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In situ effect of the combination of fluoridated toothpaste and fluoridated gel containing sodium trimetaphosphate on enamel demineralization

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ABSTRACT

Objective: This *in* situ study evaluated the effect of the association of low-F (4500 μg F/g) gel containing TMP and FT (1100 μg F/g) on enamel demineralization.

Methods: This crossover and double-blind study consisted of five phases of seven days each. Volunteers (n = 12) wore palatal appliances containing four enamel blocks. The cariogenic challenge was performed with 30% sucrose solution (six times/day). Treatments were: placebo toothpaste (PT, no fluoride/TMP); 1100 μg F/g toothpaste (FT); FT + 4500 μg F/g + 5%TMP gel (FT + TMP gel); FT + 9000 μg F/g gel (FT + 9000 gel) and FT + 12,300 μg F/g (FT + Acid gel). After topical application of treatments for one min, two blocks were removed for analysis of loosely bound fluoride (CaF_2), calcium (Ca), phosphorus (P) and firmly bound fluoride (FA) formed in enamel. After the seven-day experimental periods, the percentage of surface hardness loss (%SH), integrated subsurface hardness loss (ΔKHN), CaF_2, Ca, P and FA retained were determined. Moreover, the biofilms formed on the blocks were analyzed for F, Ca, P and insoluble extracellular polysaccharide (EPS) concentrations.

Results: FT + TMP gel promoted the lowest%SH and ΔKHN ($p < 0.001$). The highest concentration of CaF_2 formed was observed for the FT + Acid gel ($p < 0.001$), followed by FT + 9000 gel > FT + TMP gel > FT > PT. CaF_2 retained on the blocks was reduced across all groups ($p < 0.001$). Similar values were observed for the Ca/P/F and EPS in enamel and biofilm for all fluoride groups.

Conclusion: The association of FT + TMP gel significantly reduced enamel demineralization *in situ*. *Clinical Significance:* The association of treatments may be an alternative for patients with high caries risk.

1. Introduction

Dental caries is considered a multifactorial disease that requires the complex interaction of several factors to develop clinical manifestation [1]. Fluoride (F) has been the main agent used for dental caries prevention worldwide. The use of F toothpaste (FT) is considered the primary reason for the reduction in caries prevalence observed over the last decades [2,3].

Topical application of F (TAF) is often used in preventive programs as well as in patients at high risk of developing dental caries as an adjunct measure for the reduction of lesions [4]. When a product with high F concentration is applied on the tooth surface, there is deposition of calcium fluoride (CaF_2), which is covered by calcium and phosphate ions, and saliva proteins that delay the solubility of the compound [5]. Thus, it functions as a source of F, thereby interfering with the dynamics of the de-remineralization processes [6]. However, the cost of products with high F concentration can be high, especially fluoridated varnishes that are the first choice for infants with high caries risk [7–9]. This implies restricted access in terms of public health, especially in developing countries where fluoride gels are most commonly employed. Notwithstanding, the risk of ingestion and adverse events (mainly nausea and vomiting) can be present when fluoride gel is applied in children younger than six years, overcoming the potential benefits of its application [9–11].

One of the possible strategies that could be utilized to reduce the risk of acute toxicity is the reduction of F composition along with supplementation by calcium and/or phosphate salts. Among the phosphate salts with anticariogenic activity, sodium trimetaphosphate (TMP) appears to be the most effective [12]. The addition of TMP to experimental gels with lower F content (4500 μg F/g) was shown to

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reduce demineralization and promote remineralization of tooth enamel similarly to conventional gels (9000 μg F/g and 12,300 μg F/g − Acid gel), as demonstrated in *in vitro* [13] and *in situ* [14] studies.

In situations of high caries risk, the combination of two topical methods, TAF and fluoride toothpaste (FT), has been suggested [15]. A randomized controlled clinical trial demonstrated that TAF with Acid gel associated with supervised brushing with FT showed there to be the similar capacity of remineralizing initial carious lesions when compared with FT alone [16]. On the other hand, an *in situ* study [17] that evaluated the remineralization of enamel lesions after daily applications of high concentration gel (12,300 μg F/g, Acid gel) associated with FT (1450 μg F/g) indicated there was an increase in mineral volume for FT associated with acid gel compared to FT, concluding that there was an association of increased F incorporation into lesions for the FT + Acid gel group when compared to just FT. With this, there is no consensus on the additional effect of the association of F methods for dental caries control [18].

Thus, the aim of this *in situ* study was to assess the effect of the association of low-F (4500 μg F/g) gel containing TMP and FT (1100 μg F/g) on enamel demineralization. The null hypothesis was that the association of treatments with low-F/TMP gel and FT would promote a similar reduction in demineralization when compared to the association of 9000 μg F/g gel and FT treatments.

