Thickness and nanomechanical properties of protective layer formed by TiF$_4$ varnish on enamel after erosion

Maria Isabel Dantas de MEDEIROS[(a)]
Hugo Lemes CARLO[(a)]
Rogério LACERDA-SANTOS[(b)]
Bruno Alessandro Guedes de LIMA[(c)]
Frederico Barbosa de SOUSA[(a)]
Jonas Almeida RODRIGUES[(d)]
Fabiola Galbiatti de CARVALHO[(a)]

[(a)]Universidade Federal da Paraíba – UFPB, Postgraduation Program on Dentistry, João Pessoa, PB, Brazil.

[(b)]Universidade Federal de Campina Grande – UFCG, Department of Dentistry, Patos, PB, Brazil.

[(c)]Universidade Federal da Paraíba – UFPB, Department of Mechanical Tecnology, Tecnological Center, João Pessoa, PB, Brazil.

[(d)]Universidade Federal do Rio Grande do Sul – UFRGS, School of Dentistry, Department of Pediatric Dentistry, Porto Alegre, RS, Brazil.

Abstract: The layer formed by fluoride compounds on tooth surface is important to protect the underlying enamel from erosion. However, there is no investigation into the properties of protective layer formed by NaF and TiF$_4$ varnishes on eroded enamel. This study aimed to evaluate the thickness, topography, nanohardness, and elastic modulus of the protective layer formed by NaF and TiF$_4$ varnishes on enamel after erosion using nanoindentation and atomic force microscopy (AFM). Human enamel specimens were sorted into control, NaF, and TiF$_4$ varnish groups (n = 10). The initial nanohardness and elastic modulus values were obtained and varnishes were applied to the enamel and submitted to erosive challenge (10 cycles: 5 s cola drink/5 s artificial saliva). Thereafter, nanohardness and elastic modulus were measured. Both topography and thickness were evaluated by AFM. The data were subjected to ANOVA, Tukey's test and Student's t test (α = 0.05). After erosion, TiF$_4$ showed a thicker protective layer compared to the NaF group and nanohardness and elastic modulus values were significantly lower than those of the control group. It was not possible to measure nanohardness and elastic modulus in the NaF group due to the thin protective layer formed. AFM showed globular deposits, which completely covered the eroded surface in the TiF$_4$ group. After erosive challenge, the protective layer formed by TiF$_4$ varnish showed significant properties and it was thicker than the layer formed by NaF varnish.

Keywords: Tooth Erosion; Dental Enamel; Microscopy, Atomic Force.

Introduction

The prevalence of tooth erosion has been increasing among children and adolescents, mainly because of the high consumption of soft drinks.[1,2] As it is difficult to change the dietary habits of these patients, other strategies have been proposed to control dental erosion, such as the topical application of fluoride,[3,4,5] which forms a precipitation of calcium fluoride (CaF$_2$)-like deposits on the tooth surface.[4,5,6]

For caries prevention, these CaF$_2$-like deposits increase the fluoride content of plaque for several hours and some protective capability against caries has been shown.[7] However, the effectiveness of fluoride and CaF$_2$-like deposits in the prevention of tooth erosion is limited to the surface or to

the adjacent surface layer of enamel,[4,6] because there is no sheltered area in the erosive process as occurs in subsurface carious lesions.[4] Accordingly, the efficacy of other fluoride compounds with polyvalent metal ions, such as titanium tetrafluoride (TiF_4), has been evaluated against erosion. The protective effect of TiF_4 is related to the increased uptake of fluoride due to its acidic pH and to the formation of a glaze-like surface layer rich in titanium and fluoride.[8,9,10]

Acidic and high-concentration fluoride formulations, such as varnishes, have been more effective against erosion compared to toothpastes and mouth rinses.[6] The antierosive effect of TiF_4 varnish compared to conventional varnishes, such as sodium fluoride (NaF), is still contradictory.[11,12,13,14] In order to explain these contradictory results, recent speculations have been made about the deposition and thickness of the layer formed by TiF_4 varnish application,[4,15] but there is a paucity of data on the thickness and properties of the protective layer formed by TiF_4 varnish after an erosive challenge.

