In situ effect of titanium tetrafluoride varnish on enamel demineralization

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Abstract: The effect of a 4% titanium tetrafluoride (TiF_4) varnish on enamel demineralization was evaluated. Twelve volunteers participated in this double-blind, randomized crossover study. Six enamel specimens were positioned in intraoral appliances throughout four treatment stages: 4% TiF_4 varnish (experimental varnish), 5% sodium fluoride (NaF) varnish (Duraphat®), placebo varnish, and negative control (deionized water). After 24 h, the varnishes were removed and plaques were allowed to accumulate. A 20% sucrose solution was dripped onto enamel blocks (10x/day). Enamel alterations were analyzed by surface microhardness (SMH), percentage of surface loss (%SML), cross-sectional microhardness (CSMH), scanning electron microscopy (SEM), and energy dispersive X-ray spectrometry (EDS). Student's paired t-test was used for SMH analysis and repeated-measures analysis of variance (ANOVA) for %SML and CSMH (ΔZ) analyses (p-value=0.05). The TiF_4 varnish group had lower %SML than the placebo and control groups (p=0.044 and p=0.003, respectively), thus showing its capacity to inhibit surface demineralization. TiF_4 and NaF varnishes demonstrated a protective effect against mineral loss on the enamel subsurface. Both were statistically different from the control group when CSMH was analyzed (p=0.000). A titanium dioxide film was observed on enamel surfaces of the TiF_4 group SEM images. EDS confirmed the presence of titanium in all TiF_4 samples. The 4% TiF_4 varnish is a promising compound capable of reacting with enamel to protect it against surface and subsurface demineralization.

Keywords: Fluorides; Tooth Demineralization; Biofilms.

Introduction

Fluoride (F) products have been widely used in the prevention and control of caries and the fluoride ion (F⁻) present in these products has been considered the major determining factor for caries reduction in most industrialized countries.[1,2] Topical fluoride agents are known to promote remineralization and inhibit demineralization of dental hard tissues due to calcium fluoride (CaF_2) reservoir.[1,3]

The formation of CaF_2 and its protective effect on demineralization depend on pH, fluoride ion concentration, and type of fluoride salt.[3] In a cariogenic challenge, F released from the CaF_2 reservoir may diffuse into the enamel and bind to apatite.[3]

Most commercially available dental varnishes contain sodium fluoride (NaF) and some authors have investigated other F compounds such as titanium tetrafluoride (TiF$_4$) for the prevention of enamel demineralization.[4,5,6,7,8]

Titanium tetrafluoride is a fluoridated compound with low acid solubility and resistance to corrosion. It has a profound ability to protect against demineralization due to the deposition of CaF$_2$ and formation of an acid-resistant titanium dioxide (TiO$_2$) layer on the enamel.[4,9,10,11]

TiF$_4$ solution is a promising anticaries compound, but it is unstable and presents a low pH value and, because of that, it is not well accepted for clinical use.[9] To overcome this problem, some authors tested the anticaries[6] and antierosive[12] potential of a TiF$_4$ varnish. However, other studies are needed to elucidate the effect of TiF$_4$ varnish in a situation that mimics the oral environment as in an *in situ* model.

The null hypothesis of this study was that the single application of TiF$_4$ varnishes does not reduce surface and subsurface demineralization after cariogenic challenge in comparison with NaF varnish.

Methodology

Study design

This double-blind, randomized, crossover, *in situ* study was approved by the Research Ethics Committee of the Federal University of Rio de Janeiro (process number: 48/2010) and was performed with undergraduate and graduate dental students who signed an informed written consent for their participation.

Twelve healthy adult volunteers (aged 19–31 years) met the inclusion criteria proposed by Zero[13] for *in situ* studies and did not violate the main exclusion criterion, i.e., use of any antibiotics or any other medication 60 days prior to the study and throughout the experimental stages.