2. Material and methods

2.1. Experimental design

This study was approved by the Institutional Review Board of São Paulo State University (Unesp), School of Dentistry, Araçatuba, Brazil (Protocol: 50723315.1.0000.5420), and all participants read and signed an informed consent form prior to study onset. This crossover double-blind study was conducted in five phases of seven days each. The sample size of volunteers was based on a previous study [19], considering the primary outcome from surface and cross-sectional hardness analysis, in terms of the mean difference between groups (30 and 1300, respectively), standard deviation (20 and 900, respectively), an α-error of 5% and a β-error of 20%. Volunteers (n = 12) aged 20 to 30 years who were in good general and oral health [20] wore palatal appliances initially containing four bovine enamel blocks (Fig. 1A) selected by initial surface hardness (SHi). The cariogenic challenge was performed with 30% sucrose (six times/day). Treatments were: no fluoride/TMP toothpaste − Placebo (PT); 1100 μg F/g toothpaste (FT); FT and 4500 μg F/g gel containing 5%TMP (FT + TMP gel); FT and 9000 μg F/g gel (FT + 9000 gel) and FT and 12,300 μg F/g (FT + Acid gel). After topical application of treatments for one min, two blocks were removed for analysis of loosely bound (CaF_2) and firmly bound (FA) fluoride formed in enamel; and calcium (Ca) and phosphorus (P) on the same blocks. After the seven-day experimental period, the percentage of surface hardness loss (%SH), integrated subsurface hardness loss (ΔKHN), CaF_2 and FA retained as well as Ca and P in enamel were determined. Moreover, biofilms were analyzed for F, Ca, P and insoluble extracellular polysaccharide (EPS) concentrations. Data were assess using one-way and two-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test ($p < 0.001$).

2.2. Gel/toothpaste formulation and fluoride/pH assessment

An experimental gel (i.e., 100 g) with neutral pH was prepared in the laboratory with the following ingredients: 8.0 g of carboxymethyl cellulose (Synth, Diadema, São Paulo, Brazil), 0.1 g sodium saccharin (Vetec, Duque de Caxias, Rio de Janeiro, Brazil), 28.0 g glycerol (Sigma-Aldrich Co., St. Louis, MO, USA) and 0.5 g of peppermint oil (Synth, Diadema, São Paulo, Brazil) adjusted with deionized water to 100 g. F (NaF, Merck, Darmstadt, Germany) was added to the gel at concentrations of 4500 (1.0 g of NaF) or 9000 μg F/g (2.0 g of NaF). Subsequently, TMP (Sigma-Aldrich Co., St. Louis, MO, USA) was added at a 5% concentration (5.0 g) to the gel with F concentrations of 4500 μg F/g. A commercial acid gel was used as positive control (12,300 μg F/g, Acid gel, pH = 4.5, DFL Indústria e Comércio S.A., Rio de Janeiro, RJ, Brazil). In addition, toothpastes were produced with the following constituents: 0.5 g of titanium dioxide (Sigma-Aldrich Co., St. Louis, MO, USA), 1.7 g of carboxymethyl cellulose (Sigma-Aldrich Co., St. Louis, MO, USA), 0.08 g of methyl *p*-hydroxybenzoate sodium (Sigma-Aldrich Co., St. Louis, MO, USA), 0.1 g of saccharin (Vetec, Duque de Caxias, Rio de Janeiro, Brazil), 0.5 g of peppermint oil (Synth, Diadema, São Paulo, Brazil), 26.6 g of glycerol (Sigma-Aldrich Co., St. Louis, MO, USA), 10 g of abrasive silica (Tixosil 73®, Rhodia, São Paulo, Brazil) and 1.7 g of sodium lauryl sulfate (Sigma-Aldrich Co., St. Louis, MO, USA) adjusted with deionized water to 100 g. NaF (Merck, Darmstadt, Germany) was added to the F toothpaste to reach a concentration of 1100 μg F/g. A toothpaste without F (Placebo) was also prepared.

