

Effect of a resin-modified glass-ionomer with calcium on enamel demineralization inhibition: an *in vitro* study

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Abstract: We assessed the effect of a new coating material based on resin-modified glass-ionomer with calcium (Ca) in inhibiting the demineralization of underlying and adjacent areas surrounding caries-like lesions in enamel. The measures used were surface hardness (SH) and cross-sectional hardness (CSH). Thirty-six bovine enamel specimens ($3 \times 6 \times 2$ mm) were randomly allocated into three groups ($n = 12$): No treatment (NT); resin-modified glass-ionomer with Ca (Clinpro XT Varnish, 3M ESPE) (CL), and fluoride varnish (Duraphat, Colgate) (DU). The specimens were subjected to alternated immersions in demineralizing (6 h) and remineralizing solutions (18 h) for 7 days. SH measurements were conducted at standard distances of 150, 300, and 450 μm from the treatment area. CSH evaluated the mean hardness profile over the depth of the enamel surface and at standard distances from the materials. The energy-dispersive X-ray spectroscopy analysis was conducted to evaluate the demineralization bands created on the sublayer by % of calcium (Ca), phosphorus (P), and fluoride (F). Ca/P weight ratio was also calculated. Based on SH and CSH measurements, there was no difference between groups at the distances 150 μm ($p = 0.882$), 300 μm ($p = 0.995$), and 450 μm ($p = 0.998$). Up to 50 μm depth (at 150 μm from the treatment area), CL showed better performance than DU ($p < 0.05$). NT presented higher loss of Ca and P than CL and DU ($p < 0.05$). There was no significant difference in the % of F ion among the three groups. The new coating material was similar to F varnish in attenuating enamel demineralization.

Keywords: Demineralization, Tooth; Fluorides.

Introduction

Despite the decline of dental caries reported worldwide,^{1,2} caries is still considered a significant oral health problem.³ The main etiologic factor for its onset is the presence of a cariogenic biofilm and the intake of fermentable carbohydrates, promoting a dynamic process of demineralization and remineralization.⁴ Caries progresses when there is an imbalance of factors favoring demineralization, by the loss of calcium (Ca) and phosphate (P) ions from the apatite crystals of the tooth surface.⁵

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Fluoride (F) use still is the main strategy for non-invasive control of dental caries and, therefore, it has been applied through various methods, such as fluoridated toothpastes,⁶ mouthwashes, and varnishes.⁷ Among these methods, F varnishes as a tooth surface coverage seems to be a simple and effective way to protect at-risk enamel against acid⁸ provided by bacterial metabolism during a cariogenic challenge. In addition, F varnishes do not require patient's compliance. Despite the advantages of F varnishes, there is still some debate about whether this vehicle is as good for prevention as for repair of non-cavitated lesions.^{9,10,11} Therefore, improved remineralizing methods are essential for reducing enamel demineralization and the management of dental caries.⁹

Accordingly, new biofunctional materials that can be applied as thin coatings and actively release bioavailable ions have been introduced, as a strategy for aided remineralization and reinforcing dental hard tissue.⁸ Previous research has shown that a resin-modified glass-ionomer with Ca varnish used as coating material, improved in-depth protection of enamel submitted to a demineralization solution.⁸ However, this new generation of ion-releasing agents deserves further investigations simulating a real clinical condition, and a dynamic pH-cycling model could be appropriate. Furthermore, it would be important to evaluate possible features of this new material through the assessment of artificial caries-like lesions around the coated area, which could be detected by the inorganic components present in the tooth-material interface.

With the combined analyses of surface hardness (SH), cross-sectional hardness (CSH), and energy-dispersive X-ray spectroscopy (EDX), this study evaluated the effect of a varnish based on resin-modified glass-ionomer with Ca (Clinpro XT Varnish[®]) compared to a F varnish (Duraphat[®]) on demineralization inhibition of coated and uncoated surfaces and quantified the ions released from these materials in adjacent areas of the tooth-material interface. The null hypotheses tested were: (1) Covering enamel does not influence SH, CSH, and EDX results of the underlying enamel (coated area) and adjacent (uncoated) areas; (2) There is no difference in enamel

demineralization inhibition between Clinpro XT Varnish[®] and Duraphat F[®] varnish.

Methodology

Experimental design

This *in vitro* study evaluated the treatment factor, in three levels: NT - no treatment (negative control group); CL - resin-modified glass-ionomer with Ca (Clinpro XT Varnish; 3M-ESPE), and DU - fluoride varnish (Duraphat; Colgate). The experimental units were enamel specimens obtained from bovine incisors and selected by surface hardness (SH). The response variables were based on SH, CSH, and EDX. The Ca/P weight ratio was determined.

