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Research Article

Effect of a calcium glycerophosphate fluoride dentifrice formulation on enamel demineralization *in situ*

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Abstract: Purpose: To evaluate *in situ* the effect and mechanisms involved in the anticariogenic effect of a calcium glycerophosphate fluoride dentifrice. **Methods:** In a double-blind, crossover design, a non-F dentifrice (negative control), a F dentifrice and a F dentifrice containing 0.13% CaGP were compared regarding the inhibition of enamel demineralization. Both F dentifrices contained 1500 μg F/g (w/w) as sodium monofluorophosphate (MFP). Bovine enamel blocks were mounted in contact with a *S. mutans* test plaque, in palatal appliances worn by 10 volunteers. 30 minutes after treatment with the dentifrices, a sucrose rinse was performed and enamel demineralization was assessed after an additional 45 minutes. **Results:** No significant difference was observed among groups in the calcium and inorganic phosphate concentrations in the fluid phase of the test plaque 30 minutes after the dentifrice use ($P > 0.05$), but F concentration was significantly higher for both F dentifrices ($P < 0.05$). Also, the dentifrices did not differ regarding the pH before or 5 minutes after the sugar challenge ($P > 0.05$). A higher mineral loss was observed for the non-F dentifrice group ($P < 0.05$), but no significant difference was observed between the F dentifrices containing CaGP or not ($P > 0.05$). Using this *in situ* model, the findings suggested that CaGP at the concentration tested did not enhance the inhibition of enamel demineralization promoted by F dentifrice. (*Am J Dent* 2009;22:278-282).

Clinical significance: Calcium glycerophosphate used as an additive in F dentifrice does not seem to enhance its effect on inhibition of enamel demineralization.

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Introduction

Fluoride (F) dentifrice is considered mainly responsible for the dental caries decline observed in the last decades either in developed or developing countries.[1,2] However, F alone is not able to avoid enamel demineralization and strategies to enhance the anticariogenic potential of F dentifrices, such as the inclusion of anticaries additives, should be investigated.

Calcium glycerophosphate (CaGP) is an organic calcium phosphate salt that demonstrated significant anticariogenic properties when added to the diet of monkeys,[3] rats,[4,5] and hamsters.[6] In addition, one clinical trial[7] has suggested that CaGP enhances the anticaries effect of F dentifrice.

Among the proposed cariostatic mechanisms are the increase in calcium (Ca) and inorganic phosphate (P_i) concentration in dental plaque,[8] as well as a buffering effect in dental biofilm due to P_i release.[3,9] However, the availability of these ions in the plaque fluid as a result of CaGP use was never tested. Also, at present most of the dentifrices are fluoridated and it would be more realistic to evaluate if CaGP could enhance the known anticaries effect of F.

Lynch & ten Cate[10] evaluated the effect of CaGP in an *in vitro* biofilm model and suggested that its anticaries potential may be greater if it is used before a cariogenic challenge. From this assumption, we proposed to evaluate the ability of a CaGP-F dentifrice to exert a greater inhibition of enamel demineralization than a similar, F-only dentifrice, when used prior to one cariogenic challenge. This was tested using a controlled, short-term, *in situ* model,[11,12] which was successfully used to evaluate the anticaries effect of F dentifrices containing sodium monofluorophosphate (MFP).[13,14] The availability of Ca and P_i to the fluid phase of the plaque substrate and the inhibition of pH drop after a sugar challenge were also investigated.