The F concentrations in the gels and toothpastes were determined with a F ion-specific electrode (9609 BN; Orion Research Inc., Beverly, MA, USA) attached to an ion analyzer (Orion 720 A+; Orion Research

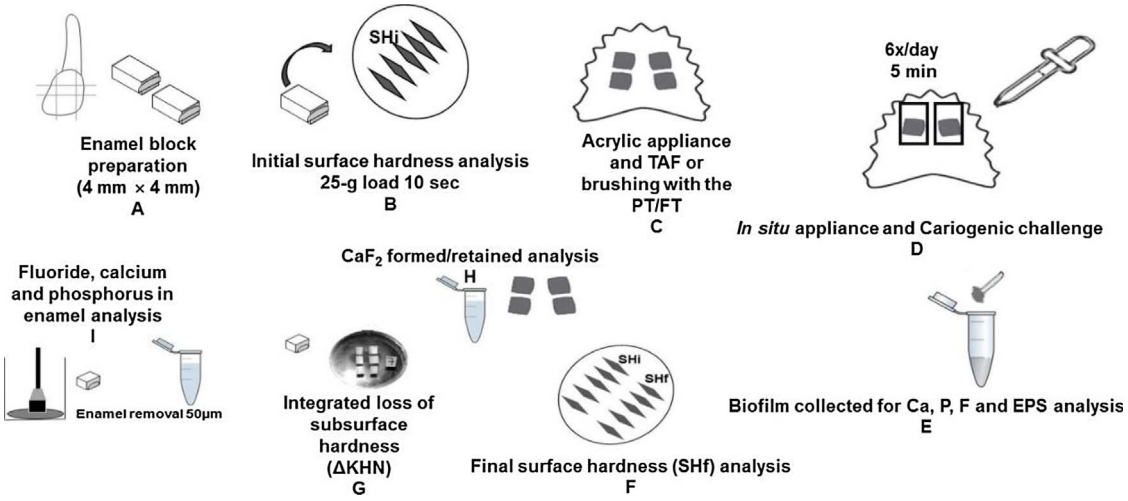

Fig. 1. A Enamel block preparation. B Initial surface hardness analysis. C Acrylic appliance and TAF or brushing with the PT/FT. D *In situ* appliance and Cariogenic challenge. E Biofilm collected for Ca, P, F and EPS analysis. F Final surface hardness analysis. G Integrated loss of subsurface hardness (ΔKHN). H CaF_2 formed/retained analysis. I Fluoride, calcium and phosphorus in enamel analysis.

Inc.) and calibrated with standards containing 0.125–2.000 μg F/g. The pH levels of the gels and toothpaste were determined with a pH electrode (2A09E, Analyser, São Paulo, Brazil) that was calibrated with standard pH levels of 7.0 and 4.0 [13,14].

2.3. Clinical phase of experimental groups

The palatal appliance was prepared in acrylic resin (Jet, Articles Classic Odontológico, São Paulo, Brazil), and initially, four enamel blocks were fixed with a different device used during each phase of the experiment. Next, prophylaxis was performed using a non-F paste and rubber cup. At the beginning of each experimental phase, an amount of gel sufficient to fill stock trays to approximately one-third of their capacity was placed. Teeth were air-dried, the trays were placed over them, and the subject was instructed to close their jaws with the trays in contact for one min [14] for treatments with gels. In toothpaste-only groups, the volunteers were instructed to brush their natural teeth for one minute with PF or FT. The same treatments (gels and/or toothpastes) were simultaneously performed on enamel blocks extraorally (Fig. 1C). To simulate the initial treatment with the toothpastes, the blocks were treated with slurries of toothpastes (toothpaste/deionized suspension, 1:3 w/w) over the course of one minute. Immediately after the treatments, two blocks from each device were removed for determination of CaF_2 and FA formed in enamel (Fig. 1H) as described subsequently. Ca and P in enamel were measured on the same blocks (Fig. 1I).

Next, to allow biofilm accumulation on the enamel blocks, a piece of plastic mesh was fixed to the acrylic appliance with the specific leaving of a one-mm space from the block surface [19–21]. To provide a cariogenic challenge able to promote the formation of subsurface lesions (Fig. 2), the volunteers were instructed to remove the device and drip 30% sucrose solution (Sucrose, Synth, Diadema, Brazil) (Fig. 1D) onto each enamel block six times/day at predetermined times (8:00 am, 11:00 am, 2:00 pm, 5:00 pm, 7:00 pm and 9:00 pm) [19,21]. Subjects were instructed to use the appliances during the entire day (including at night) except when drinking or eating. At these moments, the devices were to remain covered by gauze moistened in deionized water. The volunteers brushed their natural teeth three times/day (at 07:30, 12:30 and 21:30 h) with PT or FT during a habitual oral hygiene routine for two min as previously described for the treatments. Brushing was performed with the acrylic appliance in the oral cavity, allowing the natural saliva/toothpaste slurry to come into contact with the enamel blocks by gently swishing the slurry in the mouth [21]. During the seven-day pre-experimental period and washout periods, the volunteers brushed their teeth with toothpaste without F (Placebo).