The sample size was calculated based on a 0.8 power to detect a significant difference of 50% in mean mineral loss (ΔZ) in each treatment group (4% titanium tetrafluoride varnish - 2.45% F - experimental varnish; sodium fluoride varnish - Duraphat® - 2.26 % F - regarded as the "gold standard") compared to control (placebo varnish - no-F; vehicle control), using a one-sided test with 5% significance level (BioEstat 5.3, Instituto de Desenvolvimento Sustentável Mamirauá, Tefé, AM, Brazil) according to the results of a previous study with 4% TiF$_4$ solution.[7] For this statistical power, a minimum of nine volunteers would be required. Sample size was calculated considering a crossover *in situ* design. For an estimated loss of 25%, the sample should include 12 volunteers.

This study was performed in four 14-day stages, in which volunteers wore acrylic palatal appliances containing six sound bovine enamel blocks (three on each side) placed 1 mm below the acrylic level. The appliances containing the enamel blocks were sterilized with ethylene oxide before each stage. Volunteers were randomly allocated to each stage and received treatment accordingly (Figure 1).

Specimen preparation

Enamel blocks (4 × 4 × 2 mm^3) were obtained from bovine incisors with sound crowns using water-cooled diamond discs (Buehler, Lake Bluff, IL, USA). The enamel surface was then polished with 600- and 1200-grit silicon carbide paper (Extec, Enfield, CT, USA), followed by a 1-µm diamond abrasive slurry (Buehler, Lake Bluff, IL, USA). A total of 288 blocks were selected based on their baseline surface microhardness (SMH) (mean 341.59 ± 34.15). SMH was measured using a microhardness tester (HVS-1000; Time Group Inc., Beijing, China) with a Knoop diamond indenter under a 50 g load for 5 s, by making five indentations spaced 100 µm from one another at the center of the enamel surface.[7, 14]

Specimen treatment

At the beginning of each stage, volunteers placed the device in their mouths and kept it there for 5 min. The appliances were then removed from the volunteers' mouths, and the enamel specimens were randomly treated according to the selection made for each stage: 4% titanium tetrafluoride varnish (2.45% fluoride; experimental varnish), sodium fluoride (NaF) varnish (Duraphat®, 2.26% fluoride, regarded as the "gold standard"), placebo varnish (no-fluoride; vehicle control), and negative control (deionized water). The treatments were applied onto the enamel surface using a microbrush (Microbrush Corp., Grafton, WI, USA).

Figure 1. Schematic diagram of process steps: A - Cutting Teeth; B – Dentin/Enamel Flattening; C - Enamel Polish; D - Baseline Surface Microhardness; E – Appliance Preparation; F – Ethylene Oxide Sterilization; G (I) – Treatment of the Specimens, (II) – Treatment Removal, (III) – Carbohydrate challenge; H - Final Surface Microhardness; I – Cross-sectional Microhardness; J -Scanning Electron Microscopy.

The 4% TiF$_4$ varnish was formulated by dissolving TiF$_4$ and resin products in ethanol 96% (v/v, varnish vehicle) following a protocol from a previous study.[15] The placebo varnish was produced in the same manner, but without TiF$_4$. The NaF-Duraphat® varnish (Colgate-Palmolive Ind. e Com. Ltda., São Paulo, SP, Brazil) contained 5% NaF, 33.1% alcohol, natural resins (colophonium, mastix, shellac), wax, saccharin, and flavor. All varnishes were yellow and had a soft consistency.

In situ protocol

After treatment, volunteers wore the appliances for 24 h. Next, the varnish was removed with a scalpel and a cotton swab was soaked in acetone diluted in water (1:1).[16] Subsequently, a plastic mesh was used to cover the blocks in order to allow dental biofilm accumulation, and a 20% sucrose solution was applied to provide a carbohydrate challenge. Volunteers were instructed to remove the appliances from the oral cavity and to apply one drop of the sucrose solution on each enamel block 10 times/day[17] (Figure 1). The excess solution was removed using gauze, and the device was reinserted in the mouth 5 min later. Volunteers wore the appliances continuously, except during meals. The subjects received oral and written instructions on the schedule. They also received a diary for taking notes on the use of the device.