Specimen preparation and selection

Polished enamel specimens (3 × 6 × 2 mm) were obtained from bovine incisor teeth, which were cut using ISOMET Low Speed Saw cutting Machine (Buehler Ltd., Lake Bluff, IL, USA). Baseline SH was determined by five indentations, using a Knoop diamond indenter, spaced 100 μm from each other. Assessments were made under 25-g load for 10 s, using an HMV - 2000 (Shimadzu HMV-2; Shimadzu Corporation, Kiyamachi-Nijo, Kyoto, Japan). Enamel specimens presenting baseline SH ranging from 315 to 385 KHN were selected for the study.¹² To establish the homogeneity of the samples, specimens with average surface hardness > 10% or < 350 KHN were excluded.

Treatment of the specimens

The selected specimens were randomized according to initial SH and randomly divided into three groups (n = 12): CL, DU, and NT. The selected specimens were laterally coated with an acid-resistant varnish to allow only the exposure of the prepared surface (Red nail polish, Colorama[®]) (Figure 1). Three areas with 2-mm-width and 3-mm-length were exposed to the treatments. Before the pH-cycling protocol, 1/3 of the polished surfaces of each specimen received the treatments CL, DU, and NT. The central area was kept uncovered and was considered the adjacent-to-treatment area for the evaluation of the overall effect of ion-releasing materials. The other 1/3 of the specimen

was entirely covered with an acid-resistant varnish and used as control for the CSH analysis (Figure 1).

For the treatments with CL and DU, a thin layer of the materials was applied using a microbrush in accordance with instructions supplied by manufacturers as listed in Table.

pH-cycling regimen

The specimens were submitted to a pH-cycling regimen during 7 days at 37°C, according to

Vieira et al.¹³ The specimens were daily subjected to alternated immersions in 30 mL of demineralizing solution (2.0 mM Ca(NO₃)₂·4H₂O, 2.0 mM NaH₂PO₄·2H₂O, 0.077 mM acetate buffer, 0.02 ppm F, pH = 4.7) for 6 h and in remineralizing solution (1.5 mM Ca(NO₃)₂·4H₂O, 0.9 mM NaH₂PO₄·2H₂O, 150 mM KCl, 0.1 mol/L buffer, 0.03 ppm F, pH=7.0) for 18 h. On the last 2 days, the blocks were maintained only in remineralization solution, according to Vieira et al.¹³

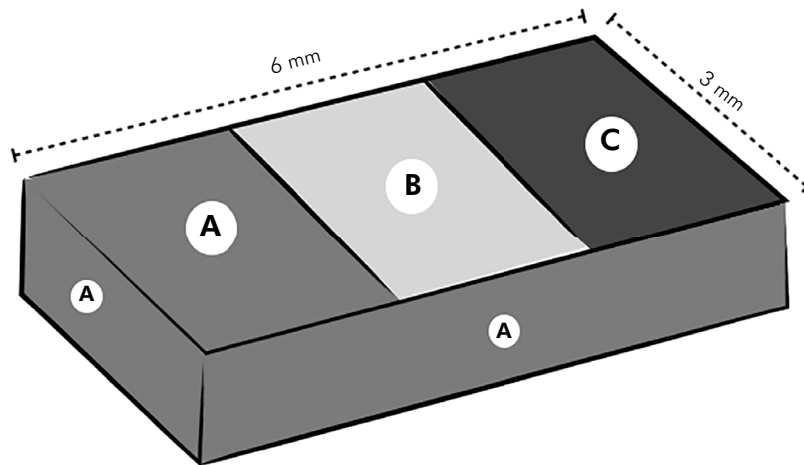


Figure 1. Surface of the enamel specimen divided into three areas (2-mm width and 3-mm length). (A), Area coated with acid-resistant varnish. (B), Central area uncovered and considered the adjacent-to-treatment area. (C), Area that received treatments (CL, DU, or NT).

Table. Composition of the materials used in this study and instructions supplied by manufactures.