2.4. Hardness analysis

Enamel surface hardness was measured before (SHi) (Fig. 1B) and after pH cycling (SHf) (Fig. 1F) using a Micromet 5114 hardness tester (Buehler, Lake Bluff, IL, USA) and Buehler OmniMet software (Buehler) [11] followed by calculation of the percentage of surface hardness loss (%SH = [(SHf-SHi)/SHi] *100). For the cross-sectional hardness measurements, the enamel blocks were longitudinally sectioned through their center and embedded in acrylic resin with the cut face exposed and gradually polished. A sequence of 14 indentations was created 100 μm apart at different distances (5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220 and 330 μm) from the outer enamel surface using a Micromet 5114 hardness tester (Buehler, Lake Bluff, USA) and Buehler OmniMet software (Buehler) with a Knoop diamond indenter under a 5 g load for 10 s [14]. Integrated hardness (KHN x μm) of the lesion into sound enamel was calculated according to the trapezoidal rule (GraphPad Prism, version 3.02) and subtracted from the integrated hardness for sound enamel to yield the integrated area of the subsurface regions in enamel, which was dubbed the integrated loss of subsurface hardness (ΔKHN; KHN x μm) (Fig. 1G) [14].

2.5. Analysis of loosely bound fluoride (CaF_2) formed and retained on enamel

The concentration of CaF_2 deposited on enamel was analyzed after treatments (CaF_2 formed) and after *in situ* challenge (CaF_2 retained). A digital caliper (Mitutoyo CD-15B, Mitutoyo Corporation, Japan) was employed to measure the surface area of enamel blocks (n = 240) [14]. The surface of each specimen, except for enamel, was coated with wax. They were then immersed in 0.5 mL of 1.0 mol/L KOH solution under constant agitation (Fig. 1H) [22]. After 24 h, the solution was neutralized and buffered with 0.5 mL of TISAB II (total ionic strength adjustment buffer) modified with HCl. F content was determined with an ion-specific electrode (9409BN, Thermo Scientific, Beverly, MA, USA) and a microelectrode reference (Analyser, São Paulo, Brazil) coupled to an ion analyzer (Orion 720 A^{plus}, Thermo Scientific, Beverly, MA, USA) calibrated by standards containing 4.00 to 64.00 μg F/mL (100 μg F/g, Orion 940907). Data obtained in mV were converted to μg F/cm^2 using Microsoft Excel.

2.6. Analysis of firmly bound fluoride (FA) formed and retained; Ca and P in enamel

After extraction of the CaF_2, enamel blocks were fixed to a modified microscope with a micrometer (Micrometer 733 MEXFLZ-50, Starret, Athol, MA, USA) to measure enamel wear. One layer of enamel (50.4 ± 0.4 μm) was removed from each block using self-adhesive

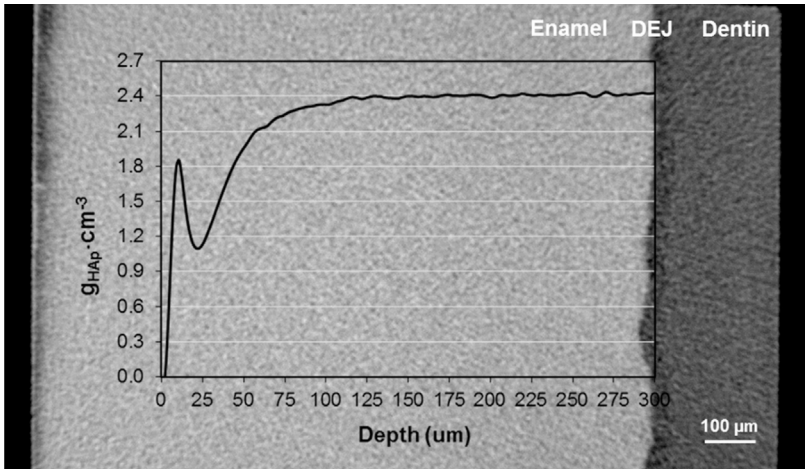

Fig. 2. Microtomography reconstructed slice from a typical specimen of the Placebo toothpaste (PT) operated at 80 kV, 125 μA, aluminum filter of 1 mm, at 1.5 μm of spatial resolution, rotation step at 0.600° and random moviment at 10 (Skyscan1272, Bruker, Kontich, Belgium). Cross-sectional profile of mineral concentration (g_{HAp} cm^{-3}) as function of depth (μm) shows a typical subsurface lesion in the enamel after 7-days of cariogenic challenge. DEJ = Dentin-enamel junction.