A one-week washout interval between the experimental stages was allowed to prevent any carryover effect. Throughout the experiment, including the washout periods, volunteers brushed their teeth and the appliance (except in the area of enamel slabs) with a fluoridated dentifrice (Colgate triple action; Colgate-Palmolive Ind. e Com. Ltda., São Paulo, SP, Brazil) containing 1,100 µg of fluoride/g (NaF) and silica as abrasive agent.[18] On the 14th day of each stage, the blocks were removed for evaluation of enamel loss. The response variables were as follows: enamel surface microhardness loss (%SML), enamel cross-sectional microhardness (CSMH), and energy dispersive X-ray spectrometry (EDS) analysis. The blocks were also analyzed qualitatively by scanning electron microscopy (SEM).

Microhardness analysis

After each stage, SMH of enamel blocks was measured again in all groups. A Knoop diamond indenter was used with a 50 g load for 5 s, and five indentations were made at a distance of 100 µm from the baseline indentations. The percentage of surface microhardness loss was calculated using the following equation: %SML= (sound SMH – SMH after *in situ* treatment)/ sound SMH * 100.[7]

Cross-sectional microhardness analysis

After the SMH test, the enamel blocks were tested for cross-sectional microhardness. All blocks were longitudinally sectioned in the middle of the fragment on a cutting machine (Buehler, Lake Bluff, IL, USA). One half was included in the resin stub and polished with silicon carbide discs (600- and 1,200-grit sandpapers, Buehler, Lake Bluff, IL, USA). The CSMH analyses were performed by a microhardness tester (HVS-1000, Time Group Inc., Beijing, China) using a Knoop diamond indenter and a static load of 25 g applied for 10 s. Two sequences of 14 indentations were made 100 mm apart at depths of 10 to 60 mm at 10-mm intervals, from 60 to 200 mm at 20-mm intervals, and from 200 to 300 mm, each at 100-mm intervals from the outer enamel surface. ΔZ (vol% x µm) was used to calculate the mineral content.[19]

Scanning electron microscopy and energy dispersive spectrometry

After sectioning the blocks in the middle, the other half of the five enamel blocks from each group was randomly selected and prepared for EDS and SEM analysis (JEOL-JSM, 6460LV, Tokyo, Japan).[16] The EDS analysis was performed with Kontron, a link and automatic image analyzer system, which provided the following double-way measurements: evaluation of the total area of the block and evaluation of three equidistant points (1-mm distance between points - center, left, and right) on each selected block. The results were represented by the mean of the measured values.

After EDS analysis, the specimens were covered with a 30-µm layer of gold in a vacuum apparatus. The specimens were examined (500x and 2,000x magnification) in low vacuum mode (45 Pa), operating at 20 kV, with analysis of backscattered electrons.

Statistical analysis

The normal distribution of data was checked for all the tested variables using the Shapiro-Wilk test. One-way ANOVA was used to compare the SMH values for all blocks before the experiment. Student's paired t-test was used to compare SMH before and after the experiment. Differences in %SML and CSMH (ΔZ) among treatments were tested using the repeated-measures ANOVA. The SPSS software (version 20.0) was used for the statistical analysis and the significance level was set at 5%.

Results

Microhardness loss

When comparing the SMH values for all enamel blocks before the experiment, no statistically significant difference was observed among groups, thus demonstrating data homogeneity (p=0.164; one-way ANOVA).

When SMH values before and after 14 days of the experiment were compared for each treatment (Table 1), a statistically significant decrease for all treatment groups was observed (p=0.000; Student's paired t-test). SMH results after the experiment showed differences between the treatments; and although all groups had a decrease in hardness, this outcome did not occur equally. The group treated with the TiF$_4$ varnish presented lower %SML (30.52 ± 10.03) than the negative control (62.58 ± 21.18) and the placebo (52.08 ± 23.35) treatments. Both differences were statistically significant according to the repeated-measures ANOVA, with p-values of 0.003 and 0.044, respectively. There was no statistical difference between NaF varnish and placebo or

negative control groups (p>0.05; repeated-measures ANOVA). In addition, no statistically differences were observed between TiF$_4$ and NaF varnishes (p = 0.085; repeated-measures ANOVA).

