surface were then averaged. Integrated hardness (KHN multiplied by distance in microns) for the lesion into sound enamel was calculated by the trapezoidal rule (GraphPad Prism, version 3.02) and subtracted from the integrated hardness for sound enamel to obtain the integrated loss of subsurface hardness (ΔKHN) [23].

Fluoride, calcium and phosphorus analyses

The other halves of the enamel slabs that were cut longitudinally were subjected to microabrasion, as described by Alves et al. [24]. For analysis of the fluoride and calcium concentrations, two specific electrodes were used, respectively: Orion 9609-BN (Orion Research, Beverly, MA), previously calibrated with a standard solution (0.09–1.44 μg/ml F); and Orion 9300 (Orion Research), previously calibrated with a standard solution (10–160 μg/ml Ca), connected to an ion analyzer (Orion 720 A; Orion Research). In order to determine fluoride and calcium concentrations, total ionic strength adjustment buffer (TISAB III; Orion Research) and ionic strength adjuster (ISA; Orion Research) were added, respectively. Phosphorus was determined by a colorimetric method [25] using a spectrophotometer (Hitachi U-1100 UV/Vis spectrophotometer; Hitachi High Technologies, Tokyo, Japan) at a wavelength of 660 nm and a standard solution of phosphorus (3 mg%). Results were expressed as micrograms per cubic millimeter.

Fluoride concentration in the water drunk by the volunteers

Fluoride concentration was measured using a specific electrode for fluoride ion (Orion 9609-BN) and a digital ion analyzer (Orion 720 A) that had been previously calibrated with a standard solution (0.1–1.6 μg/ml F) in TISAB III, the solutions being made under constant agitation (TE-081; Tecnal, Piracicaba, São Paulo, Brazil). The values were expressed in micrograms of fluoride ion per milliliter.

Statistical analysis

The mean values of SMH_1, SMH_2, SMH_3, %SMHR, ΔKHN, and F, Ca and P concentrations ($\mu g/mm^3$) were considered as variables and Groups I–V were considered as variation factors. The number of volunteers was considered as the experimental "*n*" in the study. After confirmation of homogeneity, the SMH_1,

SMH_2, SMH_3, %SMHR and ΔKHN values were submitted to two-way ANOVA and Tukey's multiple-comparison test. The fluoride, calcium and phosphorus concentrations were submitted to the Kruskal–Wallis test. All variables were submitted to Pearson's correlation analysis. Analyses were performed using the GMC statistical software package (version 2002; available at http://www.forp.usp.br/restauradora/gmc/gmc.html) at a significance level of 5%.

Results

Polarized light microscopy analysis

Figure 2 shows polarized light micrograph of a specimen after demineralization. Mean (SD) lesion depths was 47.7 (12.6) µm (range 36.4–66.5 µm). It was possible to observe a superficial enamel layer with a thickness of 9.8 (0.9) µm (range 8.3–10.8 µm).

Microhardness

The mean (SD) SMH_1 of the enamel blocks was 347.4 (3.3) KHN. Comparing the groups, the lowest mean SMH_1 was 345.4 (3.2) KHN and the highest was 348.7 (2.3) KHN ($p = 0.102$). The mean (SD) SMH_2 was 62.4 (12.9) KHN. Comparing the groups, the lowest mean SMH_2 was 54.8 (13.3) KHN and the highest was 67.2 (15.6) KHN ($p = 0.094$). Table I shows the SMH_3 and %SMHR data according to the distances of indentations from the enamel border in contact with the material and the group. Regarding the distances, the control did not show statistically significant differences. For the other groups, the 150-µm distance presented a higher %SMHR value compared to the 600-µm distance. Aegis presented higher SMH_3 and %SMHR values than ACP-F. Aegis presented similar SMH_3 values to those of the fluoride-containing sealants (Fluroshield and ESF) and similar %SMH values to those of ESF. Figure 3 shows that the control presented the highest ΔKHN, differing significantly from the other groups at all analyzed distances. For the other groups, no statistically significant differences in ΔKHN values were observed at the distances of 300 and 600 µm. However, for Fluroshield, ACP-F, Aegis and ESF, significant differences ($p < 0.05$) were observed between the 150- and 600-µm distances.