Material	Brand	Composition	Application instruction
Resin-modified glass-ionomer (CL)	Clinpro XT Varnish (3M ESPE, ST Paul, MN, USA)	Liquid: HEMA, water, camphoroquinone, calcium glycerophosphate and polyalkenoic acid	Apply acid etchant for 15s with 35% phosphoric acid. Rinse with water. Apply air for 5s. Mix paste/liquid components together rapidly for 15s (2.5 min working time). Apply thin layer to tooth surface. Light cure for 20s.
		Paste: HEMA, Bis-GMA, water, initiators and fluoroaluminosilicate glass.	Wipe the coating by moist cotton applicator
Fluoride varnish (DU)	Duraphat (Colgate, São Paulo, SP BR)	Sodium fluoride, olophony, ethyl alcohol, shellac, mastic, saccharin, aroma, white beeswax	Apply thin layer to tooth surface using a cotton applicator.
No treatment (NT)	-	-	-

HEMA: 2 hydroxyetil methacrylate; Bis-GMA: bisphenol-A-diglycidyl methacrylate.

EDX analysis

For the analysis of the % component composition of the enamel, EDX assessment was performed as described by Velo et al.¹⁴ All specimens were examined by scanning electron microscopy (SEM) (Aspex Express; Fei Europe, Eindhoven, Netherlands) at accelerating voltage of 15-20 kV before and after the pH-cycling regimen, in relative vacuum. Elemental analysis by EDX, which is fully integrated to the Aspex Express SEM, was conducted over the entire area to determine the relative amounts of Ca, P, and F by weight percentage, carried out in standardless mode.

Surface hardness analysis

At the end of the pH-cycling regimen, SH (n = 12) was again determined. Five indentations at three standard distances from the treatment area were made (150 μm , 300 μm , and 450 μm) (Figure 2). The mean values from the five indentations were calculated and compared to the baseline means.

After SH analysis, all specimens were perpendicularly sectioned, embedded, and polished. Five rows of 5 indentations each were made, one below the material at 20, 50, 90, 110 and 220 μm depths (Knoop diamond, 25 g, 10 s, HVM - 2000), one below the area protected with nail polish at depths of 20, 50, 90, 110, and 220 μm ,¹⁵ and three in the central region of the external surface at the treatment area (150, 300, and 450 μm) (Figure 2). The mean values of

the five measuring points at each distance from the surface were then averaged and expressed as Knoop hardness number (kg/mm).

Statistical analyses

Data were calculated and statistically analyzed with IBM SPSS version 17 Software (Armonk, NY, USA). Normal distribution and equality of variances were checked for all the variables using Q-Q Plots and Kolmogorov-Smirnov tests. SH was analyzed using a Generalized Linear Model adjusted for each distance in a separate way. CSH was analyzed using two-way repeated measures ANOVA and Mauchly's test. For the EDX, the amounts of Ca and the Ca/P ratio were analyzed by one-way repeated measures ANOVA and Tukey's tests. The values of F and P were analyzed by the non-parametric Kruskal-Wallis test at a significance level of 5%.

Results

SH measurements

The changes in hardness values of enamel specimens are presented in Figure 1. A significant reduction in mean SH was observed in all specimens after the pH-cycling regimen. The final SH values for CL and DU ranged from 226 to 234 (Figure 3) with no significant difference between each other at distances of 150 μm ($p = 0.882$), 300 μm ($p = 0.995$) and 450

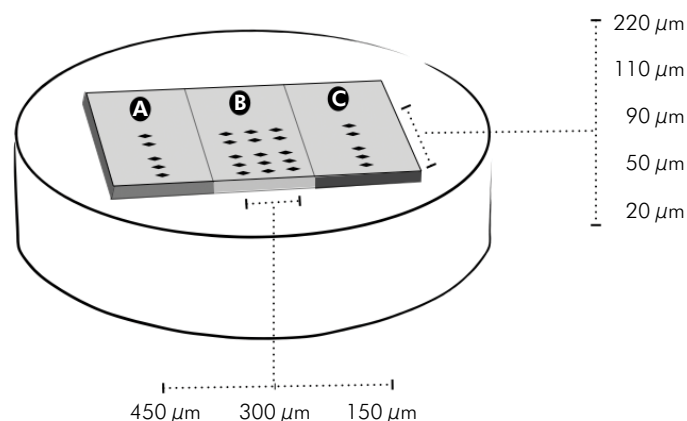


Figure 2. Distances of indentations and depth from the margin of each measured area for both SH and CSH analysis.

