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ABSTRACT

The anticaries effect of professional fluoride (F) application has been attributed to calcium-fluoride-like deposits (CaF₂) formed on enamel, but this has not been clearly demonstrated. We hypothesized that CaF₂ formed on plaque-free enamel by F application would reduce enamel demineralization due to the increase of F availability in fluid of subsequently formed plaque. We created distinct levels of CaF₂ on enamel to evaluate a dose-response effect. Enamel blocks were mounted in contact with a *S. mutans* test plaque and used *in situ* by 10 volunteers. F released to the fluid phase of this substrate ("plaque fluid") was measured before a cariogenic challenge. "Plaque fluid" F concentration was highly correlated to the enamel CaF₂ concentration ($r = 0.96$, $p < 0.001$) and to consequent enamel demineralization ($r = -0.75$, $p < 0.001$). The results suggest that F released to plaque fluid from CaF₂ formed on enamel may play a significant role in the anticaries effect of professionally applied F agents.

KEY WORDS: fluoride, topical application, plaque fluid, demineralization, calcium fluoride.

Fluoride Release from CaF₂ and Enamel Demineralization

INTRODUCTION

Professional topical fluoride (F) application is considered an effective measure for caries prevention (Marinho *et al.*, 2007). It increases the amount of tooth-bound F (fluorapatite, FAp) on enamel, which has been shown to enhance its resistance to demineralization (Takagi *et al.*, 2000). However, an important effect of topical F application seems to rely on the concomitant formation of a phosphate-contaminated calcium-fluoride deposit (calcium-fluoride-like material, hereafter referred to as CaF₂) on dental hard tissues, which would act as a F reservoir, slowly releasing F to interfere with de/remineralization events at the tooth/plaque interface (for review, see ten Cate, 1997).

Therefore, if CaF₂ formed on plaque-free enamel is an important factor responsible for the anticaries effect of professionally applied topical F treatments (Ögaard *et al.*, 1990; Ögaard, 2001), F released from CaF₂ should be found in the fluid of newly formed plaque, where it could significantly inhibit enamel demineralization. Indeed, an increase in F concentration was found in whole plaque formed on enamel treated by topical F application (Paes Leme *et al.*, 2004), but this has never been studied in plaque fluid. Also, a dose-response effect of the concentration of CaF₂ on enamel, its release to plaque fluid, and the subsequent inhibition of enamel demineralization has not been examined.

Thus, we hypothesized that if CaF₂ is an important source of F for plaque fluid, the amount of CaF₂ formed on enamel should be related to F concentration in the plaque fluid and to the consequent inhibition of enamel demineralization.

MATERIALS & METHODS

Experimental Design

This was a crossover, double-blind, short-term *in situ* study (Zero *et al.*, 1992; Cury *et al.*, 2003), approved by the Ethics Committee of the Piracicaba Dental School. Ten volunteers signed a written informed consent. In two experimental phases, they used a palatal appliance containing bovine enamel blocks with distinct levels of CaF₂, in an intra-oral demineralization test (Fig.). Enamel blocks were treated with either a non-F solution, or an acidulated 0.5 M (9500 ppm F) NaF solution. Different concentrations of CaF₂ were created on F-treated blocks by either not aging them, or by aging them for 6 hrs or 48 hrs in artificial saliva. A set of enamel blocks was used for determination of CaF₂ and FAp formed and retained on enamel by these procedures. Eight blocks were mounted in a palatal appliance, 4 from each treatment in each side of the appliance, in contact with a test plaque prepared from *S. mutans* (Fig., a). After 30 min of intra-oral exposure, test plaque from 2 blocks of each treatment was collected for determination of baseline F, calcium (Ca), and inorganic phosphorus (P_i) concentrations in the fluid phase of the bacterial test plaque ("plaque fluid") (Fig., b). The appliances were re-inserted into the volunteers' mouths, and

