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ORIGINAL ARTICLE

Fluoride and calcium concentrations in the biofilm fluid after use of fluoridated dentifrices supplemented with polyphosphate salts

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Abstract
Objectives The present study evaluated fluoride (F) and calcium (Ca) concentrations in the biofilm fluid formed in situ under cariogenic challenge after using F dentifrices supplemented or not with sodium trimetaphosphate (TMP) or calcium glycerophosphate (CaGP).
Methods Volunteers (n = 12) were randomly divided into 5 groups according to the toothpastes used: placebo (without F, CaGP or TMP), 1100 ppm F (1100F) and low-fluoride dentifrice (LFD, 550 ppm F) with no supplementation (550F) or supplemented with 1 % TMP (550F-TMP) or 0.25 % CaGP (550F-CaGP). In each phase, volunteers wore palatal appliances containing 4 bovine enamel blocks. Cariogenic challenge was performed with 30 % sucrose solution, 6 times/day. On the morning of the eigth day, biofilm samples were collected 12 h and 1 h after brushing and cariogenic challenge. F and Ca analyses in the biofilm fluid were performed with the inverted electrode after buffering with TISAB III and using the Arsenazo III method, respectively. Data were submitted to two-way ANOVA (repeated measures) and Student-Newman-Keuls test ($p < 0.05$).
Results A dose-response relationship was verified between F concentrations in the dentifrices and in the biofilm fluid. Significant differences were observed among placebo, 550F, and 1100F only 1 h after brushing, without statistical differences among 550F, 550F-TMP, and 550F-CaGP. No defined trend was observed among the groups regarding Ca concentrations, with the highest values seen for placebo and 550F-CaGP.
Conclusion The anticaries effect of LFDs supplemented with CaGP or TMP cannot be related to an increased availability of F and Ca in the biofilm fluid.
Clinical significance The better performance of LFDs containing CaGP or TMP shown in previous studies should be attributed to their ability to interact with tooth enamel and with the biofilm, rather to their effect on the biofilm fluid.

Keywords Fluoride · Dentifrice · Biofilm fluid · Sodium trimetaphosphate · Calcium glycerophosphate

Introduction

The uncertainties surrounding the efficacy of low-fluoride dentifrices (LFD, 500–550 ppm F) against dental caries when compared to conventional dentifrices (CD, 1000–1100 ppm F) has prompted to several studies attempting to increase the anticaries effects of such formulations [1, 2]. Among the strategies available, the supplementation of LFDs with calcium and/or phosphate salts has been studied in recent years [3]. In vitro and in situ studies showed that LFDs supplemented with calcium glycerophosphate (CaGP) [4, 5] or sodium trimetaphosphate (TMP) [6] have a similar anticaries effect when compared to a CD. Such effects were clinically confirmed in a recent randomized trial, in which the progression of caries lesions was shown to be significantly lower in children using the TMP-containing LFD in comparison to the conventional formulation (1100 ppm F), while the progression in the group using a CaGP-containing LFD was similar to the 1100 ppm F toothpaste [7].

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The addition of CaGP and TMP to other topically applied fluoridated products has also been shown to promote a synergistic protective effect against dental caries and erosive wear, using in vitro [8–10] and in situ models [4, 5, 11]. Little is known, however, about the mechanisms by which these salts interfere with the de- and remineralization processes of dental enamel. The use of the LFDs supplemented with CaGP or TMP was shown to promote an increase on enamel surface hardness, besides a marked effect on the depth of enamel subsurface lesions [4–6, 12]. Furthermore, F and calcium (Ca) concentrations in biofilm formed in situ in the presence of these toothpastes were significantly higher than their counterparts without calcium or phosphate salts, reaching levels similar to those attained by the use of the CD [4, 13].

Although this increase in biofilm F and Ca levels seems to explain the synergistic effect of TMP and CaGP when added to a fluoride dentifrice, the availability of these ions in the fluid phase of the biofilm remains unknown. Previous studies demonstrated that F and Ca ions may be retained in biofilm through the formation of mineral deposits [14–16], which may be released from biofilm fluid during a cariogenic challenge. The literature also reports that even when biofilm is not completely removed, F retained in this reservoir can be released into the fluid of biofilm, reducing demineralization of enamel covered by biofilm [17]. Based on the above, the assessment of the effects of LFDs containing TMP or CaGP on the mineral composition of the biofilm fluid could bring useful information for a better understanding of the mechanisms by which these salts interfere with the dynamics of dental caries.