polishing discs (13 mm in diameter, 400-grit silicon carbide, Buehler) fixed to the bottom of polystyrene crystal tubes (J-10; Injeplast, Sao Paulo, SP, Brazil) [23,24]. The tubes containing enamel powder received an addition of 0.5 mL of 1.0 mol/L HCl, and these were kept under constant stirring for one hour. The analysis of FA formed/retained was performed in 0.25 mL of that solution after addition of the same volume of TISAB II, modified with NaOH, as previously described. Ca analysis in enamel after treatments and subsequent to *in situ* challenge, considered as formed and retained, respectively, was performed according to the Arsenazo III colorimetric method [25]. The absorbance readings were recorded at 650 nm using a plate reader (Microplate Spectrophotometer EONC, Biotek, Winooski, VT, USA). P was measured according to Fiske and Subbarow [26], and the absorbance readings were recorded at 660 nm. All results were expressed as $\mu g/mm^3$ (Fig. 1I).

2.7. Analysis of dental biofilm composition

The biofilm formed on enamel was collected and stored in microcentrifuge tubes. The biofilm samples were dried in vacuum over P_2O_5 (Vetec Quimica Fina Ltda., Duque de Caxias, Rio de Janeiro, Brazil) for 12 h at room temperature. After extraction for 3 h at room temperature with 0.5 mol/L hydrochloric acid (250 µL/mg, biofilm wet weight) under constant agitation, the same volume of NaOH (0.5 mol/L) was added [19,21,27]. The samples were then centrifuged (11,000 × *g*) for 1 min and the supernatant retained for determination of F, Ca and P. F was analyzed using an ion-specific electrode (Orion 9409 BN) and a potentiometer (Orion 720 A^{plus}) [19,21]. Ca concentration was evaluated by the Arsenazo III colorimetric test [26]. P concentration was measured via the molybdate colorimetric method [25]. EPS was extracted by adding 1.0 mol/L NaOH (10 µL/mg dry weight) to the biofilm [27,28]. The amount of EPS was determined according to the phenol-sulfuric acid method [29]. The results were expressed as mol/kg of F, Ca and P; and mg/g of EPS (dry weight) (Fig. 1E).

2.8. Statistical analysis

SigmaPlot 12.0 software was used for statistical analysis and the significance level was set at 5%. All variables exhibited a normal (Shapiro–Wilk) and homogeneous (Bartlet) distribution. Data from the dental biofilm analysis (F, Ca, P and EPS) and enamel analysis (%SH and ΔKHN) were subjected to one-way repeated measures analysis of variance (ANOVA). The results of CaF_2, FA, Ca and P formed/retained (enamel analysis) were submitted to two-way repeated measures ANOVA. The Student–Newman–Keuls post hoc test was performed for multiple comparisons.

3. Results

Mean (SD) of total (TF) and ionic fluoride (IF) concentrations (µg/g), respectively, were 10.5 (0.9) and 10.0 (1.2) in the PT, and 1189.0 (33.1) and 1102.4 (28.5) in the FT. IF concentrations (µg/g) in the TMP gel, 9000 gel and Acid gel were, respectively: 4,514.6 (29.7), 9,308.2 (22.5) and 12,497.1 (12.3). The mean pH value of the toothpastes was 7.3 (0.1) ranging from 7.2 to 7.4. The pH of the neutral gels was 7.2 (0.2), ranging from 7.0 to 7.4. All gels had neutral pH except for the Acid gel (4.4 (0.6)).

The use of FT + TMP gel resulted in a 58% and 67% decrease in%SH in comparison with FT + Acid gel and FT, respectively ($p < 0.001$) (Table 1). No significant difference was observed between the FT + 9000 gel and FT + Acid gel ($p = 0.678$) group. In addition, the capacity for reducing subsurface mineral loss (ΔKHN) was ~28% higher with FT + TMP gel ($p < 0.001$) when compared to FT; FT + 9000 gel and FT + Acid gel exhibited similar values ($p > 0.001$) (Table 1).

A higher concentration of CaF_2 formed after topical application of gels/toothpaste was observed in the FT + Acid gel when compared to other groups ($p < 0.001$) followed by FT + 9000 gel > FT + TMP gel > FT > PT ($p < 0.001$). After seven days of demineralization, CaF_2 on the blocks (retained) was significantly diminished across all groups ($p < 0.001$).

Similar values of F and Ca formed/retained in enamel were observed for all groups except for the PT group, which had the lowest concentration ($p < 0.001$). No significant difference was observed among the groups regarding enamel P formed/retained concentrations ($p > 0.001$).