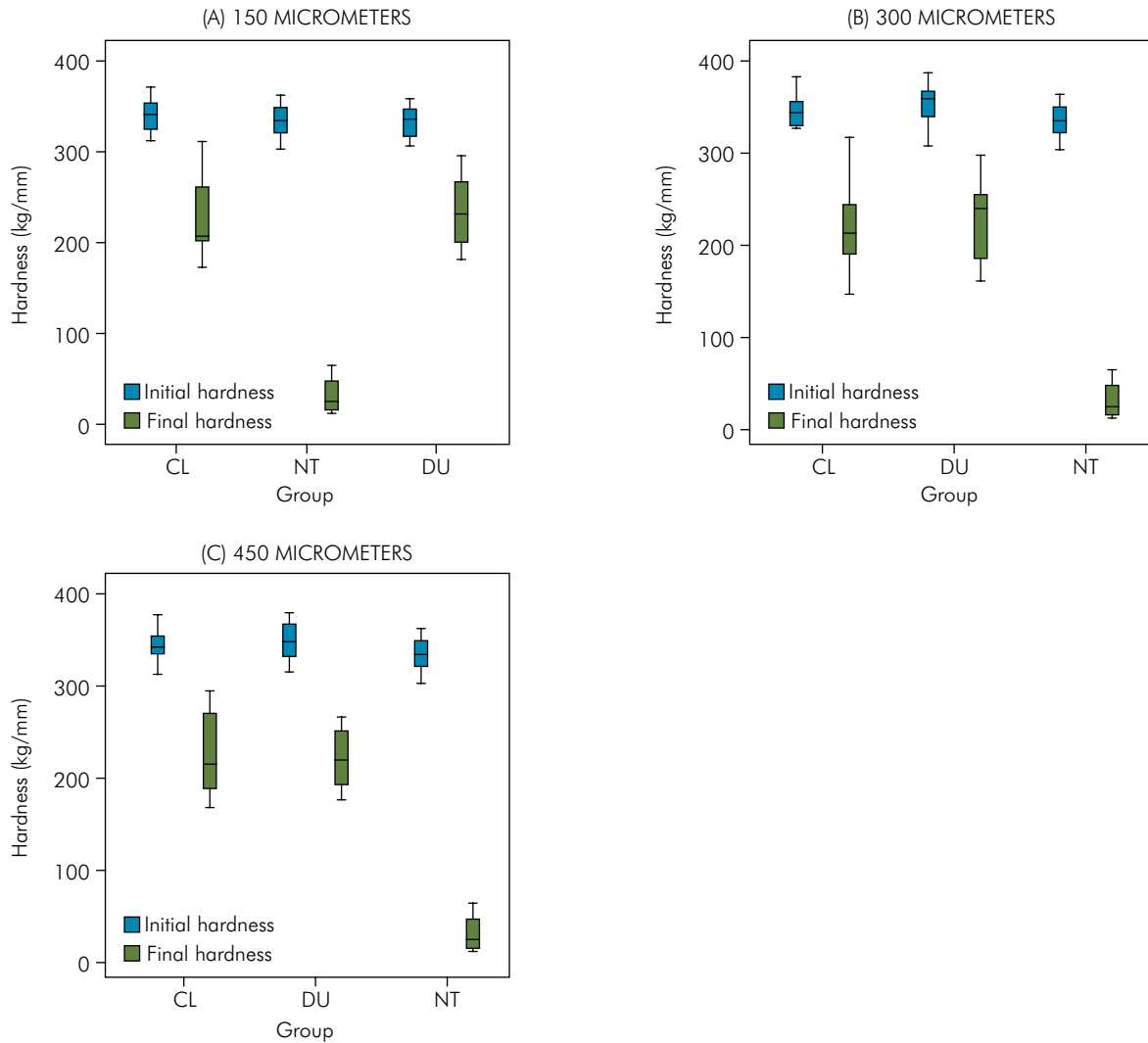


Figure 3. Mean values of initial and final surface hardness at (A) 150 µm, (B) 300 µm, and (C) 450 µm from the treatment area.

µm ($p = 0.998$). The NT group showed significantly greater surface hardness loss compared to CL and DU ($p < 0.01$). The final SH values for NT group were: 31.17 ± 18.69 at 150 µm; 33.09 ± 19.91 at 300 µm, and 30.89 ± 17.36 at 450 µm. There was no difference among the distances within each group ($p > 0.05$).

CSH measurements

Figures 4 and 5 show the mean hardness profile over the depth of the enamel surface (20, 50, 90, 110, and 220 µm) and at standard areas from the materials. The tests showed that the following interaction effects were statistically significant: Depth × Distance ($p < 0.001$; $\eta_p^2 = 0.504$), Depth × Group ($p = 0.024$; $\eta_p^2 = 0.129$) and

Distance × Group ($p = 0.010$; $\eta_p^2 = 0.148$). The values of initial depth of 20 µm were as follow: at 150 µm from the treatment area (CL: 196.33 ± 57.40 and DU: 103.65 ± 41.89); at 300 µm from the treatment area (CL: 124.83 ± 57.67 and DU: 125.31 ± 45.58); and at 450 µm from the treatment area (CL: 124.64 ± 45.16 and DU: 116.29 ± 49.57). The values of final depth of 220 µm were as follow: at 150 µm from the treatment area (CL: 311.75 ± 20.61 and DU: 305.42 ± 21.89); at 300 µm from the treatment area (CL: 311.17 ± 26.62 and DU: 297.58 ± 19.99); and at 450 µm from the treatment area (CL: 294.83 ± 24.27 and DU: 301.50 ± 41.91). The significant interactions suggested that the groups had different reactions at distinct depths and distances.