Thus, the present study aimed to evaluate the concentration of F and Ca in the biofilm fluid formed in situ associated with the use of LFDs supplemented with TMP or CaGP, under cariogenic challenge. The study's hypothesis was that the supplementation of LFDs with TMP or CaGP would significantly increase F levels in the biofilm fluid when compared to their counterpart without TMP or CaGP.

Material and methods

This study was approved by the Human Research Ethics Committee of Araçatuba Dental School (CAAE 20146313.7.0000.5420) and all participants signed an informed consent form.

The study was carried out through an in situ, double-blind, crossover, and randomized design comprising five experimental phases of 7 days each. A 7-day washout period was done prior to each experimental phase. During this period, the volunteers were instructed to use placebo toothpaste to eliminate possible residual effects from the previous treatments and not use mouthrinse or other topical fluoride agents. Twelve volunteers, regardless of gender, aged 20–34 years-old and resident in Araçatuba participated in the study. The inclusion criteria comprised good general and oral health volunteers, without systemic drugs use that might interfere with biofilm formation or salivary flow. On the other hand, individuals with active caries lesions, who received fluoride applications 2 weeks before the experiment, water activities practitioners, environment polluted by low pH components workers (industry), smokers, and volunteers diagnosed with systemic diseases (xerostomia, diabetes, autoimmune diseases, malnutrition, gastroesophageal problems) were excluded. The volunteers wore acrylic palatal appliances (24 h/day) containing four bovine enamel blocks (4 × 4 × 2 mm) placed 1 mm below the acrylic level and covered by a plastic mesh to allow dental biofilm accumulation. During each phase, the volunteers dripped a 30 % sucrose solution on the enamel blocks (6 times/day), and brushed their teeth three times a day, for 7 days, using one of the following toothpastes: (1) placebo (fluoride-free), (2) 550 ppm F, (3) 1100 ppm F, (4) 550 ppm F with 0.25 % CaGP, (5) 550 ppm F with 1 % TMP, hereafter abbreviated as placebo, 550F, 1100F, 550F-CaGP, and 550F-TMP, respectively. Biofilm samples were collected in the morning of the eigth day after overnight fasting (2 enamel blocks), and 60 min after tooth brushing and exposure to sucrose. A sample of 11 volunteers was calculated based on a previous in situ study conducted with a similar protocol, assessing whole biofilm fluoride concentrations after the use of placebo and 550 ppm F toothpastes (mean difference = 0.16 μM F/Kg, standard deviation =0.1) [4], considering α - error of 5 % and β-error of 20 % (SigmaPlot, version 12.0). Assuming a dropout rate of 20 %, sample size was then determined as 14 volunteers.

The experimental dentifrices were produced in the laboratory of Pediatric Dentistry from Araçatuba Dental School, using the same basic formulation (except for F, CaGP, and TMP concentrations) with the following components: titanium dioxide, carboxymethyl cellulose, methyl p-hydroxybenzoate, sodium saccharine, oil peppermint, glycerin, silica abrasive, sodium lauryl sulfate, and water. Formulations without F (placebo) and containing F (NaF—Merck®, Germany) in the concentrations of 550 and 1100 mg F/g were obtained. Also, CaGP, or TMP (Sigma®-Aldrich, USA) were added to the 550 ppm F dentifrice at concentrations of 0.25 and 1 %, respectively. The CaGP concentration was determined based on studies of Amaral et al. (2013) [4] and Freire et al. (2016) [7], which used in situ and in vivo protocols, respectively. TMP concentration was determined based on the study of Freire et al. (2016) [7]. Fluoride concentrations in the toothpastes were determined using an ion-specific electrode (9409 BN) connected with an ion analyzer (Orion 720 Aplus), previously calibrated with 5 standards (0.125, 0.25, 0, 5, 1.0, and 2.2 mg F/mL) [18].