As for the biofilm composition, all treatments demonstrated higher retention of Ca and F when compared with PT ($p < 0.001$) (Table 2), while P concentrations were similar among all treatments ($p = 0.052$). All treatments had the lowest values for alkali-soluble EPS concentration when compared with PT ($p < 0.001$). Similar concentrations were observed for the FT, FT + TMP gel, FT + 9000 gel and FT + Acid gel treatments ($p = 0.709$), which were significantly less than that of the PT group ($p < 0.001$).

4. Discussion

The use of products with high F content in association with F toothpastes is recommended for patients at high risk of developing dental caries [5]. Thus, it is important to perform *in vitro* and *in situ* studies to evaluate the therapeutic effects of novel gel formulations on enamel demineralization connected with daily use of fluoridated toothpaste prior to clinical studies. The present results indicate that the association of FT + TMP gel treatments significantly decreased enamel demineralization, representing superior protective effects when compared to FT + 9000 gel. Therefore, the null hypothesis was rejected.

The association of two modalities of topical F application (i.e., professionally- and self-applied) in the present study deserves two separate considerations. First, regarding conventional gels (i.e., not supplemented with TMP), the literature features conflicting evidence pertaining to the association of this modality with daily use of FT, which seems to be related to the different study protocols. While Lagerweij and Cate [17] showed a significant increase in mineral volume for FT (1450 µg F/g) associated with Acid gel (12,300 µg F/g) applied daily, in comparison with FT exclusively (possibly owing to the high frequency of gel application), Paes Leme et al. [30] demonstrated there was no reduction in%SH and ΔKHN when comparing FT (three times/day, 14 days) + Acid gel (applied only once) with the FT regimen. The present study (seven-day *in situ* protocol) showed additional benefits of the 9000 and Acid gels in combination with FT on the enamel surface only (%SH) with no apparent effect on the enamel subsurface (ΔKHN). A possible explanation for the lack of effect of the combination of FT and the gels compared with FT alone in the study of Paes Leme et al. [30] and in the present study might be based on the higher frequency of FT use (thus providing F at frequent intervals and sufficiently adequate to shift the balance from de- to remineralization), while the gels were applied only once. As well, the discrepancy between the results of the present study and that of Paes Leme et al. [30] in terms of surface hardness might be related to the different durations of the *in situ* phase. It is possible that the effects of CaF_2 deposition on enamel surface hardness were still visible at the end of the seven-day *in situ* phase in the present study, but the effects of such reservoirs for longer *in situ* periods (as in the aforementioned study) would be markedly diminished because of F and Ca release upon cariogenic challenges. Second, regarding the TMP-containing gel, it was noteworthy that surface hardness loss (%SH) and integrated subsurface hardness loss (ΔKHN) were significantly lower for this gel when compared to conventional gels (neutral or acidic), potentially indicating important clinical implications as discussed subsequently. The affinity of TMP for enamel and its capacity to reduce acid diffusion are the main reasons explaining the superior results obtained for the TMP-containing gel despite the two- to three-fold lower F content when compared with 9000 and Acid gels,

Table 1
Mean (SD) valu (%SH), integrated loss of subsurface hardness (ΔKHN), loosely bound fluoride (CaF_2), firmly bound fluoride (FA), calcium (Ca) and phosphorus (P) in the enamel analyzed according to the toothpastes/gels treatments.