Nanoindentation, which measures indentation load–displacement response behavior, is a well-established technique for determining the mechanical properties of thin protective layers.[16] A diamond indenter is used to apply small loads on the order of nN.[17] Nanoindentation testing can quantify the nanohardness and elastic modulus of protective layers formed by fluoride agents on the tooth structure or determine the demineralization of the outermost softened layer of an eroded enamel surface. This test also allows accurate determination and control of indentation force and accurate measurement of indentation depth.[16,17]

According to Lussi and Carvalho,[4] to fully protect the enamel surface against erosion, the protective layer formed by fluoride compounds should be dense enough to build up a physical barrier that protects the underlying enamel from erosive acids and it should also be stable enough against erosive dissolution. As there is no investigation into the thickness and nanoproperties of the protective layer formed by NaF and TiF_4 varnishes on eroded enamel, the following hypotheses were tested in the present study: (1) The nanoindentation test would reveal differences in the nanohardness and elastic modulus of the protective layer formed by NaF and TiF_4 varnishes on enamel surface after the erosive challenge; (2) Atomic force microscopy (AFM) would show differences between the thickness and topography of the protective layer formed by NaF and TiF_4 varnishes on eroded enamel.

Methodology

Specimen preparation

After obtaining approval from the Research Ethics Committee (Protocol no. 0056/13), 40 sound human third molars were selected for this study. The teeth were stored in 0.1% thymol at 4°C and used within 1 month after extraction. Two enamel specimens (4 × 4 × 3 mm) from each tooth were cut with a flexible diamond disc (7016, KG Sorensen, Barueri, Brazil) at low speed under water cooling, and 50 specimens were obtained. They were embedded in acrylic resin and the enamel surfaces were ground flat with SiC abrasive paper discs (400, 600 and 1200 grit) and polished with 1 μm alumina suspension (Erios Corp., São Paulo, Brazil). Afterwards, the specimens were checked for the presence of cracks and fractures using a microscope (Nikon 88286, Tokyo, Japan) at 40× magnification. The baseline nanohardness of the enamel surface was determined and specimens with 4.85 GPa ± 20% of this value were selected for standardization of the initial hardness.[18] Specimens without the predetermined values were discarded. Thirty specimens were selected and allocated to three groups (n = 10), according to fluoride varnish application: (1) Control group – no varnish application; (2) NaF group – NaF varnish application (Duraphat, Colgate-Palmolive Ltda., São Bernardo do Campo, Brazil) and 3. TiF_4 group – experimental TiF_4 varnish application (FGM, Joinville, Brazil) (Table 1). Afterwards, 10 extra specimens were selected for evaluation of the thickness of the protective layer formed by NaF and TiF_4 varnishes (n = 5 for each).

Fluoride varnish application

The specimens were immersed in artificial saliva for 24 h[19] and the following method was used

Table 1. Composition of fluoride varnishes tested in the study.

Material	Composition (batch number)
Duraphat (Colgate-Palmolive, Ind. Com. Ltda., São Bernardo do Campo, Brazil)	2.26% sodium fluoride, alcohol, natural resins, wax, saccharine, flavor – pH 4.5 (51001)
Experimental TiF$_4$ varnish (FGM, Joinville, Brazil)	2.26% titanium tetrafluoride, alcohol, synthetic resin, and natural resin – pH 3.4 (200313)

for the production of artificial saliva (in g:L): 2 g of methyl p-hydroxybenzoate, 0.625 g of sodium carboxymethylcellulose, 0.059 g MgCl$_2$ – 6H$_2$O, 0.166 g of CaCl$_2$ 2H$_2$O, 0.804 g of K$_2$HPO$_4$, and 0.326 g of KH$_2$PO$_4$ adjusted to pH 6.75 with KOH.[20] After that, the varnish was applied to each specimen, in each of the groups. Each varnish was individually drawn into a 0.3 mL insulin syringe (BD Ultra-fine, Franklin Lakes, USA) for standardization of the amount of agent applied. For the NaF and TiF$_4$ groups, 20 μL of each varnish was applied to the enamel surface and spread with a microbrush. The specimens were immersed in artificial saliva for 6 h for clinical simulation of the contact time of the varnish with the tooth surface.[11,14] Thereafter, the varnishes were carefully removed from the surface with acetone using a scalpel blade.[9,11,14] Total removal of the layer was checked microscopically. The varnishes were removed to evaluate their chemical effect rather than their mechanical protection, and also to simulate the clinical situation in which varnishes might be removed post-application through regular toothbrushing and mastication.[12,21] In the control group, no product was applied and the specimens were immersed in artificial saliva for 6 h. The varnishes were applied only once before the erosive challenge.[11,12,14]