Two hundred eighty enamel blocks measuring 4 × 4 × 2 mm were obtained from bovine incisors previously

stored in 2 % formaldehyde solution (pH 7.0) for 1 month. Enamel blocks were serially polished and selected according to their surface hardness (SH, 369.0 ± 3.1 KHN). Each acrylic palatal appliance had 4 enamel blocks, which were randomly assigned into the 5 experimental groups ($p = 0.97$). A 4.0-mm-deep space was created in the appliances, leaving 1.0-mm space for dental biofilm accumulation on the enamel blocks. A plastic mesh was fixed in acrylic resin to avoid mechanical disturbance and to induce dental biofilm formation [4].

The cariogenic challenge was produced by the use of a 30 % sucrose solution (Synth, Brazil), which was replaced every 48 h. The volunteers were instructed to remove the appliance from the oral cavity before dropping two drops of sucrose solution on each block (sufficient amount to fill out the space of 1.0 mm), 6 times a day, at predetermined times (8:00 am, 11:00 am, 02:00 pm, 05:00 pm, 07:00 pm, 09:00 pm). After dripping, the appliances were left to rest for 5 min before being returned to the oral cavity, in order to allow the diffusion of the sucrose in the biofilm. Treatment with dentifrices was performed 3 times a day, for 7 days. The volunteers used the transverse technique to standardize the amount of dentifrice and brushed their natural teeth with the device in the oral cavity, therefore producing natural dentifrice/saliva slurry; this was later swished in the oral cavity during 30 s, spat out, and the mouth was gently rinsed with tap water. The volunteers were instructed to use the appliances 24 h a day and remove only during meals. They were instructed not to use systemic drugs that might interfere with biofilm formation or salivary flow and topical fluoride products throughout the entire experiment.

Biofilm samples were collected in the morning of the eighth day of each experimental phase, at 2 moments (biofilm from 2 enamel blocks each time), with volunteers fasting overnight. The first sample was collected about 12 h after the last treatment with the toothpastes done on the previous night. Following, the volunteers brushed their teeth with the provided dentifrice and the cariogenic challenge was performed 5 min later. The second sample was then collected 60 min after tooth brushing. The biofilm was collected with a plastic spatula and weighed in preweighed microcentrifuge cap tubes filled with mineral oil. Tubes were then centrifuged (21,023*g*, 5 min, 4 °C) in order to separate the biomass from the fluid phase. After centrifugation, a small fraction of the fluid was collected with a micropipette, also filled with mineral oil.

After biofilm fluid separation from its solid phase, it was transferred to the surface of an inverted ion-specific electrode, immersed in mineral oil. Through this micro-analysis technique, multiple samples were placed simultaneously on the electrode [19]. The samples were placed on drops of TISAB III (Orion) previously placed on the electrode membrane, in a ratio of 10:1 (sample: TISAB) and were read by the positioning of the reference microelectrode within each sample in order to close the circuit. This electrode was calibrated with standard solutions of known fluoride concentrations (ranging from 1 to 100 μM F).

Calcium analyses of biofilm fluid samples were performed by spectrophotometry. A quartz nanopipette of approximately 1 μL was used, allowing standardized volumes of calcium standards (ranging from 40 to 200 mM Ca) and samples. Arsenazo III was used as colorimetric reagent [20], and sample readings were performed on a microplate reader (Biotek Eon). Fifty microliters of deionized water and 1 μL of the samples/standard were added in each microplate well. Then, 50 μL of the colorimetric reagent was added in each plate well, and the plates were shaken during 60 s in the microplate reader, allowing the reaction between sample and Arsenazo III prior to obtaining the resulting absorbance. The absorbance reader was performed in 610 nm.

Statistical analysis was performed on the software SigmaPlot version 12.0, at a significance level of 5 %. Data analysis considered the types of experimental toothpastes and the time of sample collection. Fluoride (Log_{10} transformed) and calcium (raw) data passed normality (Shapiro-Wilk) and homogeneity tests (Bartlett) and were submitted to two-way, repeated-measures ANOVA, followed by the Student-Newman-Keuls test.