Analysis		Treatments				
		PT	FT	FT + TMP gel	FT + 9000 gel	FT + Acid gel
%SH (KHN)		-58.3^{a} (6.3)	-27.4^{b} (5.0)	-9.2^{d} (6.7)	-20.7^{c} (8.0)	-21.7^{c} (7.3)
ΔKHN (KHN × μm)		6,413.8[a] (1,265.7)	2,753.9[b] (568.7)	1,992.1[c] (618.6)	2,768.6[b] (604.9)	2,648.5[b] (646.1)
CaF_2 (μg/cm^2)	Formed	$0.56^{a,A}$ (0.06)	$1.27^{b,A}$ (0.15)	$3.96^{c,A}$ (1.92)	$14.13^{d,A}$ (5.19)	$29.17^{e,A}$ (5.86)
	Retained	$0.34^{a,B}$ (0.10)	$0.81^{b,B}$ (0.32)	$1.77^{c,B}$ (0.85)	$2.59^{d,B}$ (1.44)	$6.33^{e,B}$ (3.94)
FA (μg/mm^3)	Formed	$0.19^{a,A}$ (0.05)	$0.36^{b,A}$ (0.11)	$0.35^{b,A}$ (0.07)	$0.33^{b,A}$ (0.06)	$0.33^{b,A}$ (0.05)
	Retained	$0.22^{a,A}$ (0.07)	$0.36^{b,A}$ (0.07)	$0.45^{b,A}$ (0.31)	$0.47^{b,B}$ (0.19)	$0.45^{b,B}$ (0.18)
Ca (μg/mm^3)	Formed	$379.2^{a,A}$ (78.2)	457.9^{aA} (105.1)	$441.3^{a,A}$ (86.1)	$479.5^{a,A}$ (104.5)	$433.9^{a,A}$ (157.5)
	Retained	$198.7^{a,B}$ (36.7)	$274.0^{a,b,B}$ (53.8)	$326.6^{b,B}$ (98.6)	$278.5^{a,b,B}$ (87.1)	$286.8^{a,b,B}$ (102.1)
P (μg/mm^3)	Formed	$215.9^{a,A}$ (52.4)	$223.1^{a,A}$ (73.3)	$228.5^{a,A}$ (44.7)	$223.9^{a,A}$ (62.4)	$218.9^{a,A}$ (64.3)
	Retained	$147.6^{a,B}$ (33.1)	$174.1^{a,b,B}$ (47.3)	$218.9^{b,A}$ (48.2)	$158.8^{a,B}$ (54.1)	$186.8^{a,b,A}$ (55.8)

Lowercase letters indicate differences between groups in each analysis and capital letters indicate the differences between formed and retained (Student-Newman-Keuls's test; $p < 0.001$).

Table 2
Mean (SD) values of fluoride (F), calcium (Ca), phosphorus (P) and insoluble extracellular polysaccharide (EPS) in the biofilm according to the toothpastes/gels treatments.

Analysis	Treatments				
	PT	FT	FT + TMP gel	FT + 9000 gel	FT + Acid gel
F (mol/kg)	2.49E-04[a] (3.54E-05)	2.93E-03[b] (3.85E-03)	1.17E-03[b] (1.26E-03)	1.22E-03[b] (1.10E-03)	1.39E-03[b] (1.74E-03)[b]
Ca (mol/kg)	8.47E-02[a] (1.57E-02)	1.33E-01[b] (4.89E-02)	1.39E-01[b] (3.23E-02)	1.29E-01[b] (3.94E-02)	1.39E-01[b] (3.23E-02)
P (mol/kg)	3.90E-02[a] (1.75E-02)	7.12E-02[a] (4.09E-02)	6.00E-02[a] (3.00E-02)	5.49E-02[a] (3.27E-02)	6.00E-02[a] (3.36E-02)
EPS (mg/g)	2.23[a] (0.79)	1.62[b] (0.57)	1.33[b] (0.63)	1.29[b] (0.75)	1.25[b] (0.81)

Distinct superscript letters indicate statistical significance among the toothpastes/gels for each analysis (Student Newman-Keuls's test; $p < 0.001$). Values between parentheses indicate the standard deviation of the mean.

respectively.

Based on previous studies [13,14] and in line with the present findings, it is known that the association of TMP and F (at suitable proportions) reduces mineral loss, and its effect can be understood by the adsorption of TMP on enamel, even in the presence of biofilm. When TMP is adsorbed on enamel, this interaction may alter the selective permeability and diffusion of ions into the enamel interior [13,14,31]. In this sense, TMP is believed to retain positively charged ions of CaF^+ and Ca^{2+}, replacing the Na^+ of the cyclic structure and promoting reduction in acid diffusion [30]. In the case of a pH drop, it has been suggested that the release of those ions from TMP increase the ionic activity of neutral species ($CaHPO_4^0$ and HF^0) [Souza et al., 2017]. Besides their higher diffusion coefficient into enamel, the neutral species would not be detained by the enamel surface [32], but instead could penetrate into enamel, hence diminishing enamel demineralization [14,21,31] and enhancing remineralization [13,31]. The treatment with FT + TMP gel promoted reduction in the subsurface lesion (ΔKHN) by ~ 28% when compared to the FT + 9000 gel and by 25% in relation to the FT + Acid gel (Table 1). The more pronounced effect of FT + TMP gel on ΔKHN suggests that, under clinical conditions, a subsurface lesion would take longer to develop compared to conventional gels (9000 gel and Acid gel). This is extremely significant from a clinical perspective considering that a cavity could take longer to arise, especially when associated with other preventive measures.