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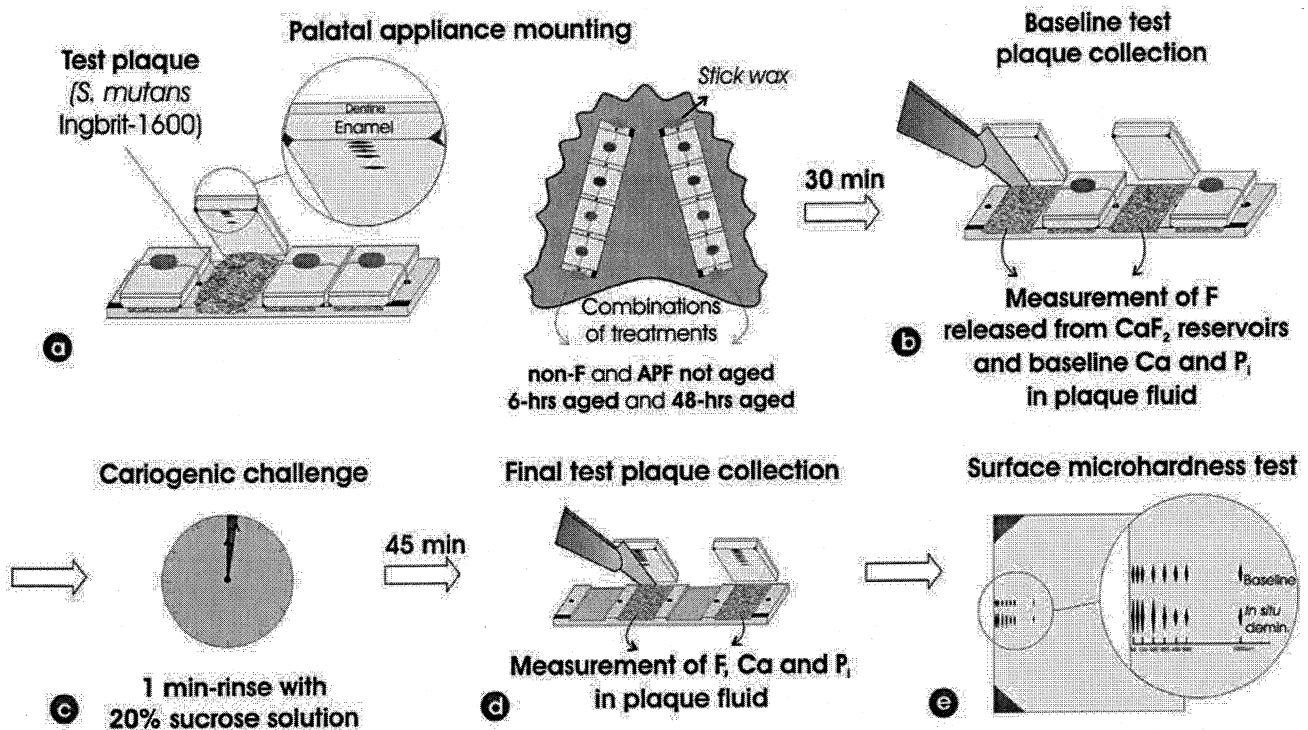


Figure. Illustration of the experiment. (a) Enamel blocks with known surface microhardness were fixed in acrylic holders, in contact with a test plaque prepared from *S. mutans* IB1600; each holder contained blocks from the same treatment, placed at each side of the appliance, in a split-mouth design. (b) After 30 min inside the mouth, 2 enamel blocks from each holder were removed, and the test plaque was collected for analysis of F, Ca, and P_i in "plaque fluid". (c) A one-minute rinse with sucrose solution was conducted, and 45 min after the rinse, the experiment was completed. (d) Test plaque from the remaining blocks was collected for fluid analysis. (e) We again determined surface microhardness on enamel blocks, to calculate the percentage of surface microhardness change (mineral loss).

a cariogenic challenge was conducted by gentle rinsing with a 20% sucrose solution during 1 min (Fig., c). Forty-five min after the cariogenic challenge, the test plaque and the other 2 blocks remaining from each treatment were collected (Fig., d). Inorganic ions were measured in the test "plaque fluid" as chemical indicators of enamel demineralization, and mineral loss was also evaluated by the percentage of loss in enamel surface microhardness (SMH) (Fig., e). CaF₂ and FAp remaining in enamel were determined.