When CSMH (ΔZ) was analyzed after 14 days, there were statistically significant differences between the TiF$_4$ varnish and control groups (p = 0.0001; repeated-measures ANOVA) or between the TiF$_4$ varnish and placebo groups (p = 0.003; repeated-measures ANOVA). The NaF varnish group also differed from the control and placebo groups (p = 0.0001 and p = 0.001, respectively; repeated-measures ANOVA). No statistically significant difference was found between the placebo and negative control groups (p > 0.05; repeated-measures ANOVA).

Only TiF$_4$ varnish was able to inhibit surface demineralization (p < 0.05) (Table 1). However, TiF$_4$ varnish had similar results to those of NaF varnish regarding the decrease in subsurface demineralization (p > 0.05; repeated-measures ANOVA) (Table 1).

SEM/EDS analysis

The enamel specimens from all groups were analyzed descriptively based on SEM findings, which allowed observing the characteristics of the surface enamel.
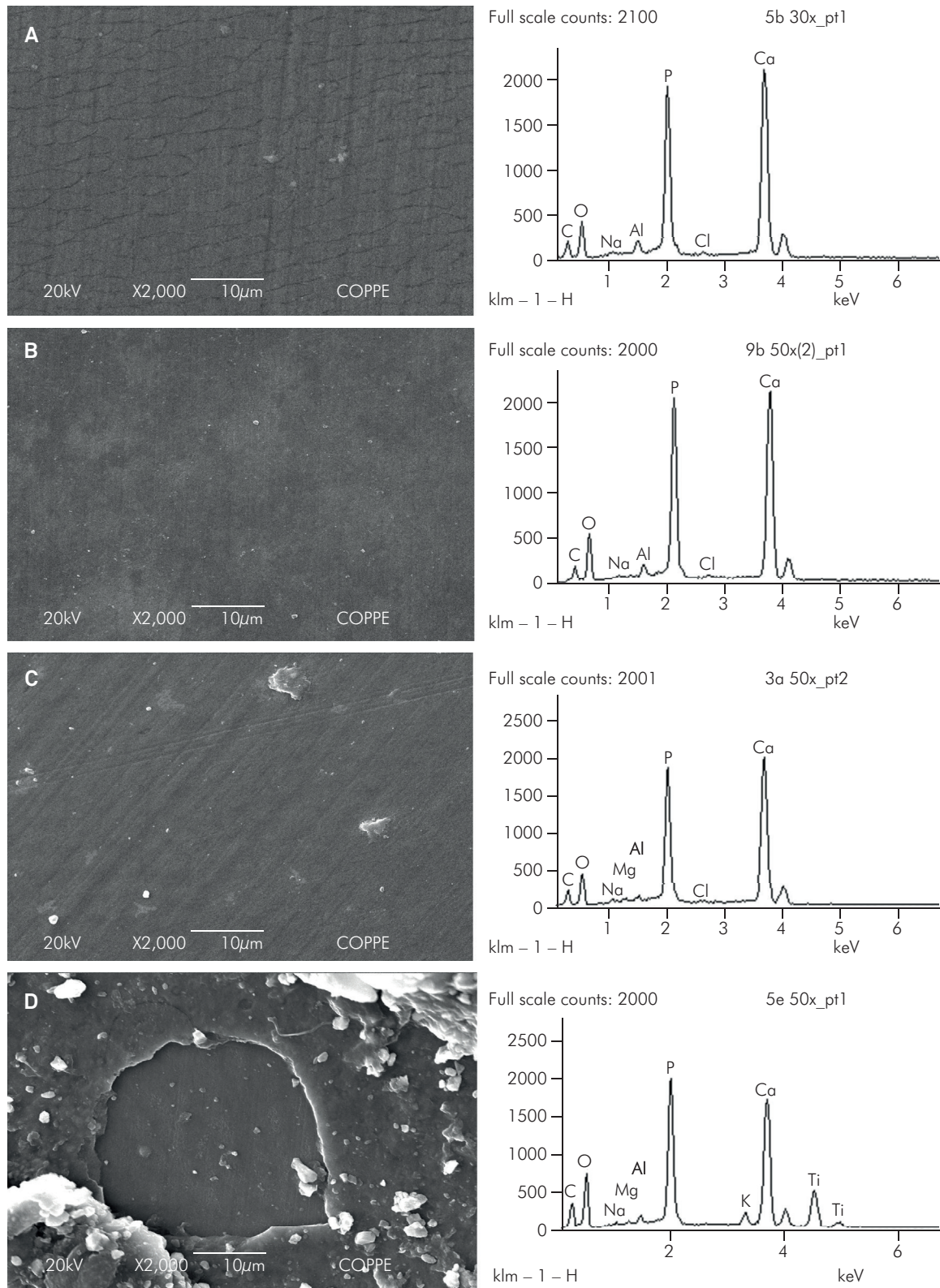
The images of the negative control and placebo groups (Figures 2A and 2B) showed more porous enamel surfaces characterized by surface mineral loss, differing from the images obtained for the NaF and TiF$_4$ groups (Figures 2C and 2D). However, the images obtained for the control group showed more mineral loss than in the placebo group (Figure 2A).