Materials and Methods

Experimental design - The study involved an *in situ*, crossover, double-blind design. Ethical approval was obtained from the Research and Ethics Committee of Piracicaba Dental School, and volunteers signed a written, informed consent. Dentifrice formulations tested were a negative control without F or CaGP; an active control, F formulation, containing 1,500 μg F/g as sodium monofluorophosphate (MFP), silica as abrasive; and a test dentifrice, with the same composition of the F dentifrice, but containing 0.13% CaGP. Only 2% of total F in the F dentifrices was already as F ion. In order to evaluate the inhibition of enamel demineralization by the tested dentifrices, 10 healthy volunteers, five males and five females, wore a palatal appliance containing four bovine enamel blocks, with known baseline surface microhardness (SMH). The blocks were covered with a layer of bacteria (test plaque) obtained from a culture of *Streptococcus mutans* and fixed on the palatal appliance using acrylic holders[12,13] (Fig. 1A,B). Immediately before the intraoral test, appliances were immersed in a slurry (1:3, w/w in distilled water) of the assigned dentifrice for 1 minute; during this time volunteers brushed their teeth with the same dentifrice (Fig. 1C). Volunteers kept the appliances inside the mouth for 30 minutes, followed by a gentle rinse during 1 minute with a 20% sucrose solution. Acidogenicity of the test plaque was evaluated before and 5 minutes after rinsing (Fig. 1D). Forty-five minutes after the sugar challenge, enamel blocks and test plaque were collected. F (as MFP and as ionic F), Ca and P_i