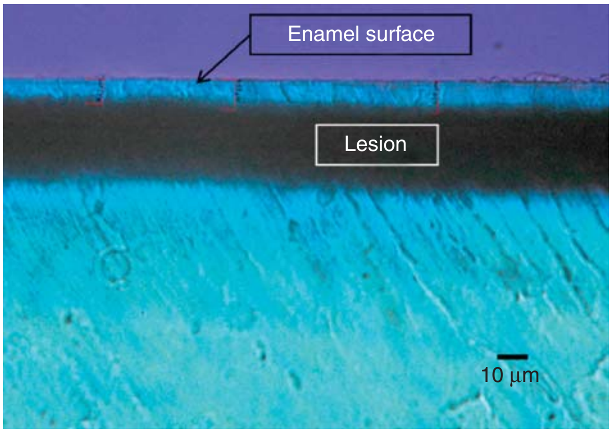

Figure 2. Polarized light photomicrograph of lesion formed after immersion in demineralizing solution.

Fluoride, calcium and phosphorus contents in enamel

ESF, ACP-F and Fluroshield yielded the highest fluoride concentrations in enamel, being statistically similar to each other (Figure 4A). ACP-F was statistically similar to Aegis. The control group was statistically similar to the Aegis group and presented the lowest fluoride concentration, differing significantly from the other groups. Fluroshield, Aegis and ESF showed the highest calcium concentrations in enamel, being statistically similar to each other (Figure 4B). ACP-F presented statistically similar results to those of Aegis and ESF. The control group presented the lowest calcium concentration, differing significantly from the other groups. Regarding phosphorus concentration in enamel (Figure 4C), ACP-F showed the lowest values and was statistically similar to Aegis. Fluroshield was similar to ESF and showed intermediate values ($p > 0.05$). The control presented the highest phosphorus concentration, differing significantly from the other groups.

Fluoride content in the water

The analysis of the water drunk by the volunteers during the course of the study showed a mean fluoride concentration of 0.72 ppm (range 0.03–1.29 ppm). There was no statistically significant correlation between fluoride concentration in the water and %SMHR ($r = -0.092$; $p = 0.801$) or between fluoride concentration in the water and fluoride concentration in enamel ($r = -0.256$; $p = 0.065$) for the control group.

Correlations

From the tested variables (SMH_3, %SMHR, ΔKHN, and fluoride, calcium and phosphorus concentrations), correlations were observed between SMH_3 and ΔKHN ($r = 0.907$; $p < 0.001$), SMH_3 and fluoride ($r = 0.346$; $p = 0.014$), SMH_3 and calcium ($r = 0.292$; $p = 0.039$), SMH_3 and phosphorus ($r = -0.503$; $p < 0.001$), %SMHR and ΔKHN ($r = -0.800$; $p < 0.001$), %SMHR and fluoride ($r = 0.379$; $p = 0.007$), %SMHR and calcium ($r = 0.322$; $p = 0.023$), %SMHR and

Table I. SMH_3 and %SMHR (n=10) according to the distance of indentation from the enamel border in contact with the material and the group.