Only in the specimens treated with the TiF$_4$ varnish, there was a titanium dioxide film on most of the

Table 1. Surface microhardness analysis before and after the experiments, percentage of enamel surface microhardness loss and cross-sectional microhardness analysis according to the treatments – 14 days after the treatments (means ± standard deviation)

Treatments	SMH analysis			CSMH analysis
	SMH Before	SMH After	%SML	ΔZ
NaF varnish	345.63 ± 17.87[A,a]	180.95 ± 67.93[B,a]	47.13 ± 20.73[a,b]	720.34 ± 635.40[a]
TiF$_4$ varnish	336.14 ± 15.99[A,a]	233.44 ± 35.85[B,b]	30.52 ± 9.93[b]	519.73 ± 478.00[a]
Placebo varnish	334.31 ± 13.83[A,a]	124.82 ± 68.37[B,a,c]	52.08 ± 21.10[a]	1,603.00 ± 1225.40[b]
Negative control	344.72 ± 11.74[A,a]	165.24 ± 82.28[B,c]	62.58 ± 23.27[a]	1,638.39 ± 748.33[b]

Means followed by different letters are statistically different (p < 0.05). Uppercase letters show differences before and after the experiment in each group, and lowercase letters in the same column show differences between the treatments.

Figure 2. Surface SEM images of enamel samples after 14 days of treatment at 2000x (left) and EDS analysis (right). (a) Negative control, (b) Placebo varnish, (c) NaF varnish and (d) TiF$_4$ varnish.

surface (Figure 2D). When comparing the images of the specimens treated with NaF and TiF_4 (Figures 2C and 2D), enamel specimens treated with TiF_4 had less damage on their surfaces than those treated with NaF.

The chemical analysis (EDS) revealed the presence of titanium (Ti) in all samples after TiF_4 treatment (Figure 2D). Fluoride was not found after the experiment in samples treated with TiF_4 and NaF varnishes. Carbon, oxygen, sodium, phosphorus, and calcium basically kept the same percentages (Table 2).

Discussion

In cases of high caries risk, professional topical fluoride application has been an excellent alternative because of its high fluoride ion concentration.[20,21] Fluoride varnishes are considered to be an efficient delivery system, used in clinical dentistry to minimize the risks of fluoride toxicity. An advantage of varnishes is their ability to keep the active compound in contact with tooth enamel while allowing for safe use of high fluoride concentrations.[20] Clinical studies with fluoride varnishes have demonstrated a reduction in the incidence of dental caries.[20] In the present study, a severe cariogenic challenge was performed in order to promote fast enamel demineralization and simulate high caries risk.

Two different fluoride varnishes were used: one with sodium fluoride (a commercial NaF varnish - Duraphat®, 2.26% F) and another one with titanium tetrafluoride (TiF_4 varnish, 2.45% F). Furthermore, a placebo varnish with the same composition as the TiF_4 varnish but without fluoride was also used. Nobrega et al.[15] observed this TiF_4 varnish maintained fluorapatite (FA) and CaF_2 levels after one year of storage; however, there was an initial decrease in CaF_2 formation.