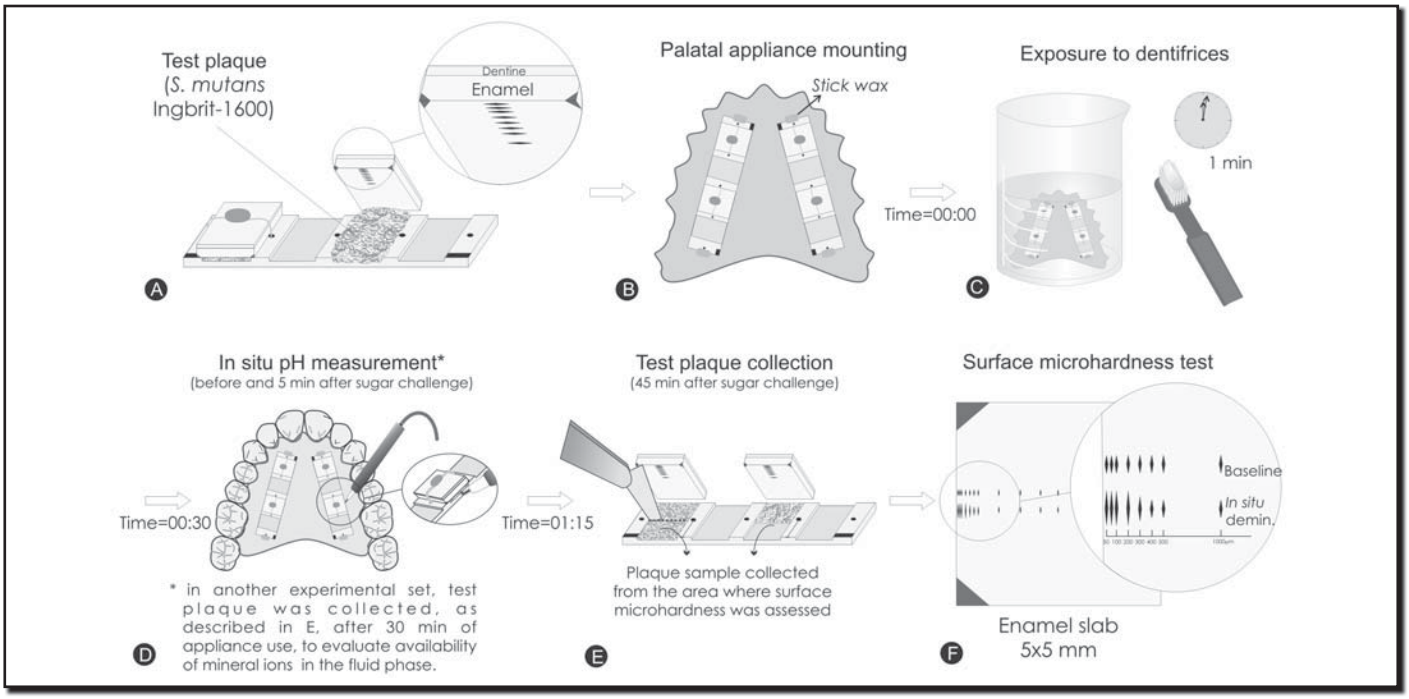

Fig. 1. Illustration of the experiment. **A.** Enamel blocks of known baseline surface microhardness (SMH) were fixed in acrylic holders, in contact with a *S. mutans* IB1600 test plaque. **B.** Holders were fixed in a palatal appliance, with the side of the enamel block where the SMH was determined towards the center of the palate. **C.** After mounting, the appliance was kept for 1 minute in a slurry of the dentifrice being tested, and volunteers brushed their own teeth with the dentifrice at the same time (time 00:00). **D.** After 30 minutes inside the mouth (time 00:30), the baseline pH of the test plaque was determined. A 1-minute rinse with sucrose solution was conducted and after additional 4 minutes, the pH drop was determined. 45 five minutes after the rinse (time 01:15), the experiment was completed. In another experimental set, test plaque was collected after 30 minutes of appliance use, to evaluate mineral ions in the fluid phase. **E.** Test plaque was collected using a plastic spatula, from the area underneath the SMH indentations. **F.** SMH was again determined, for estimation of mineral loss.

concentrations were determined in the fluid phase of the test plaque (Fig. 1E). Inhibition of enamel demineralization by the dentifrices was assessed by change of SMH (Fig. 1F). In a separate experiment, the availability of the mineral ions in the fluid phase of the test plaque after 30 minutes of intraoral appliance use was evaluated. Seven days of lead-in and wash-out periods were allowed before all experimental phases, when volunteers used the dentifrice assigned for the next phase.

Preparation of enamel blocks and baseline SMH determination - Enamel blocks of 5 x 5 x 2 mm were prepared from bovine incisors, which had been stored in 2% formaldehyde solution, pH 7.0, for at least 1 month.[13] The enamel surface of each block was polished plane-parallel as described by Zero *et al.*[15] SMH was determined from one side of the block, at distances of 50, 75, 100, 200, 300, 400, 500, 1,000, 1,500, 2,000 and 2,500 μm from its edge. These measurement sites correspond to test plaque thicknesses of 50 μm up to 2,500 μm.[16] Indentations were made with a Knoop indenter, with a 50g-load for 5 seconds in a Future-Tech FM[a] microhardness tester coupled to FM-ARS[a] software.

Palatal appliance mounting - Test plaque was prepared from *S. mutans* Ingbritt-1600, as described by Cury *et al.*[13] Palatal appliances capable of carrying two plastic holders were constructed for each volunteer. Two enamel blocks were mounted in each holder, with enamel surface in contact with the test plaque (Fig. 1A). The plastic holders were fixed to the palatal appliance (Fig. 1B), with the marked side of the enamel block where the baseline measurements were made facing the center of the palatal appliance. Further details can be found in Cury *et al.*[13]

Intraoral test - Immediately after mounting, each appliance was kept for 1 minute in a slurry (1:3, w/w) of the assigned dentifrice, followed by gentle soaking in deionized water for rinsing. During this time, volunteers brushed their own teeth with the same dentifrice and subsequently rinsed with 15 mL of deionized water (Fig. 1C). Then, the appliance was inserted in the mouth. After 30 minutes, each volunteer was asked to perform a gentle rinse with 15 mL of a 20% sucrose solution during 1 minute, without removing the appliance. The pH of the test plaque was measured *in situ*, before and 5 minutes after this rinse (Fig. 1D). Forty-five minutes after the rinse, the appliance was removed, test plaque was collected (Fig. 1E) for analysis of F, Ca and P_i in the fluid phase, and the enamel was analyzed for SMH (Fig. 1F). In another set of experiments, the availability of mineral ions in the fluid phase of the test plaque was measured after 30 minutes of *in situ* appliance use. During the intraoral tests, subjects were instructed to refrain from talking, drinking or eating.