Erosive challenge

The erosive challenge was based to simulate the drinking of a can of cola beverage (325 mL – pH 2.6; Coca-Cola, Porto Real, Brazil) by an individual according to Wongkhantee et al.[22] The specimens were immersed in 32.5 mL of cola drink for 5 s at room temperature, rinsed in deionized water, and then immersed in 32.5 mL of artificial saliva at room temperature for 5 seconds. This cycle was repeated 10 times. After that, the specimens were kept at 100% humidity until the nanoindentation test was performed.

Nanoindentation test

Both nanohardness and elastic modulus were evaluated by dynamic ultramicrohardness testing (DUH-211S, Shimadzu, Japan) with a Vickers indenter. A peak load of 5 mN was used, with loading and unloading rates of 0.3113 mN/s and a holding period of 10 s, and the minimum load was 0.02 mN.[23] Each specimen was loaded at one loading rate and one unloading rate. In each readout, five indentations were made in each specimen, at least 50 μm apart. The mean value was taken to represent the specimen's nanohardness and elastic modulus. Measurements were taken at baseline and after the erosive challenge. Each testing cycle consisted of three segments: (a) the loading segment, (b) the peak load holding segment, and (c) the unloading segment.

The method described by Oliver and Pharr[24] was used to calculate the hardness (H) and elastic modulus (E), using the formulas:

$$H = P_{max} / A_c$$
$$1/E_r = (1 – v^2)/E + (1 – v_i^2)/E_i,$$

where P_{max} is the peak load, A_c is the contact area, E and v are Young's modulus and Poisson's ratio for the specimen, and E_i and v_i are the same parameters for the indenter (1.14 GPa and 0.07, respectively). Poisson's ratio for the enamel was assumed to be 0.4.[25]

The depth of indentation for each specimen after varnish application and erosive challenge was given by the ultramicrohardness software program as maximum height (nm), by the formula: $H = 3.8584 \times F / h^2$, where H is the hardness, F is the peak load, and h is the maximum height. All values were recorded and a mean depth of indentation (nm) was calculated for each group.

Optical microscopy

Five specimens from NaF and TiF$_4$ groups were randomly selected for the analysis of enamel surface coverage after varnish application and erosive

challenge using an optical microscope (Axiotech 100, Carl Zeiss, Toronto, Canada) at 100× magnification.

Atomic force microscopy

The same five specimens evaluated by optical microscopy were analyzed by atomic force microscopy (AFM; SPM-9600, Shimadzu, Kyoto, Japan) to assess the topography of the protective layer formed by NaF and TiF_4 varnish application. Each specimen was fixed to the microscope stage on a stub (2 × 3 mm). The block surface morphology was probed in "contact mode". Imaging was performed with standard geometry silicon nitride Micro-Cantilever (OMCL-TR, Olympus, Tokyo, Japan) and probed with 0.15 N/m elastic constant and 24 KHz resonant frequency. Images with 30 × 30 μm, a resolution of 256 × 256 pixels, and operating point of 1.5 V were collected at a very low scan rate.

To evaluate the thickness of the protective layer formed by TiF_4 and NaF varnishes, five extra specimens were prepared for each varnish group. The enamel surface was divided into two equal parts: one covered with two layers of acid-resistant nail varnish (control surface – without varnish application) and one covered with fluoride varnish. The varnishes were carefully removed from the surface with acetone using a scalpel blade and the specimens were submitted to the erosive challenge, as described. Afterwards, the nail varnish was removed with a scalpel blade and the post-erosion thickness of the protective layer was measured by AFM using the "Height Trace" tool. After image capture, four parallel lines were drawn on each specimen. Each outline started on the control half (without varnish application) and finished on the half to which the fluoride varnish was applied. The difference in height of each line was recorded by the "Height Trace" tool and the thickness of the protective layer was measured. The mean thickness (nm) of the protective layer was obtained for each group. The depth of indentation was compared with the thickness values obtained for the protective layer formed to investigate whether the indentation had been made on the enamel surface or on the protective layer.