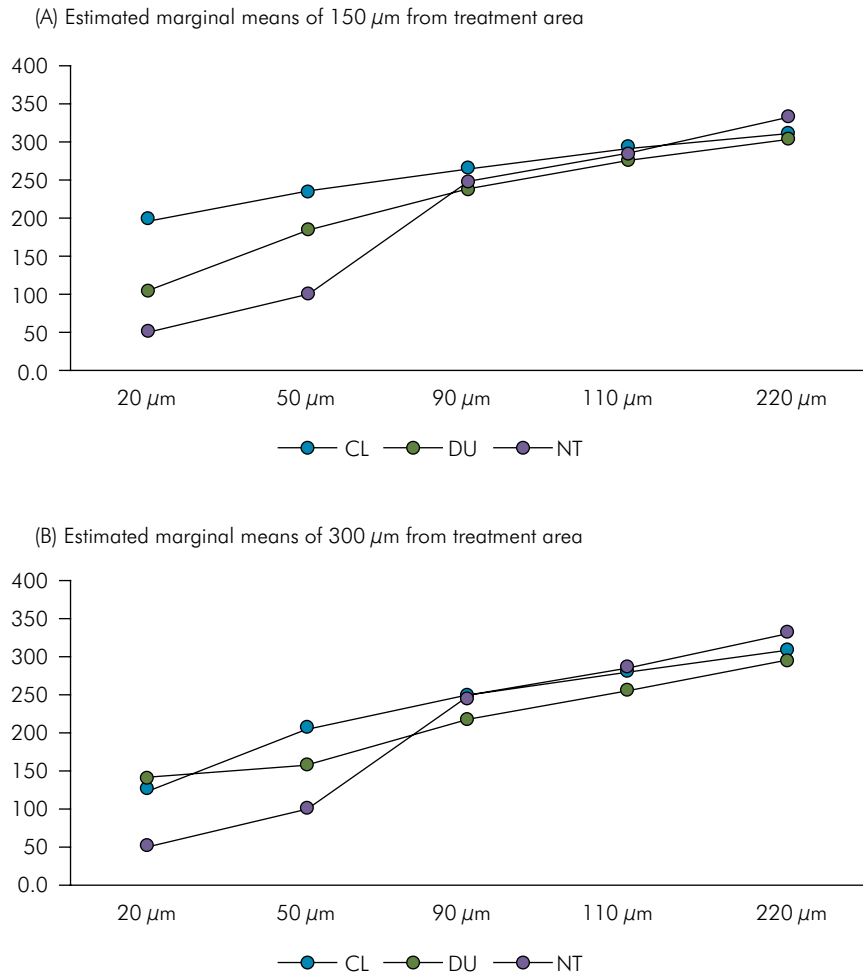


Figure 4. Mean values of enamel Knoop hardness (Kg/mm) and distance (µm) at (A) 150 µm, (B) 300 µm, and (C) 450 µm from the surface in the treatment areas.

EDX

The atomic percentages of Ca, P, and F on the specimens were determined by EDX (Figure 6). The groups presented no significant difference in increasing or reducing F and P levels ($p < 0.05$). The atomic percent of Ca tended to decrease in the group without treatment, NT ($p < 0.05$) (Figure 6).

The Ca/P weight ratio on the enamel specimens were also determined. The means and standard deviations before and after the pH-cycling regimen, respectively, were as follow: NT (1.42 ± 0.03^a , 0.49 ± 0.14^b); DU (1.41 ± 0.02^a , 1.43 ± 0.03^a); CL (1.44 ± 0.11^a , 1.42 ± 0.05^a). Distinct lowercase letters indicate significant difference among the treatments ($p < 0.05$). Overall, the final Ca/P molar ratio (after the pH-cycling regimen) decreased for the NT group ($p < 0.05$).