Test plaque acidogenicity assessment - The acidogenicity of the test plaque was assessed immediately before the sucrose rinse, and after 5 minutes. Test plaque pH was determined using a contact micro-electrode (WPI, NMPH3[b]), and a reference electrode (Orion, 9002[c]), connected to a pH meter (Orion, 720-A[c]). The reference electrode tip was immersed in 3 M KCl in a Petri dish, where the volunteers placed one finger to close the circuit. The micro-electrode was inserted up to 2.5 mm into the test plaque, measuring the pH from test plaque with a thickness from 0 to 2.5 mm.[12] One block in each side of the appliance was used for pH determination, and the mean value of these two measurements was used for statistical analysis.

Collection of plaque and analysis of mineral ions in the fluid phase - Mineral ions were evaluated in the fluid phase of the

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Table. Fluoride (as F ion and MFP), calcium (Ca) and inorganic phosphate (P_i) concentration in the fluid phase of the test plaque according to dentifrice formulations used and time of plaque collection (Mean ± SD, n=10).

Time of plaque collection	Treatment groups	F (μM)		Ca (mM)	P_i (mM)
		F ion	MFP		
30 minutes after dentifrice use, at the moment of the cariogenic challenge	Negative control	2.5 ± 0.6 [a]	0.8 ± 0.6 [a]	2.1 ± 0.8 [a]	6.5 ± 2.6 [a]
	MFP	113.0 ± 52.3 [b]	268.9 ± 167.0 [b]	1.8 ± 0.4 [a]	5.9 ± 1.2 [a]
	MFP + CaGP	130.2 ± 53.2 [b]	364.8 ± 247.1 [b]	1.8 ± 0.5 [a]	6.2 ± 2.0 [a]
75 minutes after dentifrice use (45 min after cariogenic challenge)	Negative control	2.3 ± 0.4 [a]	0.7 ± 0.4 [a]	18.3 ± 4.8 [a]	14.0 ± 2.1 [a]
	MFP	29.3 ± 26.3 [b]	93.0 ± 113.6 [b]	13.4 ± 3.5 [b]	11.8 ± 1.9 [b]
	MFP + CaGP	24.6 ± 17.4 [b]	70.8 ± 79.2 [b]	13.4 ± 4.9 [b]	11.4 ± 2.0 [b]

At each time of plaque collection, means followed by different letters designate treatments that are statistically different (P< 0.05).

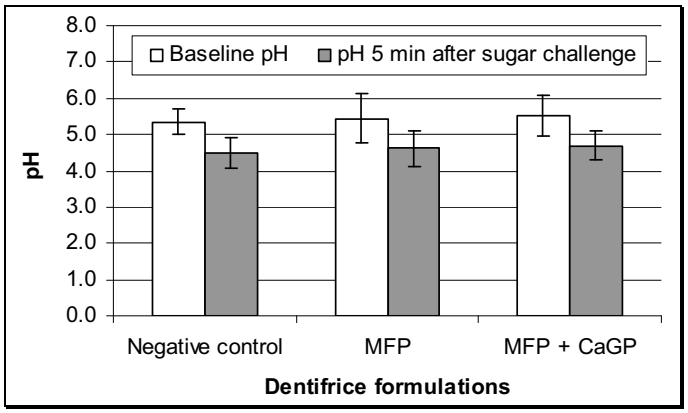

Fig. 2. Mean (n=10) of test plaque acidogenicity assessment (pH), according to the dentifrice formulations. Bars indicate standard deviation. The pH drop was statistically significant (P< 0.05), irrespective of the treatments, but difference among them was not observed (P> 0.05), for baseline pH nor for pH 5 minutes after sugar challenge.