Sample size calculations

The sample size for testing the nanoproperties of varnishes on eroded enamel (n = 10) with ANOVA was based on the following parameters: 80% power, 5% significance level, effect size "f" of 0.60, and degrees of freedom (number of population means -1) of 2 (Table 8.4.4 in Cohen[26]).

To test the thickness of the protective layer formed by each varnish, the sample size was calculated based on a two-tailed significance level of 5%, an effect size "d" of 2, and an 80% power, using the formula:[26] $[(1571)/100 × 2^2)] + 1 = 5$

Statistical analysis

Statistical power and 95% confidence intervals were calculated using the respective equations published in the literature.[26] For the ANOVA, statistical power was calculated using an alpha error of 5%, 2 degrees of freedom, and the experimental effect size "f". For the paired t test, a two-tailed alpha error of 5% and the effect size "d" were used. The data were analyzed by GraphPad Instat, version 2.0 (GraphPad software program, La Jolla, USA), at a level of significance of α = 0.05. Since all the tested variables satisfied the assumptions of equality and normal distribution (Bartlett and Kolmogorov-Smirnov tests, respectively), two-way ANOVA and Tukey's test were carried out for statistical comparisons of nanohardness and elastic modulus, and of indentation depth and thickness of protective layers among groups. The paired t test was used to compare baseline and post-erosion nanohardness and elastic modulus within the same group.

Results

The experimental effect size f was 1.38 and the statistical power of ANOVA was > 99%. The p-values for ANOVA are statistically significant even after the Bonferroni correction (which results in a significance level of 0.05/3 = 0.0167 or 1.67%). Regarding the comparison of the thickness of the protective layer, the effect size "d" was 4.98 and the statistical power was >99% for a two-tailed alpha error of 5%.

The mean values (±SD) of nanohardness and elastic modulus for each group are reported in Table 2. There were no statistically significant differences among

groups regarding baseline nanohardness and elastic modulus values (p = 0.73 and p = 0.81, respectively). After the erosive challenge, the TiF_4 group statistically showed the lowest nanohardness and elastic modulus values (p = 0.02) whereas the NaF group showed the highest values (p = 0.03). The statistical comparison before (baseline) and after erosion for each group showed that only the control and TiF_4 groups exhibited significant differences in nanohardness (p = 0.001 and p = 0.002, respectively) and elastic modulus (p = 0.008 and p = 0.001, respectively).

The mean thickness (±SD) of the layer formed by NaF and TiF_4 varnishes and the mean values (±SD) of indentation depth for each group are reported in Table 3. As the post-erosion indentation depth value for the TiF_4 group (296.9 ± 49.2 nm) was lower than the protective layer thickness (953 ± 55.7 nm), the nanohardness and elastic modulus values obtained were related to the protective layer. Conversely, indentation depth for the NaF group (213.7 ± 17.1 nm) was higher than the protective layer thickness (53.1 ± 3.7 nm), and the nanoproperties measured were related to enamel. The protective layer thickness of the TiF_4 group was significantly higher than that of the NaF group (p = 0.001).

Figures 1, 2 and 3 show AFM images of the protective layer formed by NaF and TiF_4 varnishes.

The control group showed areas of eroded enamel without a protective layer (Figures 1A and 1B). The NaF group showed globular deposits, but the enamel surface was not entirely covered with a protective layer (Figures 2A and 2B). The TiF_4 group showed continuous globular deposition, forming a protective layer that completely covered the enamel surface (Figures 3A and 3B). Similarly, optical microscopy images showed that the NaF group showed an irregular protective layer on the enamel (Figure 4A) and that the TiF_4 group formed a continuous layer, covering the enamel surface thoroughly (Figure 4B).

Discussion

The protective layer formed by NaF and TiF_4 varnishes was investigated using an erosive challenge designed to simulate the drinking of a can of soft drink (325 mL) by an individual, as described in other studies,[22,27] with time comparable to that of the intake of a single drink. According to Attin and Wegehaupt,[17] in the oral cavity, the contact of the enamel with acidic beverages is usually limited to a few seconds before clearance by the saliva. Thus, the detection of small changes would allow reducing the contact of acidic beverages with the tooth surface in *in vitro* experiments to a time period that mimics intraoral conditions.[17] Furthermore, a mild or an aggressive

Table 2. Nanohardness and elastic modulus of groups after varnish application and erosive challenge.