Results

Table 1 shows mean F concentrations in the experimental dentifrices, which presented a maximum variation within 10 % according to the allowed for the products.

A dose-response relationship was observed between fluoride concentrations in the dentifrices and the resulting levels in the biofilm fluid 60 min after the treatment with the toothpastes (Table 2). Significant differences were observed among the dentifrices ($F = 16.7$, $p < 0.001$) and time after brushing ($F = 38.0$, $p < 0.001$), with a significant interaction between

Table 1 Mean (SD) concentrations (μg/g) in the experimental fluoride toothpastes

Dentifrices	Concentration (μg/g)	
	Total fluoride	Ionic fluoride
Placebo	11.6 (1.5)	11.1 (1.2)
550F	521.3 (27.7)	550.0 (12.6)
550F-CaGP	523.1 (21,8)	549.5 (5.3)
550F-TMP	553.1 (4.4)	558.6 (11.3)
1100F	1100 (48.6)	1119.5 (19.6)

Table 2 Mean (SD) fluoride concentrations (μM) in the biofilm fluid formed in situ 12 h after brushing and 1 h after brushing and cariogenic challenge (30 % sucrose solution), according to the dentifrices used in each experimental phase

Dentifrices	Time after brushing	
	12 h	1 h
Placebo	5.8 (3.1)	5.1[a] (3.3)
550F	14.5 (12.9)	31.2[b] (26.4)
550F-CaGP	8.3 (6.5)	23.5[b] (11.5)
550F-TMP	11.8 (7.5)	24.2[b] (22.7)
1100	13.5 (10.0)	45.4[c] (22.8)

Lowercase superscript letters indicate significant differences among the dentifrices 1 h after brushing. No significant differences were observed among the groups 12 h after brushing. Two-way, repeated measures ANOVA (data log transformed), and Student-Newman-Keuls test ($p < 0.05$), $n = 12$

the two variables (F = 4.2, $p = 0.003$). For samples obtained 1 h after brushing (mean ± SD), significant differences were observed among placebo (5.1 ± 3.3), 550 (31.2 ± 26.4), and 1100F (45.4 ± 22.8) toothpastes, without significant differences among 550F (31.2 ± 26.4), 550F-CaGP (23.5 ± 11.5), and 550F-TMP (24.2 ± 22.7). For samples collected 12 h after brushing, no significant differences were observed among the dentifrices.

As for Ca concentrations in the biofilm fluid, significant differences were observed only among the dentifrices (F = 3.59, $p < 0.013$) with no significant differences for time after brushing (F = 2.37, $p < 0.151$) and no interaction between the two variables (F = 1.90, $p = 0123$), as shown in Table 3. No defined trend was observed among the groups, with the highest values seen for placebo and 550F-CaGP. Significant differences were observed among placebo and 1100F and placebo and 550F-TMP, with no differences among 550F, 1100F, 550 TMPF, and 550F-CaGP.

Table 3 Mean (SD) calcium concentrations (mM) in the biofilm fluid formed in situ 12 h after brushing and 1 h after brushing and cariogenic challenge (30 % sucrose solution), according to the dentifrices used in each experimental phase

Dentifrices	Time after brushing	
	12 h	1 h
Placebo[a]	123.1 (63.0)	148.7 (58.0)
550F[a,b]	112.6 (68.5)	93.4 (34.0)
550F-CaGP[a,b]	119.9 (42.3)	135.0 (34.1)
550F-TMP[b]	76.2 (31.5)	106.4 (27.4)
1100[b]	86.6 (32.3)	100.5 (35.4)

Lowercase superscript letters indicate significant differences among the dentifrices. Two-way, repeated measures ANOVA, and Student-Newman-Keuls test ($p < 0.05$), $n = 12$

Discussion

The addition of calcium and/or phosphate salts to fluoridated toothpastes has been proposed to reduce F concentration in the products without compromising their anticaries effect when compared to a conventional dentifrice, in order to minimize F intake from this source and consequently reducing the risk of dental fluorosis. There is considerable in vitro and in situ evidence on the effects of dental products supplemented with TMP [6, 8, 21] and CaGP [4, 5, 12] on the dynamics of dental caries using different methods, but information about the mechanism of action of these compounds is still lacking. The present study showed that the addition of TMP or CaGP to low-fluoride toothpastes did not increase the availability of F and Ca in the fluid phase of biofilms formed in situ under cariogenic challenge, leading to the rejection of the study's hypothesis.