The effect of conventional fluoridated products (i.e., not supplemented with TMP) applied at high concentrations on the reduction of enamel demineralization is related to the deposition of CaF_2 on enamel, which is known to be concentration- and pH-dependent [6]. CaF_2 concentrations measured immediately after gel application or brushing with toothpaste (CaF_2 formed) was directly related to the F concentration present in the treatments, the highest value being observed for the FT + Acid gel (Table 1) followed by the FT + 9000 gel. Furthermore, the remaining concentration of CaF_2 after the *in situ* phase (CaF_2 retained) for FT + 9000 or FT + Acid gel was significantly higher than the FT values (Table 1), demonstrating that the effect of the high F concentration gels is prolonged, even under severe cariogenic challenges. An *in situ* study showed that the presence of F can reduce enamel demineralization if sucrose consumption is not greater than six times/day, but such a protective effect is diminished for more frequent challenges [33]. As for the TMP-containing gel, TMP was not demonstrated to enhance the precipitation of CaF_2 on enamel, what is in agreement with previous observations [13,14], confirming that its effects can be predominantly attributed to the reduction of acid diffusion into enamel [14,31]. In addition, TMP is believed to retain F and Ca from the treatment (i.e., gels or FT) and from the oral environment (i.e., saliva) to produce a direct impact on the incorporation of F and Ca into enamel (Table 1). The hypothesis relies on the possibility of TMP binding to ions Ca^{2+} and CaF^+ as described earlier.

With regard to the effects of treatments on the biofilms formed during cariogenic challenges, the significant effect of FT (Table 2) in comparison with PT can be attributed to the daily use of F toothpaste, similar to what has been discussed for enamel hardness. Furthermore, the significant effect of FT + gels (Table 2) compared with PT may be attributed to the release of CaF_2 formed on enamel (Table 1) by both the pre-treatment with the gels [34,35] and the frequent exposure to FT. It is notable, however, that F concentrations in dental biofilm resulting from the combination of FT and gels was not significantly higher than FT alone (Table 2), which is in line with the ΔKHN data. Despite the reasons for this pattern not being apparent, especially when taking into account the complex interactions between different treatment modalities as well as hardness and biofilm data, the results of biofilm F concentrations seem to confirm that the main effect of conventional F therapies is more related to frequent exposure to low levels of F than infrequent exposure to high F concentrations [36].

The use of FT + F gels or FT alone promoted significant changes in biofilm composition. It is interesting that an increase in F concentrations of biofilm is often correlated with Ca concentrations [37–39], just as observed for all treatment groups when compared to PT. In the

groups treated with FT, the biofilm served as a reservoir of F and Ca ions, which could contribute to minimizing the mineral loss from enamel during large-scale cariogenic challenges, being greater for the FT + TMP gel group. It is likely that F concentrations in the biofilm were higher for groups treated with F gels in the first days of the experimental phases (not determined) as CaF_2 formed on enamel was greater than treatments with FT alone (Table 1). This may have produced an effect on%SH, which was observed even after seven days of challenge. As F and Ca were intensely consumed owing to the exceptional cariogenic challenge [36], it was not possible to verify differences in the secondary results (F, Ca and P) between FT + gels and FT only (Tables 1 and 2) after seven days. Nevertheless, only the TMP gel group presented higher Ca and P in enamel compared with the PT group (Table 1). This seems to indicate a higher capacity of the TMP gel to reduce mineral loss, even with lower F content in the formulation, just as previously mentioned.

Regarding alkali-soluble EPS concentrations, our results showed there to be a marked reduction with F treatments. The correlation between the increase of F/Ca and the reduction of EPS is a point that must be considered. TMP could act on the biofilm indirectly – high F and Ca concentrations in biofilm alter bacterial metabolism and biological activity as well as EPS formation [40]. These data are based on a single *in situ* demineralization protocol, so that other studies with biofilm accumulation at different cariogenic challenges (frequency x exposure to sucrose) would be instructive to simulate patients with varied levels of caries activity. The understanding of the inorganic composition of saliva and biofilm fluid resulting from the combination of FT and gels, as well as the use of *in situ* remineralization protocol, and *in vivo* studies would be of great value to better understand the real benefits of the true additional benefits of FT + conventional gels as well as FT + TMP-containing gels.

We concluded that the association of FT + TMP gel was more efficient in reducing enamel demineralization *in situ* when compared to conventional treatments (i.e., daily use of F toothpaste and/or association with conventional F gels). This association could be an alternative for individuals of all ages, but especially for children under six years of age at high caries risk because this combination was proven to be more effective in reducing enamel demineralization than conventional therapies while minimizing the possibility of side effects.

Declaration of interest

The author Alberto Carlos Botazzo Delbem has a patent for a product used in the study, by the National Institute of Industrial Property − INPI/SP, on April 11, 2017 under number C1 0801811-1.

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