Groups (n = 10)	Nanohardness			Elastic modulus		
	Baseline	Post-erosion	95%CI**	Baseline	Post-erosion	95%CI**
Control	4.9 ± 0.3[a*, A**]	3.4 ± 0.4[b, B]	3.6–3.2	124.2 ± 15.5[a, A]	104.0 ± 8.5[b, B]	104.1–103.8
NaF	4.8 ± 0.5[a, A]	4.4 ± 0.5[a, A]	4.6–4.2	127.4 ± 10.3[a, A]	121.7 ± 15.3[a, A]	121.9–121.5
TiF_4	5.0 ± 0.3[a, A]	2.4 ± 0.8[b, C]	2.6–2.2	128.5 ± 13.3[a, A]	82.2 ± 14.3[b, C]	82.4–82.0

*Values expressed in GPa (mean ± standard deviation). The same lowercase letters indicate no significant difference between baseline and post-erosion values (paired t test, p >.05). **The same uppercase letters indicate no significant difference among groups in each experimental period (baseline and post-erosion) (Two-way ANOVA and Tukey's test, p >.05). 95%CI values = upper bound; lower bound.

Table 3. Depth of indentation and thickness of protective layer formed by varnishes after erosive challenge.

Groups (n = 5)	Indentation Depth (nm)	Thickness of protective layer (nm)	
			95%CI*
Control	237.5 ± 19.7[*B]	-	-
NaF	213.7 ± 17.1[C]	53.1 ± 3.7[B]	60.2–45.7
TiF_4	296.9 ± 49.2[A]	953 ± 255.7[A]	1002.4–903.5

*Values expressed in nm (mean ± standard deviation). The same uppercase letters indicate no significant difference among groups (Two-way ANOVA and Tukey's test, p >.05). 95%CI values: upper bound; lower bound.
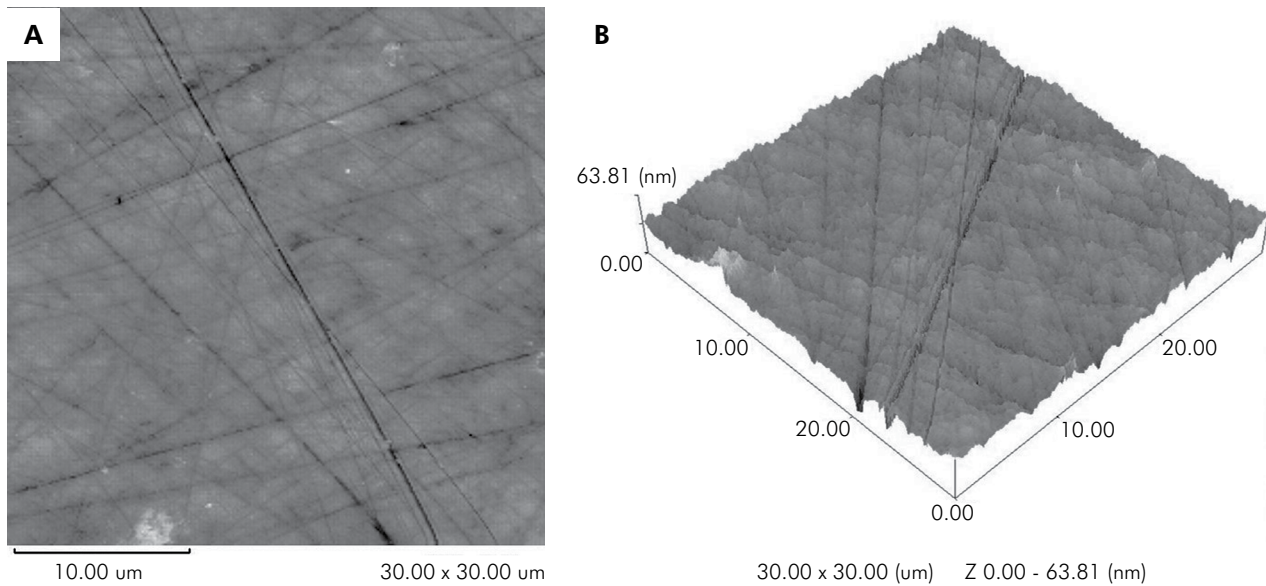
Figure 1. AFM image of enamel surface after erosive challenge and no-varnish application. (A) "Straches" lines are caused by the polishing procedure. (B) Rough surface without protection layer.