	Distance of indentation (µm)					
	SMH_3			%SMHR		
Group	150	300	600	150	300	600
Control	$^{A}115.8^{a}$ (15.6)	$^{A}116.8^{a}$ (15.5)	$^{A}117.2^{a}$ (14.3)	$^{A}18.7^{a}$ (2.5)	$^{A}18.5^{a}$ (2.6)	$^{A}18.2^{a}$ (2.5)
Fluroshield	$^{A}156.4^{b}$ (16.8)	$^{A,B}155.1^{b}$ (15.8)	$^{B}151.8^{b}$ (18.2)	$^{A}32.8^{b}$ (3.8)	$^{B}31.7^{b}$ (4.0)	$^{C}30.3^{b}$ (4.6)
ACP-F	$^{A}148.5^{c}$ (22.5)	$^{A}148.3^{c}$ (20.8)	$^{A}147.2^{c}$ (18.6)	$^{A}32.5^{b}$ (5.2)	$^{A,B}31.6^{b}$ (4.8)	$^{B}31.0^{b}$ (4.4)
Aegis	$^{A}157.6^{b}$ (11.3)	$^{A}155.8^{b}$ (11.7)	$^{A}154.0^{b}$ (10.9)	$^{A}33.9^{c}$ (3.9)	$^{A}33.0^{c}$ (4.0)	$^{B}32.0^{c}$ (4.0)
ESF	$^{A}159.4^{b}$ (18.5)	$^{A}156.6^{b}$ (18.6)	$^{A}155.3^{b}$ (18.4)	$^{A}34.3^{c}$ (4.8)	$^{B}32.9^{c}$ (4.6)	$^{B}32.0^{c}$ (4.3)

Values with different letters on either side are significantly different in each analysis (SMH_3 and %SMHR). Lowercase letters indicate comparison among the groups at each distance and uppercase letters indicate comparison among the distances within each group. SMH_3: two-way ANOVA, p = 0.022; %SMHR: two-way ANOVA, p < 0.001.

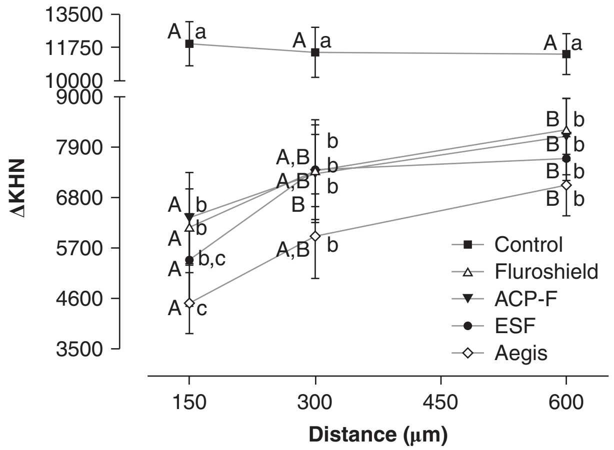

Figure 3. Integrated mineral recovery area (ΔZ) (mean ± standard error; n = 10) according to the distance of indentation from the enamel border in contact with the material. Values indicated by two different letters are significantly different (p < 0.05). Lowercase letters indicate comparison among the groups at each distance. Uppercase letters indicate comparison among the distances for each group.

phosphorus (r = –0.609; p < 0.001), ΔKHN and fluoride (r = –0.373; p = 0.007), ΔKHN and calcium (r = –0.403; p = 0.004), ΔKHN and phosphorus (r = 0.740; p < 0.001), and fluoride and calcium (r = 0.400; p = 0.004).

Discussion

In view of the continuous introduction of materials into dental practice, it is important not only to investigate and confirm their properties, but also to propose modifications or associations that may contribute to improve their performance. New pit and fissure sealants with the capacity to release calcium and phosphate due to the presence of ACP are now commercially available on the dental market. However, to the best of our knowledge, there have