Topical Fluoride Application and Baseline Surface Microhardness Determination

Enamel blocks (5 x 5 x 2 mm) were prepared from bovine incisors, and the enamel surface was polished flat (Zero *et al.*, 1990). Those with a mean Knoop hardness of 332.2 ± 18.3 were randomly divided into 4 groups of 56 specimens each, comprising the different groups/treatments. Blocks from a negative control group were individually immersed for 4 min in 0.1 M phosphate-buffered solution, pH 3.5 (10 mL/block). The remaining blocks were treated for 4 min with acidulated phosphate F (APF) solution containing 0.5 M NaF in 0.1 M phosphate, pH 3.5. After treatments, blocks were washed for 30 sec with a stream of distilled water. F-treated blocks were either not aged (group APF) or aged for 6 hrs (group APF, 6-hrs-aged) and 48 hrs (group APF, 48-hrs-aged) in a flow (0.5 mL/min) of artificial saliva (remineralization solution of ten Cate and Duijsters, 1982, without F) at a volume of 6 mL/mm², to simulate the dissolution of CaF₂ that occurs in the mouth after F treatment (Dijkman *et al.*, 1983). Enamel blocks were treated and aged just before being used in the next phase. Sixteen blocks from each group were used for determination of the baseline CaF₂ and

FAp concentrations, and remaining blocks were used in the *in situ* test.

Before the intra-oral test, we determined baseline SMH on enamel blocks to be used *in situ* by making 8 indentations at distances of 50 to 1000 μm from one edge of the block, with a 50-g load, for 5 sec in a Future-Tech FM microhardness tester coupled to FM-ARS software (Future-Tech Corp., Kawasaki, Kanagawa, Japan). This edge was marked for subsequent mounting reference in the appliance (for details, see Cury *et al.*, 2003).

Palatal Appliance Mounting

Test plaque was prepared from *S. mutans* Ingbritt-1600 and mounted in contact with enamel blocks in 2 plastic holders fixed to the palatal appliance (Fig., a). In this design, saliva accessed the test plaque through a 0.5 x 3 mm area delimited by the holder groove, simulating an interproximal plaque stagnation area. The marked side of the enamel block where the baseline hardness measurements were made was mounted to the center of the palatal appliance (for details, see Cury *et al.*, 2003). Utilizing a split-mouth design, we combined negative control and APF groups into one experimental phase, and groups aged for 6 and 48 hrs were combined in the other phase (Fig., a).

Analysis of Mineral Ions in "Plaque Fluid"

Test plaque samples collected from the appliance were immediately placed inside an oil-filled centrifuge tube to separate the fluid from the plaque solids (Vogel *et al.*, 1997). The fluid was immediately analyzed for F concentration, with an oil-covered inverted F electrode (Orion 94-09, Thermo Scientific Inc., Waltham, MA,

Table 1. Summary of the Analyses Made, According to the Treatment/Groups [mean (SD), n]