erosive challenge might not be adequate to evaluate the nanomechanical properties of protective layers formed by varnishes because the nanoindentation test has a limited use for longer exposure times than the one evaluated in the present study.[23]

The standardization of baseline nanohardness values (Table 2) made it possible to establish nanomechanical comparisons among the groups after treatment. A significant decrease in nanohardness and elastic modulus was found for the control group after erosion. This information is clinically relevant because the erosive challenge used demonstrated that a very short time of exposure to a cola beverage was enough to cause changes in the nanohardness
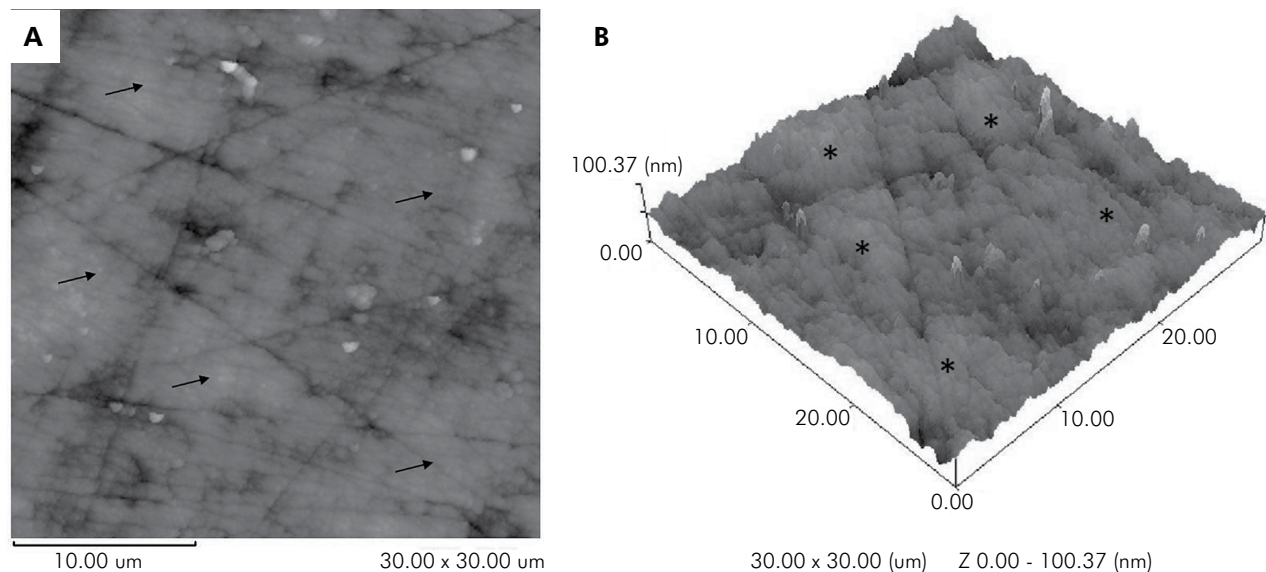