The varnishes were applied only once and were removed after 24 h to simulate the clinical situation in which a single professional application had been applied and the material had been removed during toothbrushing or mastication approximately 24 h thereafter. Nevertheless, in all varnish-treated groups, there were traces of varnish, even in the placebo group. This outcome is consistent with previous studies that found varnish remnants even within several days after the end of the experiment.[21]

In the literature, some studies showed TiF_4[7,8,10] was efficient against dental caries. The present *in situ* study demonstrated the TiF_4 varnish could inhibit enamel surface and subsurface demineralization under severe cariogenic challenge.

The TiF_4 varnish was effective in protecting the enamel from acid dissolution after 14 days, as it had a lower %SML after high cariogenic challenge. Notwithstanding, the results demonstrated the NaF varnish was unable to protect the dental enamel from surface mineral loss. Conversely, Magalhaes et al.[6] showed 4% TiF_4 varnish was less effective than NaF varnish in preventing surface demineralization.

However, our results confirmed that NaF varnish was able to decrease subsurface enamel demineralization to a similar degree as that of the TiF_4 varnish, in agreement with Magalhaes et al.[6] and Comar et al.[5]

It has been speculated that titanium ions might substitute calcium in the apatite lattice and form a titanium phosphate compound, leading to higher acid resistance of dental hard tissues.[22] In addition, TiF_4 has the ability to form an acid-resistant titanium dioxide surface film when in contact with the dental enamel surface.[4,9,10,11]

The protective effect of the amorphous acid-resistant film might be mainly related to its ability to act as

Table 2. EDS analysis of the surface of enamel blocks; chemical elements are shown according to the treatments (means ± standard deviation).

Treatments	C	O	F	Na	P	Ca	Ti
NaF varnish	30.5 ± 6.9	29.7 ± 2.6	0.0	0.5 ± 0.1	13.1 ± 1.6	25.5 ± 3.0	0.0
TiF_4 varnish	36.5 ± 9.6	29.1 ± 5.0	0.0	0.4 ± 0.1	10.7 ± 1.4	19.0 ± 3.5	4.8 ± 2.0
Placebo varnish	24.2 ± 4.3	35.5 ± 2.9	0.0	0.4 ± 0.1	13.6 ± 0.8	25.5 ± 0.9	0.0
Negative control	21.3 ± 3.0	36.7 ± 3.2	0.0	0.4 ± 0.1	14.1 ± 0.4	26.3 ± 1.1	0.0

a physical diffusion barrier. Moreover, the layer is rich in titanium and fluoride, and it might also act as a reservoir for fluoride ions.[4,10,11] SEM images and EDS analysis showed all samples treated with TiF$_4$ varnish were able to increase titanium concentration on the enamel surface. Although the incorporated titanium had been lost during the experimental period, Tveit et al.[11] suggest the TiO$_2$ layer slowed down and hindered demineralization, possibly increasing resistance of tooth enamel against acid challenges.

The formation of TiO$_2$ combines with the presence of CaF$_2$, likely reducing demineralization. Topical fluorides can react with enamel to form reaction products, which can be loosely (CaF$_2$) or firmly (fluorapatite) bound to enamel and could interfere with the demineralization and remineralization during the carious process.[3,11] Though fluoride was not found in the EDS analysis, TiF$_4$ hydrolysis can result in the formation of many fluoride complexes.[23] Nóbrega et al.[15] show the capacity of this TiF$_4$ varnish to form reaction products such as FA and CaF$_2$. Probably due to the mineral exchange process that occurs between the tooth and the biofilm,[18] loss of fluoride may have occurred on the biofilm, not allowing its measurement on the surface of the dental element after 14 days.

The low pH of the TiF$_4$ products could be a hindrance and their use by patients should be discouraged, owing to their side effects on soft tissues. Even though the TiF$_4$ varnish remains on the enamel surface for 24 h, longer application time of varnish products compared with TiF$_4$ solution could have offered possible differences in fluoride precipitation,[5] since a short application time might limit CaF$_2$ and TiO$_2$ formation. In addition, varnish products have a smaller contact with adjacent tissues due to their adhesion to the dental surface. However, caution should be taken when drawing conclusions from *in vivo* studies; more investigations are needed to ratify these findings.

Based on the results of this study, the null hypothesis was rejected, since the 4% TiF$_4$ varnish was able to reduce surface and subsurface enamel demineralization under cariogenic challenge.

Acknowledgments

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