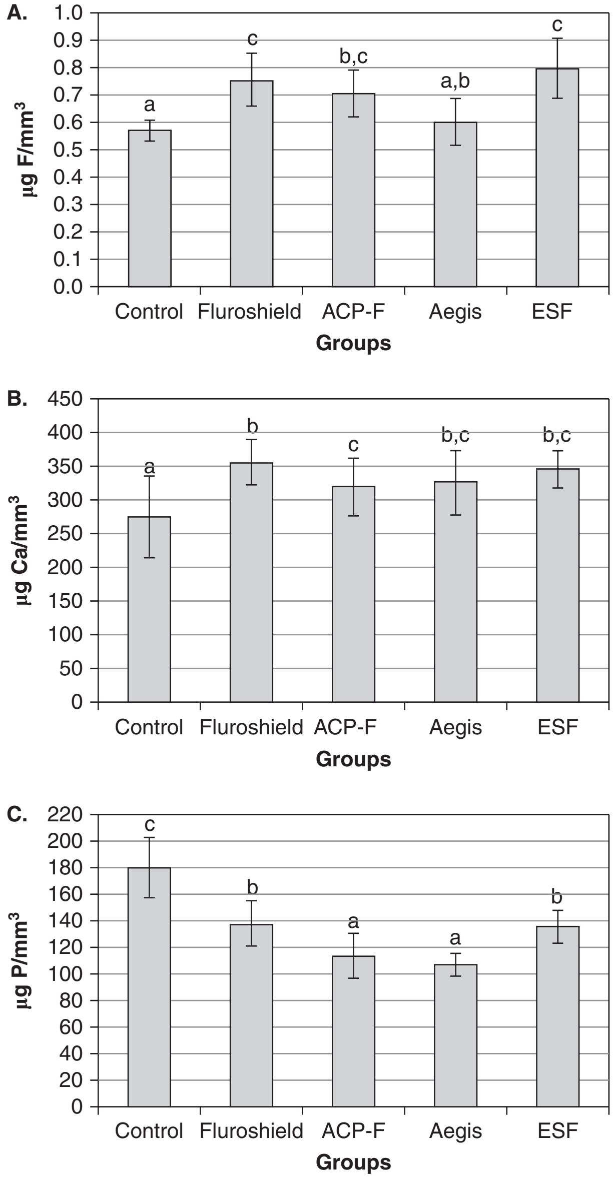

Figure 4. Concentrations of (A) fluoride, (B) calcium and (C) phosphate (mean ± standard error; n = 10) in enamel after the experimental period for each group. Values indicated by two different letters are significantly different (p < 0.05).

been no studies to date investigating the remineralizing capacity of these materials under oral conditions.

Unlike previous studies [26,27] that prepared conventional cavities on enamel for testing restorative materials, in the present study, sealant specimens were fabricated and attached to the border of the enamel slabs (Figure 1). Placing the material in a cavity leads to covering of the initial and post-demineralization indentations. This would not allow final SMH testing to be carried out. The current methodology allowed measurement of both SMH and CSMH and determination of Ca, P and F readings at a precise distance from the margin of the material. Another fact is that, although the sealants are usually applied to demineralized (acid-etched) enamel surfaces, in the present study no demineralization was done because it was necessary to have standardized caries lesions (Figure 2) in order to reduce variations of the results, as observed in Table I.

The mean values of fluoride concentration in the ingested water were constant for most volunteers during the course of the study, with variations among the volunteers. Only one volunteer presented a mean ingestion of fluoride of 0.05 (0.02) ppm, and the other two volunteers presented mean ingestions of fluoride of 0.36 (0.34) and 0.35 (0.20) ppm, respectively, which were below the mean obtained from all volunteers. This fact may be attributed to ingestion of water from different sources (mineral water or public water supply). The mean %SMHR values (considering the three distances) and F concentrations in enamel for the above-mentioned volunteers were, respectively: 19.0%/0.59 ppm F, 20.1%/0.64 ppm F and 16.6%/0.63 ppm F. The results do not suggest a major influence of fluoride on enamel remineralization since the control presented lower %SMHR values, ΔKHN and fluoride concentration in enamel (Table I, Figures 3 and 4A).