Figure 2. AFM image of enamel surface after NaF varnish application and erosive challenge. (A) (→) Interspersed globular deposits on surface. (B) *Enamel surface partially covered by a layer of globular deposits.
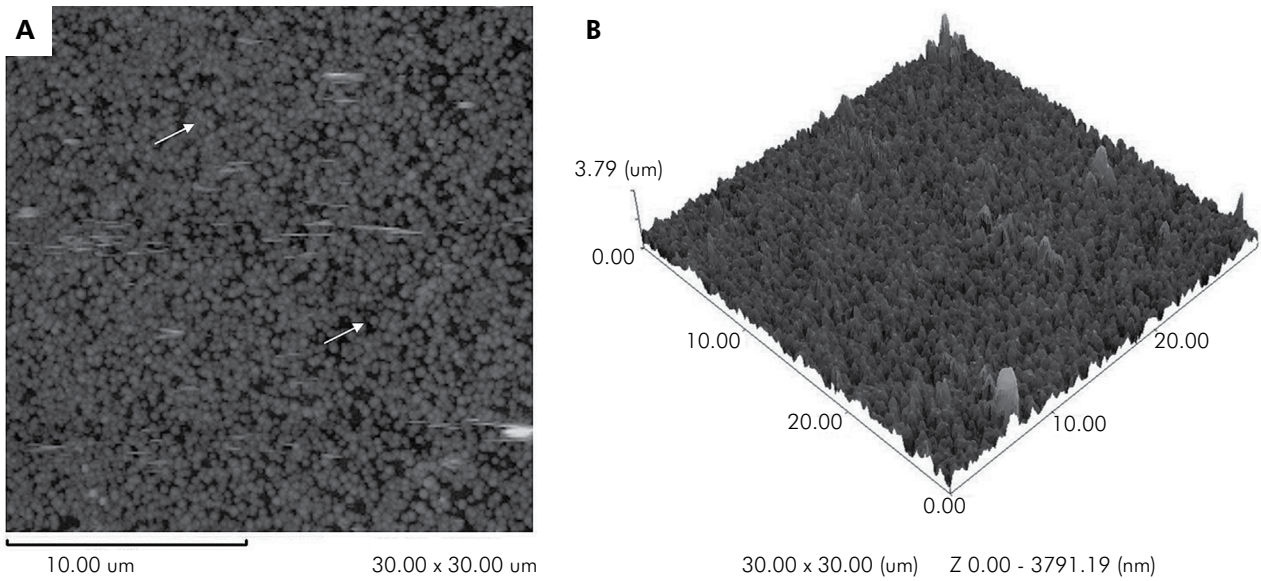
Figure 3. AFM image of enamel surface after TiF$_4$ varnish application and erosive challenge. (A) (→) Continuous globular deposits on surface. (B) Enamel surface completely covered by a layer of globular deposits.

and elastic modulus of the enamel. This result emphasizes the importance of prevention of dental erosion even in patients who have not yet developed the disease, but who are at risk, such as those who habitually consume soft drinks. Similarly, it was demonstrated by the nanoindentation test that there was significant enamel softening after a 30 s exposure to the soft drink.[28]

Fluoride varnishes have the capacity to adhere to the tooth surface, allowing for an increased contact time with the tooth and providing a positive effect on erosion prevention.[8,12,14] The antierosive effect of experimental TiF$_4$ varnish is mainly compared to that of the conventional NaF varnish, and studies have prompted speculations about the thickness and properties of protective layers formed by NaF and TiF$_4$ varnishes.[12,13,14,15] Levy et al.[12] speculated that the CaF$_2$ layer is less resistant to erosion compared with metal-rich surface precipitates. Comar et al.[15] speculated that the glaze-like surface layer produced
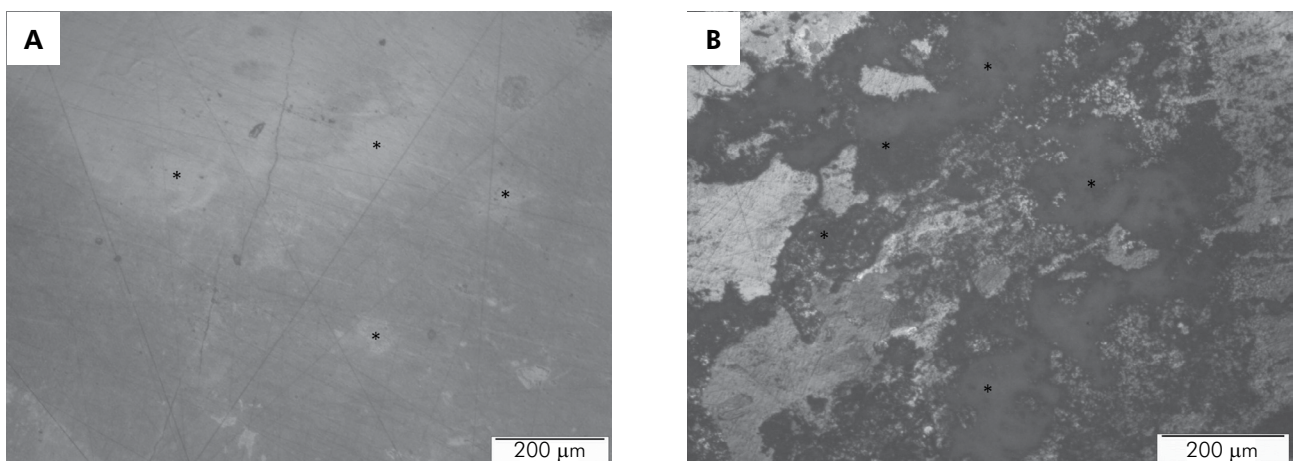

Figure 4. Optical microscopy image (100×) of NaF and TiF$_4$ groups. (A) NaF group with interspersed layer formation on surface. (B) TiF$_4$ group with continuous layer formation on surface. *Layer formed after varnish application.

by TiF$_4$ might be too thin. Based on these speculations, the present study was the first to demonstrate the thickness and nanomechanical properties of the protective layer formed by TiF$_4$ varnish after an erosive challenge.