Literature has shown that the supplementation of low-fluoride toothpastes with TMP or CaGP has a marked effect on enamel hardness, leading to a higher degree of surface hardness and decreased loss of integrated subsurface hardness. The use of a 500-μg F/g toothpaste associated with TMP at concentrations higher than 0.5 % increased surface hardness and decreased ΔKHN when compared to the negative control, with the greatest effect observed for the low-F toothpaste containing 3 % TMP (190 % higher) [6]. As for CaGP, the addition of 0.25 % of this salt to a low-fluoride dentifrice decreased mineral loss by 132 %, reaching greater protective effect than that seen for the positive control in vitro [12]. The same toothpaste resulted in a 44 % decrease in enamel surface hardness change in situ in comparison with its counterpart without CaGP, having a similar effect than a 1100 ppm F toothpaste [4].

The information above is in line with a mechanism recently proposed, according to which TMP seems to act as a partial barrier to acids, by binding to enamel and forming a "network" able to retain fluoride compounds that are released during subsequent cariogenic challenges [10]. The authors also demonstrated that while the effects of TMP alone are negligible, it has a synergistic effect with fluoride, which gives support to the above-mentioned hypothesis. The same trend was later confirmed by another investigation showing that gels containing 1 % NaF and 5 % TMP were able to inhibit enamel demineralization similarly to a 2 % NaF gel, while TMP alone did not reduce demineralization [8]. Regarding CaGP, the possible anticaries mechanism has been suggested to be related to interactions with enamel during the de/remineralizing process in a similar pattern as TMP [4, 5, 12]. It has been also suggested that the availability of Ca and F in the enamel from the fluoridated dentifrices associated with CaGP was the main factor to improve the ability of remineralization [5].

Despite the growing body of evidence on the synergistic effects of fluoride and TMP or CaGP when added to

toothpastes on enamel hardness composition, little is known about the effects of these salts on the dental biofilm. The use of low-fluoride formulations containing these salts was shown to significantly reduce the formation of extracellular polysaccharides, as well as to significantly raise F and Ca concentrations in biofilms formed in situ, in comparison to the 550 ppm F without TMP [13] or CaGP [4]. These positive results led to the assumption that the increased levels of F and Ca in the biofilm would be reflected in a higher availability of these ions in the fluid phase of the biofilm, but such effect was not confirmed in the present study. Although significant differences were seen in F levels in the biofilm fluid 1 h after brushing with placebo, 550F, and 1100F dentifrices (what validates the method used), no significant differences were observed among toothpastes containing 550F, regardless the addition or not of CaGP or TMP. These findings are in line with previous data on the effects of CaGP when added to a conventional dentifrice (1500 μg F/g), showing no significant differences between toothpastes with or without CaGP at 0.13 % regarding F, Ca, and inorganic P concentrations in the biofilm fluid [22]. Although the literature reports an increase in the total biofilm mineral ions after the addition of Ca, Pi, and F supplements [23], this increase was not observed for the biofilm fluid, which might suggest the existence of equilibrium or a homeostatic mechanism that maintains ion concentration in the biofilm fluid, regardless of its concentration in the whole biofilm, as previously hypothesized [20].

For samples collected 12 h after brushing, no significant differences were noted among the dentifrices, indicating that all fluoride retained in the biofilm after brushing was slowly released over time, returning to baseline levels afterwards. This result is consistent with previous data showing higher F concentrations in whole biofilm samples 1 h after brushing with F dentifrices, which returned to baseline (placebo) values 12 h after the use of a conventional toothpaste [24]. Also, fluoride concentrations in saliva and dental biofilm collected 8 h after the last use of fluoride products (dentifrices and fluoride solution) did not differ among treatments (placebo, 1100 ppm F, and 1100 ppm F + fluoride solution), suggesting that F products for home-use have no long-term effect on fluoride concentrations in saliva and in dental biofilm, mainly in residents of an area with a fluoridated water supply [25, 26].