test plaque collected after 30 minutes *in situ* (at the moment of the cariogenic challenge) and at the end of the experiment. Enamel blocks were removed from the holder and test plaque samples were collected using a plastic spatula, and immediately placed inside an oil-filled centrifuge tube.[17] Site of collection was chosen to correspond to test plaque that was in contact with the area of enamel blocks where surface microhardness was evaluated (Fig. 1E). The tube was centrifuged for 10 minutes (21,000 g) at 4°C to separate the fluid from the plaque solids. The fluid was recovered with oil-filled capillary micropipettes, and immediately analyzed for F concentration as MFP and ionic F. The remaining fluid was deposited under mineral oil on the bottom of a plastic Petri dish until the analyses of Ca and P_i.

Ionic F in the fluid phase of the plaque was analyzed using an inverted F electrode.[17] Samples were applied on the surface of the oil-covered F electrode and diluted with TISAB III (1:10) under microscope. A micro-reference electrode was used to close the circuit, and the signal was read using a high-impedance electrometer (WPI, FD223[b]), and graphically observed using the program Plot 1.[d] Subsequently to the direct determination of F ion in samples, acid phosphatase (Sigma, P-1435[e]) was added to each sample drop in order to hydrolyze MFP.[18] After 5-10 minutes, total F in the sample was determined and the concentration of F as MFP was calculated by subtraction from the previous F ion values.

For Ca and P_i analyses in the fluid, colorimetric reagents Arsenazo III and malachite green were used, respectively.[19] Quartz nanoliter volume pipettes[20] were used to deposit standardized volumes of the samples or standards into the reagents. The absorbance of the mixtures, after mixing, was then read using a micro-cuvette (Hellma, 105.202[f]), in a Beckman DU-70[g] spectrophotometer.

Surface microhardness analysis - Enamel blocks removed from the holders were washed with deionized water and SMH was measured again, at 100 μm from the initial indentations, at the same distances from the block edge. From this block edge, sucrose solution and saliva had access to the enamel surface covered by test plaque, simulating a dental plaque thickness of up to 2.5 mm.[16] The percentage of surface microhardness change (%SMC) was calculated [%SMC = (SMH after demineralization – baseline SMH) x 100/baseline SMH]. The results found in the four blocks at each distance for each volunteer were averaged and submitted to statistical analysis.

Statistical analysis - A randomized block design was used for the statistical analyses, considering the volunteers as statistical block. All data were analyzed using ANOVA, except for %SMC, which was analyzed using split-plot ANOVA, considering the dentifrice formulations as plots and test plaque thickness as subplot. Significant differences were compared using Tukey's test. In order to fit the assumption of equality of variances and normal distribution of errors, data of baseline plaque pH, ionic F and MFP, Ca and P_i in the fluid phase of the test plaque at 30 minutes and ionic F and MFP at the end of the test were transformed to the $\log^{10}$; and data of pH after the challenge were analyzed after inverse transformation. To check for a significant decrease in pH after the sugar challenge, the two pH measurements were compared within each dentifrice formulation using a paired *t*-test. For all statistical analyses, SAS System 6.11[h] software was used and the significance limit was set at 5%.

Results

Although a significant decrease in pH was observed in the test plaque treated with the three dentifrice formulations 5 minutes after the sugar challenge, as compared to baseline pH (P< 0.05), no significant difference among the three formulations was observed at baseline nor 5 minutes after the sugar challenge (P> 0.05) (Fig. 2).

The availability of mineral ions in the fluid phase of the test plaque 30 minutes after dentifrice use showed no significant difference for Ca and P_i concentrations among the three tested dentifrices (P> 0.05) (Table). On the other hand, significantly higher F concentrations, either ionic or as MFP, were observed in the fluid phase of the test plaque treated with both F denti-