It is known that the glaze-like layer is assumed to be formed from a new compound (hydrated hydrogen titanium phosphate) or organometallic complexes through the replacement of calcium with titanium ions in the apatite lattice.[8,9,10] The layer formed by TiF$_4$ was thicker than the NaF layer (Table 3), with globular deposition completely covering the enamel surface (Figures 3 and 4B). In addition, the 953 ± 55.7 nm thickness found in the present study can be another factor related to the antierosive effect of the TiF$_4$ varnish. Probably, the thicker the layer is, the less the diffusion of the acids will be through the layer, building up a physical barrier that protects the underlying enamel from acid attack. Furthermore, the TiF$_4$ varnish showed similar fluoride concentration (2.26%), but lower pH (3.4) in comparison with the NaF varnish (4.5) (Table 1). The low pH might also contribute to the higher precipitation of TiF$_4$ deposits. The thickness of the protective layer formed by fluoride compounds and post-erosion enamel loss were also evaluated by Stenhagen et al.[29] The average thickness of the glaze-like layer formed by TiF$_4$ was 600 nm,[29] but a TiF$_4$ solution was used. Since TiF$_4$ formulations and methodologies differed between the two studies, the results cannot be directly compared.

Besides the thick glaze-like layer formed by application of the TiF$_4$ varnish, Figures 3 and 4B show that this layer completely covered the eroded enamel surface. The NaF group showed interspersed globular deposits, not entirely covering the surface (Figures 2 and 4A). Similarly, Koeser et al.[30] and Lussi and Carvalho[4] found, under optimized conditions for CaF$_2$-like precipitation, no more than 40% of enamel surface coverage with the CaF$_2$ layer.

Although this study found lower nanohardness and elastic modulus values for the TiF$_4$ group (Table 2), these results were related to the layer formed by the TiF$_4$ varnish. This is the first demonstration of the nanomechanical properties of the protective layer formed by TiF$_4$ varnish after an erosive challenge.

Clinically, these values might also be associated with the positive antierosive effects of TiF$_4$ varnish and its better ability to adhere to enamel compared to NaF, since the numerical difference between eroded enamel and the TiF$_4$ layer was approximately 1 GPa for nanohardness and 21.8 GPa for the elastic modulus.

On the other hand, it was not possible to measure the nanoproperties of the CaF$_2$ layer, because the average post-erosion depth of indentation in the NaF group was 213.7 ± 17.1 nm and the thickness of the protective layer was 53.1 ± 3.7 nm (Table 3). Thus, the post-erosion nanohardness and elastic modulus values were related to the enamel surface treatment. However, the NaF group showed no statistically significant difference before and after the erosive challenge (Table 2), demonstrating that this varnish was effective in preventing the initial demineralization of enamel caused by exposure to the cola beverage. This protective effect of the NaF varnish has also been observed in previous studies.[14,31]

Therefore, the glaze-like layer formed by TiF$_4$ was thick, dense, and completely covered the eroded enamel surface. These properties of the protective layer formed by fluoridated compounds were considered necessary by Lussi and Carvalho[4] in order to fully protect the enamel surface against erosion. The two hypotheses tested were accepted because there were differences in nanohardness, elastic modulus, and thickness values and topography of the protective layer formed by NaF and TiF$_4$ varnishes on the enamel surface after the erosive challenge. However, investigations are necessary to test the maintenance of mechanical properties of the protective layer on the enamel surface using longer experimental periods of erosive challenge and wear.

Conclusion

After a short time of exposure to a cola beverage, the TiF$_4$ varnish formed a thicker protective layer and completely covered the eroded enamel surface, compared to the NaF varnish.

Acknowledgements

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Author's name
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