Treatment/ Groups	F in Enamel Blocks after Treatments/Aging ^a		Mineral Ions in the "Plaque Fluid"							F in Enamel Blocks after the <i>in situ</i> Test	
	CaF ₂ ($\mu\text{g F/cm}^2$)	FAP ^e ($\mu\text{g F/g}$)	After 30 min <i>in situ</i> , before Cariogenic Challenge			45 min after Cariogenic Challenge, at the End of the <i>in situ</i> Test			% SMC	CaF ₂ ($\mu\text{g F/cm}^2$)	FAP ^g ($\mu\text{g F/g}$)
			F (μM)	Ca (mM)	P _i (mM)	F (μM)	Ca (mM)	P _i (mM)			
Negative control	0.1 A ^b (0.03), n = 16	94.9 A (59.6), n = 16	2.1 A (0.8), n = 10	2.3 A (0.9), n = 10	5.4 A (1.2), n = 10	1.5 A (0.6), n = 10	19.4 A (5.5), n = 10	15.3 A (1.9), n = 10	37.4 A (9.7), n = 10	0.2 A ^f (0.02), n = 9 ^d	109.0 A (58.0), n = 10
APF	21.7 B (15.1), n = 15 ^c	727.9 B (233.7), n = 15 ^c	413.7 B (85.1), n = 10	1.8 A (0.9), n = 10	4.3 B (1.1), n = 10	395.4 B (84.1), n = 10	2.7 B (1.6), n = 10	5.3 B (1.5), n = 10	1.0 B (3.9), n = 10	10.8 B ^f (4.4), n = 10	1093.4 B ^f (232.9), n = 10
APF, 6-hrs- aged	13.4 B (6.7), n = 14 ^{c,d}	721.7 B (135.2), n = 15 ^c	230.2 C (67.2), n = 9 ^d	2.3 A (1.1), n = 10	4.2 B (1.1), n = 9 ^d	222.1 C (78.2), n = 10	4.1 B (2.3), n = 10	4.6 B (1.2), n = 10	5.3 BC (6.3), n = 10	6.5 C ^f (3.0), n = 10	844.0 C ^f (118.9), n = 10
APF, 48-hrs- aged	2.6 C (1.0), n = 16	803.2 B (275.5), n = 16	22.9 D (7.1), n = 10	2.2 A (0.9), n = 10	4.9 AB (1.3), n = 10	43.3 D (16.1), n = 10	7.8 C (2.4), n = 10	7.5 C (1.5), n = 10	14.4 C (11.1), n = 10	1.9 D (0.7), n = 10	660.0 D (155.9), n = 10

^a Determined from an extra set of enamel blocks not used *in situ*.

^b Distinct capital letters are used to indicate treatment/groups which are significantly different from each other within each variable ($p < 0.05$). For statistical analysis, data for CaF₂ and F in "plaque fluid" were log-transformed; data for FAP and Ca and P_i in the fluid 45 min after the challenge were transformed to the square root; and data for Ca in the fluid at 30 min were transformed to the inverse.

^c One enamel block was lost during *in vitro* procedures.

^d One value indicated by the SAS/LAB (SAS Software) as an outlier was removed. Outliers removed: CaF₂ after treatment/aging, APF 6 hrs-aged = 70.8 $\mu\text{g F/cm}^2$; F in fluid, APF 6 hrs-aged = 18.6 μM ; P_i in fluid, APF 6 hrs-aged = 8.3 mM; CaF₂ after *in situ*, negative control = 0.06 $\mu\text{g F/cm}^2$.

^e In $\mu\text{g F/cm}^2$, means (SD) of FAP values after treatments/aging were 0.26 (0.15), 2.11 (0.89), 2.12 (0.71), 2.31 (0.82), for the groups control, APF, 6-hrs aged, and 48-hrs aged, respectively.

^f Significantly different from the initial values of CaF₂ or FAP in enamel, after treatments/aging ($p < 0.05$).

^g Due to differences in the enamel layer removed (ranging from 9.1 to 12.3 μm), the layer of enamel dissolved was used as a co-variable in the statistical analysis.

USA) under a microscope (D.F. Vasconcellos, São Paulo, SP, Brazil), and Ca and P_i were determined by colorimetric reactions (Vogel et al., 1983; Tenuta et al., 2006).

Analysis of Enamel Mineral Loss

SMH was measured in enamel blocks used *in situ*, at 100 μm from the initial indentations, at the same distance points 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 1.0 mm (Zero, 1995). The percentage

Table 2. Correlations between F Availability in Different Reservoirs in Enamel, that Released to the "Plaque Fluid", and the Different Indicators of Mineral Loss [Spearman coefficient of correlation (p value)]

Variables	F in Enamel Blocks after Treatments/Aging		F in "Plaque Fluid" before the Cariogenic Challenge
	CaF ₂	FAP	
F in "plaque fluid" before the cariogenic challenge	0.96 (< 0.001)	0.38 (0.02)	-----
Ca in "plaque fluid" after the cariogenic challenge	-0.86 (< 0.001)	-0.38 (0.02)	-0.81 (< 0.001)
P _i in "plaque fluid" after the cariogenic challenge	-0.80 (< 0.001)	-0.31 (0.05)	-0.80 (< 0.001)
%SMC*	-0.80 (< 0.001)	-0.40 (0.01)	-0.75 (< 0.001)

* Percentage of surface microhardness change.

of SMH change (%SMC) was calculated from the mean hardness values of each enamel block before and after the *in situ* test [%SMC = (SMH after *in situ* test - baseline SMH) x 100/baseline SMH].