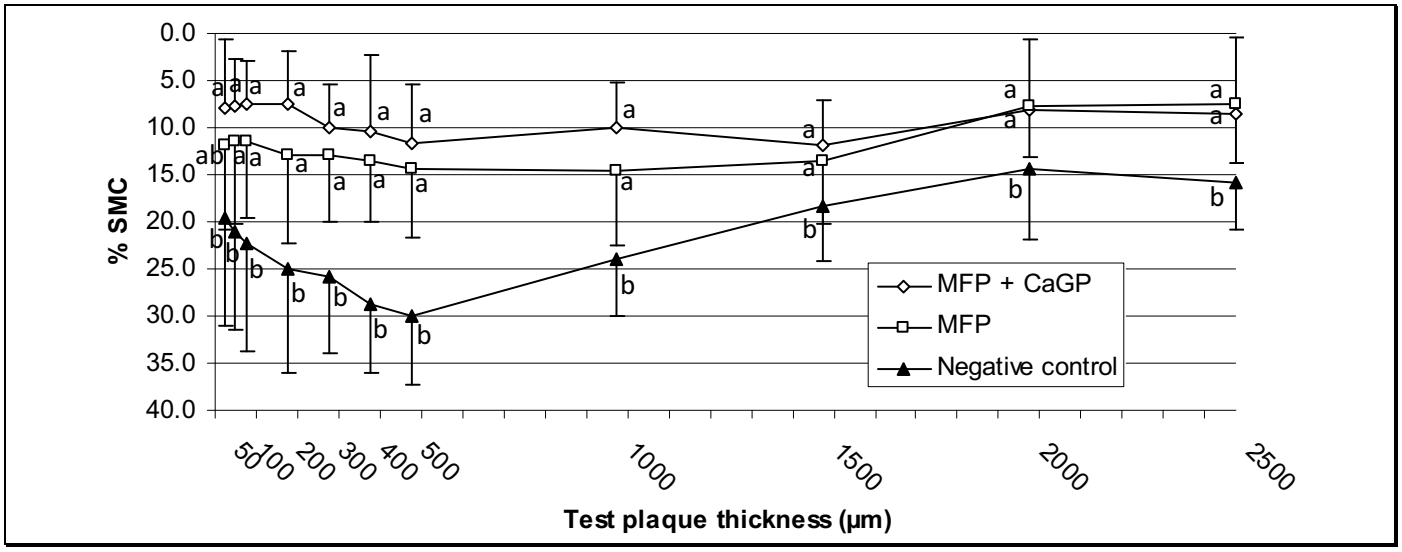

Fig. 3. Mean (n=10) percentage of enamel surface microhardness change (%SMC), according to the dentifrice formulations used and test plaque thickness (μm). Bars indicate standard deviation. Significant differences (P< 0.05) among dentifrices at each distance are represented by distinct letters.

frices when compared to the non-F dentifrice (P< 0.05), and without significant differences between them (P> 0.05) (Table).

At the end of the *in situ* test, F concentration in the fluid was still higher for both groups treated with F dentifrices (P< 0.05) and a significantly lower Ca and P_i concentration was found for these groups (P< 0.05), without significant differences between them (P> 0.05) (Table).

Figure 3 shows the mineral loss of enamel blocks according to the dentifrices used. No significant difference was observed between both F dentifrices, either with or without CaGP (P> 0.05). A higher mineral loss was observed for the non-F dentifrice, at all thicknesses of the test plaque (P< 0.05).

Discussion

While it is well recognized that F dentifrice is related to the worldwide decrease in dental caries prevalence,[1,2] the addition of other preventive agents could further improve its anticaries properties. The results showed, however, that CaGP at the concentration used in the present study does not enhance the effect of a dentifrice containing 1,500 μg F/g as MFP on reducing enamel demineralization. This finding contrasts with that of Mainwaring & Naylor,[7] who observed a significant effect of a CaGP-MFP dentifrice in a 4-year clinical trial when compared to a MFP-only dentifrice. This discrepancy could be explained by the difference in composition of the F dentifrice tested, which in their study was a 1,000 μg F/g dentifrice, calcium carbonate-based. Since a dose-response for the increase in F concentration in MFP dentifrices from 1,000 to 1,500 μg F/g was already observed,[21] the higher strength dentifrice could not benefit from the CaGP additive. Additionally, it is possible that the CaGP additive presents an effect enhancing remineralization of previously demineralized enamel, and in the present study only the inhibition of enamel demineralization was tested.