Discussion

The aim of this study was to evaluate the demineralization inhibition of a varnish based on resin-modified glass-ionomer with Ca compared to a fluoride varnish on coated and uncoated areas and the elemental inorganic content of enamel after treatments. Previous study has shown that this material improved in-depth protection of enamel submitted to a demineralization solution.⁸ However, in order to evaluate the demineralization inhibition and chemical composition of caries-like lesions, our study reproduced clinical situations through a pH-cycling regimen followed by SH, CSH and EDX analysis.

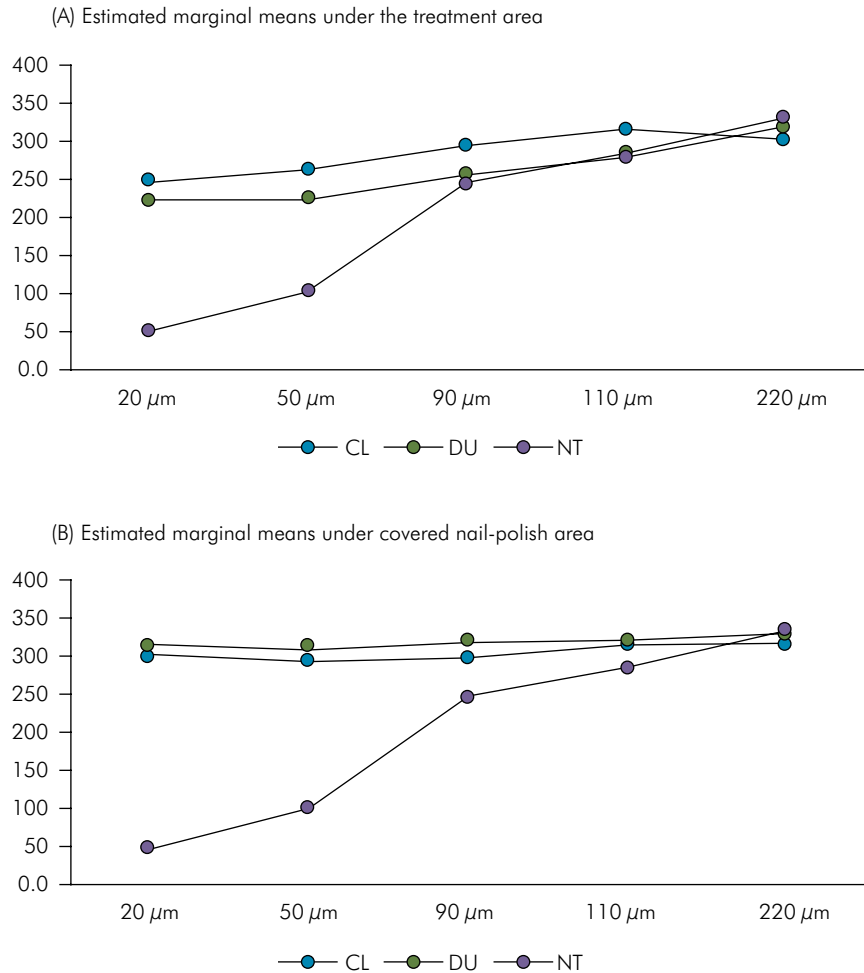


Figure 5. Mean values of enamel Knoop hardness (Kg/mm) and distance (μm) from the surface under the treatment areas (A) and under covered area (B).

A series of experiments were conducted in order to simulate clinical situations using a dynamic pH-cycling regimen with predominance of remineralization ($Re > De$), which simulates a patient not under caries activity¹⁶ and suitable for the main purpose of the study to evaluate the demineralization inhibition (preventive effect). The pH-cycling regimen chosen was adapted from a previous study using bovine enamel as substrate.¹³ This model was able to simulate the demineralizing and remineralizing episodes that occur in oral cavity; the demineralizing solution was not saturated regarding Ca and P ions in order to simulate the plaque fluid conditions allowing the formation of initial enamel lesions.¹³

All treatments were conducted in the same specimens to evaluate the experimental conditions. Thus, it was possible to evaluate the effect of both materials in the adjacent area and in an intact area with sound enamel (covered by nail polish). For a more conclusive analysis, enamel SH and CSH were measured, considering their high correlation with microradiography analysis, considered the gold standard.^{15,17,18} The CSH was performed since it gives important evidence regarding the mechanical in-depth resilience of the demineralized enamel due to the penetration of an indenter (physical strength), which might be indirectly related to its mineral content.¹⁵

The first hypothesis was partially accepted. Based on SH results, CL and DU did not inhibit the