Although it is not possible to make a direct comparison between whole biofilm F concentrations after the use of LFDs supplemented with CaGP [4] or TMP [13] with the present results in the biofilm fluid, the discrepancies between the present results and the abovementioned studies are evident. The only methodological difference in the study protocol was the mode of application of the dentifrices. While a dentifrice:water slurry (1:3) was dripped ex vivo directly on enamel blocks during 1 min [4], in the present study, volunteers were instructed to brush their teeth with the device in the oral cavity in order to produce a natural dentifrice:saliva slurry, which was later swished in the oral cavity during 30 s. Given that penetration of fluoride into biofilms is highly dependent on biofilm's thickness [27] and that fluoride is mainly restricted to the biofilm/saliva interface after brushing with conventional or low-fluoride toothpastes in thick biofilms [28] (as in the present study), it is likely that the mode of application (dentifrice foam versus dentifrice slurry) and exposure time (30 versus 60 s) might have played a major role in the results obtained in the present study. This aspect is of outermost relevance and raises questions on the in situ models currently used to assess the effects of fluoride toothpastes on the dynamics of dental caries.

Another considerable aspect was the timing of sample collection. Biofilm samples were collected 60 min after the treatment with the toothpastes and 55 min after the cariogenic challenge. It is known that the decline in biofilm fluid pH to levels lower than 5 following exposure to sucrose lasts about 20 min [29, 30], during which F concentrations in the biofilm fluid increases due to the dissolution of CaF_2 and the release of F bound to sites on bacteria [16, 20]. After 40 min, biofilm fluid pH then rises to 6 or more due to the clearance of sucrose by salivary flow and buffering of hydrogen ions, and this increase is accompanied by a decrease in biofilm fluid F concentration due to the formation and precipitation of the relatively insoluble F salts and binding on bacteria. Therefore, performing a cariogenic challenge immediately after the use of the toothpastes (60 min collection) is likely to have affected the results, so that any possible influence of the test toothpastes on biofilm fluid F levels could not be verified. In this sense, it is noteworthy that the high intensity of the cariogenic challenge used in the present study (30 % sucrose solution) might have produced an intense depleting effect on fluoride levels in the dental biofilm. As for Ca concentrations in the biofilm fluid, no significant differences were observed for time after brushing. Surprisingly, the highest values of Ca concentration were obtained for placebo and 550F-CaGP, lowest were found for 550F-TMP and 1100. The reasons for this discrepancy are not known. However, considering that F is retained on the biofilm mediated by Ca bindings on bacteria walls [15], it is possible that brushing the teeth with a conventional dentifrice (1100 ppm) allowed F binding to Ca present in the biomass (mainly bacterial surfaces), further allowing ionic Ca present in biofilm fluid to bind to the biomass as well, which would, in turn, reduce Ca concentrations in the biofilm fluid. On the other hand, as placebo has no fluoride, the abovementioned mechanism would not occur, so that ionic Ca would remain free in the fluid phase of the biofilm. Moreover, the high values found for the 550F-CaGP dentifrice suggest that CaGP can be considered as a source of free calcium [31], what may help to explain the anticaries effect of this toothpaste in caries progression in children [7].

This investigation provided additional information for better understanding of the mechanisms of F and Ca

uptake by biofilm fluid. In this sense, the lack of synergistic effect between F and TMP or CaGP in the biofilm fluid seen in the present study along with previous in vitro and in situ data indicates that the anticaries effects of LFDs supplemented with these salts may be more related to the interaction of these salts with tooth enamel than with an increased availability of F and Ca ions in the biofilm fluid. Further studies should be carried out to complement this in situ investigation, in order to clearly verify the clinical benefits provided by the supplementation of LFDs with phosphate salts, as well as to provide stronger evidence on the mechanisms of action of toothpastes containing TMP or CaGP.

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Compliance with ethical standards All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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Informed consent Informed consent was obtained from all individual participants included in the study.

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