Our results of mineral loss are in accordance with the data of test plaque acidogenicity and availability of mineral ions in the fluid phase of the plaque. No significant differences were observed among the three tested dentifrices in the post-sucrose pH (Fig. 2) or in the Ca and P_i concentration at the moment of the cariogenic challenge (30 minutes after dentifrice use). The buffering effect of phosphate from CaGP was first proposed by Bowen[9] but using CaGP at 0.25% and 1%, applied directly with a sucrose solution to the teeth of monkeys. In the present study, the concentration of CaGP in the dentifrice was lower, 0.13%, and a water slurry was prepared for treatment of the appliance in order to simulate the dilution by saliva. In fact, Lynch & ten Cate[10] did not find any buffering effect of CaGP used at concentrations up to 0.5% in an *in vitro* biofilm model, although they did not evaluate the pH inside the biofilm mass, but at the planktonic phase. While deficient P_i release from incomplete glycerophosphate hydrolysis could account for the lack of buffering capacity found, the Ca concentration was also similar for the three dentifrices tested, suggesting that 30 minutes after dentifrice use the additional mineral ions provided by the CaGP would already be leveled off to the baseline fluid concentrations. Moreover, the hydrolysis of MFP (Table) confirms that the bacterial plaque used presents phosphatase activity, and would also hydrolyze glycerophosphate.

In fact, at both times of plaque collection, one fourth of the total F in the fluid phase of the plaque was present as F ion, suggesting that release of F ions from MFP is continuous throughout the experimental phase. The optimum pH for phosphate release by plaque phosphatases is around 8.[22] In the present study, after 30 minutes inside the mouth, test plaque pH had decreased to around 5.5, probably due to production of acids from endogenous substrates by the bacteria.[23] This low pH could explain the higher proportion of MFP to F ion found in the fluid phase of the plaque in this experiment, when compared to a more efficient hydrolysis that would occur in natural plaque.[18]

Irrespective of the CaGP additive, both F dentifrices significantly reduced enamel demineralization in the present model, which is supported by the higher F concentration found in test plaque fluid compared with the negative control group treatment (Table). The loss in surface microhardness was around 10%, in accordance with previous results using a similar MFP/silica formulation.[13] The analysis of the plaque after the intraoral test suggests that it could also be a feasible indicator of enamel demineralization, since higher concentration of Ca and

P_i were found in the fluid phase of plaque treated with the non-F dentifrice.

In conclusion, using a short-term *in situ* model, no additional effect on the inhibition of enamel demineralization was observed for a F dentifrice containing CaGP when compared to a F-only dentifrice, since it was not able to persistently increase the availability of Ca and P_i to the fluid phase of the test plaque. The effect of the CaGP additive on enamel remineralization should be further investigated.

a. Future-Tech Corp., Fujisaki, Kawasaki-ku, Kawasaki-City, Kanagawa, Japan.
b. World Precision Instruments, Sarasota, FL, USA.
c. Orion, Boston, MA, USA.
d. Paffenbarger Research Center/ADA Foundation, Gaithersburg, MD, USA.
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f. Hellma, Müllheim, Germany.
g. Beckman, Fullerton, CA, USA.
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Dr. Tenuta is Assistant Professor, Dr. Del Bel Cury and Dr. J.A. Cury are Full Professors, Dr. Tabchoury is Associate Professor, and Dr. Moi is a PhD student, Piracicaba Dental School, UNICAMP, Piracicaba, São Paulo, Brazil. Dr. Cenci and Dr. Pereira-Cenci are Assistant Professors, School of Dentistry, Federal University of Pelotas, Pelotas, Rio Grande do Sul, Brazil.

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