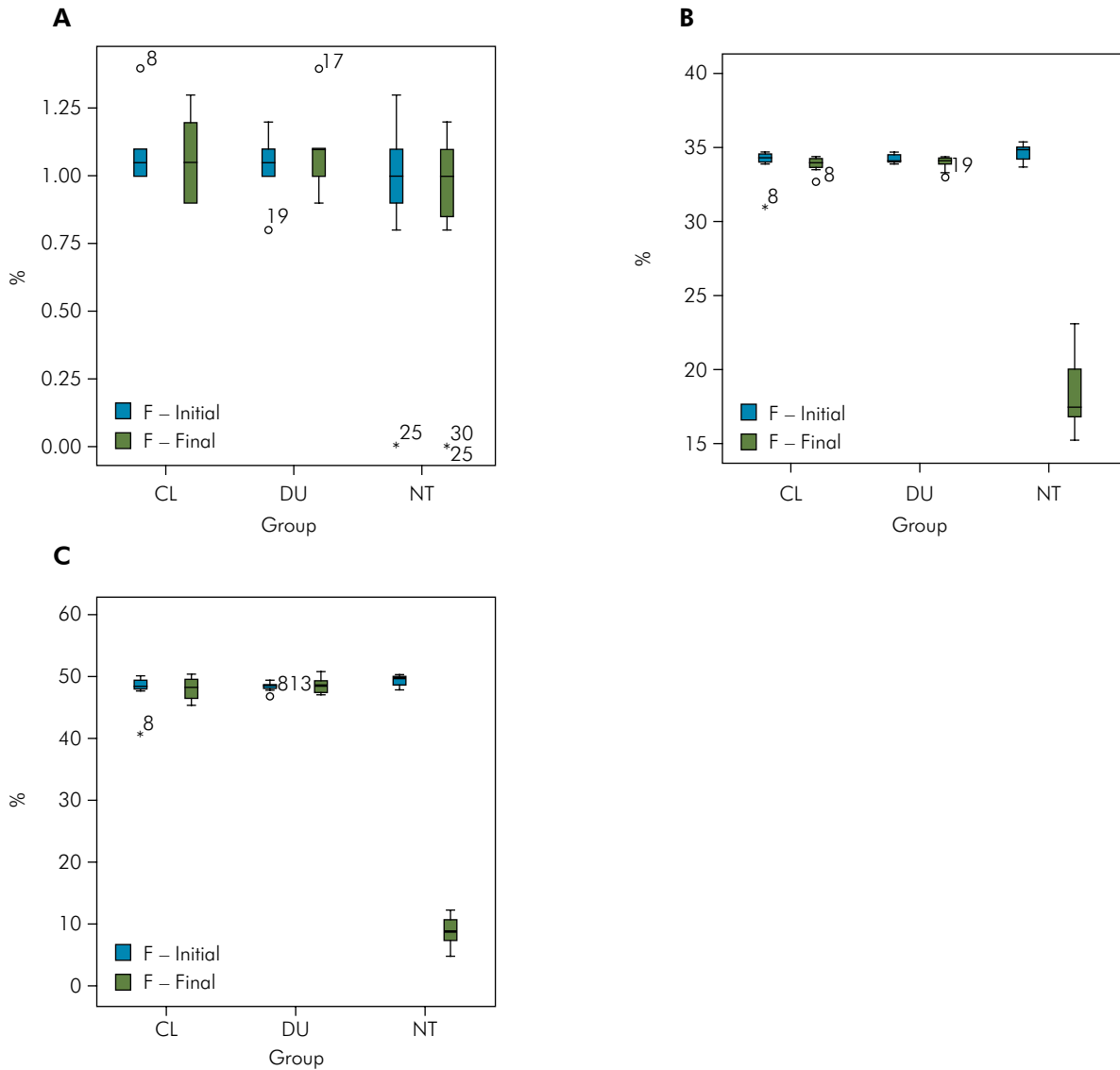


Figure 6. Atomic percentages of F (A), P (B), and Ca (C) on the enamel determined by EDX.

initial demineralization promoted by the pH-cycling regimen in the treated area (Figure 3) and these data are consistent with the current knowledge about the physicochemical effect of F on caries control, reducing demineralization and not avoiding it.¹⁹ Meanwhile, both varnishes were able to inhibit the demineralization of enamel more than the NT group (Figures 1 and 2). According to previous studies, the effect of Ca released from the coating material might influence in re-hardening of the enamel surface although there are few evidences about its benefits.²⁰ Unlike the highest remineralization

showed by Clinpro™ varnish in a previous study by Elkassas et al.,²¹ our results showed similar SH values from CL and DU after the pH-cycling regimen (Figure 3). However, it is important to highlight that herein we evaluated the demineralization inhibition (preventive effect) of such varnish and not enamel remineralization as reported before.²¹