All groups, including the control, promoted enamel remineralization, which demonstrates that the in situ model used in this study was capable of initiating the remineralization process. However, the control showed a lower remineralizing capacity than that of the other groups, with greater phosphorus content and lower fluoride and calcium contents in enamel (Figure 4), with a Ca/P ratio of 1.2, resulting in a mineral with lower hardness (Table I). The results of the other groups are due to the release of ions from the materials that favored enamel remineralization [6,28]. Therefore, both fluoride and ACP successfully promoted remineralization, as demonstrated in Table I and Figure 3. When sealants containing only fluoride were used after enamel demineralization, in addition to the %SMHR and ΔKHN results, there was a greater concentration of fluoride and calcium and a lower concentration of phosphorus in enamel, compared to the control group (Figure 4), showing a Ca/P ratio of 2.0. However, this does not mean that calcium phosphates precipitated with this Ca/P ratio, but rather that it is more likely that precipitation of calcium phosphates occurred with a Ca/P ratio closer to that of hydroxyapatite (1.5–1.6), in a greater amount associated with fluoride. The fluoride released by the sealant acted as a catalyst accelerating remineralization and also resulted in the formation of fluoride-rich apatite crystals and calcium fluoride, which are more mineralized [29,30] and harder (Table I). The correlation between the hardness data (SMH_3, %SMHR and ΔKHN) and fluoride in enamel corroborates this hypothesis. In the Aegis group, lower fluoride and phosphorus concentrations were observed, but calcium concentration was similar to that obtained with the other sealants. In addition to the %SMHR and ΔKHN results, ACP promoted an oversaturation of the calcium and phosphorus levels, showing a Ca/P ratio of 2.4, which was demonstrated by the greater enamel concentration of calcium compared to phosphorus (Figures 4B and 4C). This increases the probability of deposition of calcium phosphates with a Ca/P ratio closer to that of hydroxyapatite (carbonated hydroxyapatite) and incorporation of amorphous calcium phosphate [31].

The %SMHR and ΔKHN data corroborate this hypothesis because the Aegis group produced higher surface hardness values and a smaller subsurface lesion area (Table I, Figure 3). This may also be confirmed by observing the correlations between the hardness data (SMH_3, %SMHR and ΔKHN) and the Ca and P concentrations in enamel. However, the lack of correlation between the Ca and P data makes it difficult to extrapolate these results to determine the type of calcium phosphate that was formed and, in a way, explains the Ca/P ratio found in this study.

ACP-F was not more effective than the other materials (Table I, Figure 3), since a synergism was expected between the fluoride and ACP released by this material. However, it was not observed in the present study, although fluoride, calcium and phosphorus concentrations in enamel were similar to that of Aegis (Figure 4), which exhibited a greater remineralizing potential, and similar to those of ESF (Table I, Figure 3). With the addition of fluoride, the Ca/P ratio dropped to 2.2 when compared to Aegis (2.4), indicating lower precipitation of ACP and an increase in fluoridated apatites with a Ca/P ratio closer to that of hydroxyapatite, as well as calcium fluoride, which confirms the increase in remineralization in this group (Table I, Figure 3).

The margins and neighboring areas of a sealant are always critical to the sealing technique because biofilm accumulation may occur and make these regions more acidic than more distant areas, which may interfere with the remineralizing capacity of saliva [15]. The results of this study indicate, however, that greater enamel remineralization occurred in the regions closer to the border of the enamel slab

(150 μm) that remained in contact with the material (Table I, Figure 3). These findings may be attributed to the greater ion concentration in this region and consequent incorporation of ions by the enamel [6,26,28,32], although a distant effect of the material may also promote carious lesion remineralization [28].

According to the proposed methodology, this in situ study showed that both fluoride and ACP were effective in providing a remineralizing capacity to the tested materials in the oral environment, even presenting different forms of apatite deposition. Other experimental sealants with different ratios of ACP and F must be tested, aiming to provide a formulation that provides synergism of ions for the enhancement of enamel remineralization.

Conclusions

The sealants containing ACP and/or fluoride were able to promote in situ remineralization of artificially induced carious lesions on smooth enamel surfaces. This in situ methodology was able to distinguish the effect of fluoride- and calcium phosphate-releasing materials using different analyses and may be applied in tests of new formulations of dental materials.

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