For CSH analysis, the second hypothesis was accepted, since the extent of the demineralization process in group NT was higher at different surface depths when compared to DU and C, showing the greater protective effect of both products. Up to 50 μm

depth (at 150 μm from the treatment area, Figure 4A), CL showed better performance than DU. This fact could have occurred due to the chemical bond between glass ionomer of Clinpro XT Varnish and the tooth surface, which allows longer maintenance time. On the other hand, some studies claim that the addition of Ca and P ions to a glass ionomer-based material increases their availability for binding to the released F ions.^{21,22} This is in accordance with the concept that a high initial concentration of F is better for inhibiting the formation of new lesions, while lower initial concentration is most effective for remineralization and control of the progression of lesions.²³ In addition, we hypothesize that a resin-modified glass-ionomer with Ca used as coating material could be effective in the long term since it acts mainly in the subsurface of the lesion in adjacent area of the material as showed by CSH values (Figure 4A), but further studies are needed to elucidate this. The findings suggest a synergic effect of F and the released ions, promoting greater protection for enamel.²⁴ However, a limitation of this study must be highlighted. Ideally, the first depth of CSH measured should be performed as close as possible to the outer enamel surface. Considering the size of the micro-indenter used herein, the first measure was conducted at 20- μm depth and a nano-indenter should be used to evaluate shorter distances to obtain more reliable results.

Based on the results showed by EDX analysis, the most evident finding was the lower percentages of Ca and P in the NT group compared to CL and DU after the pH-cycling regimen (Figure 6). This result confirms the dissolution of hydroxyapatite and loss of minerals in the absence of remineralizing materials.¹⁶ In contrast, the percentages of F ions were not different among groups, showing that there was no incorporation of F into the enamel substrate (Figure 6). However, this data must be interpreted with caution owing to the fact that calcium fluoride-like particles (CaF_2) are the most abundant source of free ions during cariogenic challenges, forming a reservoir of F, which are subsequently incorporated into enamel as hydroxyfluorapatite or fluorapatite.²⁵ During a pH-cycling regimen, due to the continued de-remineralization episodes, most

of this reservoir is lost; therefore, CaF_2 -like particles might act as a reservoir on the enamel surface, and F is lost during pH-cycling. We hypothesized that, in a clinical condition, CaF_2 -like particles are formed and behave as a mineral reservoir releasing F to the biofilm and tooth surface.

Our results also showed a decrease in the Ca/P weight ratio in the NT group, which indicates that the tested treatments (DU and CL) altered the inorganic components of enamel, once the Ca/P weight ratio determines the rate of hydroxyapatite mineralization.¹⁴ This is a relevant parameter, as the mechanical properties of the substrate depends on hydroxyapatite mineralization; this data corroborated the SH and CSH results (Figures 3 and 4). The Ca/P ratio was calculated for stoichiometric hydroxyapatite as 2.151.²⁶ The lower values of the Ca/P of the NT group ($p < 0.05$) indicated that these specimens were less mineralized with respect to Ca content than the other groups (treated with DU and CL). In addition, there was no difference between DU and CL regarding the Ca/P ratio ($p > 0.05$).

The ions release from both coating materials can shift the interaction of cariogenic acids with different layers of enamel and may reinforce dental hard tissue against caries. Therefore, the use of the glass ionomer-based sealant (Clinpro XT-Varnish[®]) cannot be proposed as a superior option to conventional materials, but as an alternative for solving the progress of incipient carious lesions, avoiding the use of self-applied F products that requires patient cooperation. The data presented herein are relevant since the introduction of biofunctional materials such as resin-modified glass-ionomer with Ca might not be a cost-benefit strategy. As mentioned earlier, this new generation of varnishes could be effective in the long term since it acts mainly in the subsurface of the lesion in adjacent area of the material as showed by CSH values (Figure 4A), but further studies are needed to elucidate this.

Based on our results, clinicians can be encouraged to use the Clinpro XT Varnish to avoid treatment protocols that require multiple patient visits, since this material promotes similar results to the conventional F varnish. Therefore, further detailed studies are required to establish the cost-benefit ratio between

strategies that produce similar results in terms of prevention and health care.

Conclusions

In summary, the fluoride varnish Duraphat® and the glass ionomer-based sealant with Ca (Clinpro XT Varnish®) promoted partial inhibition of enamel demineralization by acid challenge in underneath and in adjacent areas. We hypothesize that the new

generation of varnishes with Ca could be effective in the long term since it acts mainly in the subsurface of the lesion, adjacent to the material as shown by CSH values.

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