Determination of CaF₂ and FAP in Enamel

For determination of CaF₂, blocks were isolated by wax, leaving only the enamel surface exposed, and individually immersed in 1.0 M KOH (1.0 mL per block) for 24 hrs (Caslavskaya et al., 1975). After blocks were buffered with TISAB II containing 1.0 M HCl, F was measured with an ion-selective electrode (Orion 96-09, Thermo Scientific, Inc.) and an ion analyzer (Orion EA-940, Thermo Scientific, Inc.), and the concentration of CaF₂ was expressed as $\mu\text{g F/cm}^2$.

After KOH extraction, we estimated FAP (here to represent a mixture of F-containing apatite minerals dissolved by acid) by removing 1 enamel layer in 0.75 mL of 0.5 M HCl for 30 sec under agitation. After neutralization with TISAB II containing 0.5 M NaOH, F was determined as described above. P_i was measured in the acid extract (Fiske and Subbarow, 1925), and the amount of enamel dissolved was calculated based on a P_i concentration in enamel of 17.4% and a density of 2.92. Thus, FAP concentration was expressed as $\mu\text{g F/g}$ of enamel.

Statistical Analysis

All data were analyzed by ANOVA, with the volunteers considered a source of variation (statistical blocks). Data which did not fit the assumptions of normal distribution of errors and equality of variances were transformed (Box et al., 1978). The Tukey test was used for post-ANOVA comparisons. F in enamel before and after the *in situ* test was compared by a *t* test. Correlation between variables was studied by the Spearman coefficient of correlation, since they failed the normality test. All analyses were performed

with SAS software (SAS Institute Inc., version 8.01, Cary, NC, USA), with p level fixed at 5%.

RESULTS

Pre-treatment of enamel blocks with an APF solution significantly increased CaF₂ and FAp concentrations in enamel (Table 1, columns 2 and 3). The aging process decreased the CaF₂ concentration, but did not affect FAp values.

After 30 min *in situ*, a significantly higher F concentration in "plaque fluid" was observed for the F-treated blocks, when compared with the negative control group (Table 1, column 4). The highest ($p < 0.05$) F concentration was found in "plaque fluid" in contact with APF-treated, not aged, enamel blocks, and significantly lower F concentrations for groups aged for 6 and 48 hrs. Ca concentration in "plaque fluid" at this moment was similar for all groups, but P_i concentration was significantly higher for the negative control group when compared with groups APF and APF aged for 6 hrs (Table 1, columns 5 and 6).

Forty-five min after the cariogenic challenge, F concentration in the "plaque fluid" was still high in all F-treated groups, and the differences among the groups were maintained. Ca and P_i concentrations increased significantly after the cariogenic challenge for all groups (columns 8 and 9), and were higher for the negative control group, followed by the groups APF aged for 48 hrs, APF aged for 6 hrs, and APF not aged; these last two groups did not differ statistically from each other.

Analysis of the %SMC data (Table 1, column 10) showed that the highest demineralization was observed for the negative control group and the lowest for the APF group, not aged, with intermediate demineralization levels for groups aged for 6 and 48 hrs.

CaF₂ significantly decreased and FAp significantly increased after the *in situ* test for groups APF and APF aged for 6 hrs when compared with the values after treatment/aging (Table 1, columns 11 and 12). For the negative control group, a significant increase in CaF₂ was observed after the *in situ* test, but the values were still significantly lower than in all other groups.

F released to the "plaque fluid" before cariogenic challenge was highly correlated to the concentration of CaF₂ formed and retained on enamel ($r = 0.96$, $p < 0.001$) (Table 2), but correlation with FAp was weak ($r = 0.38$, $p = 0.02$). Correlation between CaF₂ and F released to the "plaque fluid" before cariogenic challenge and those variables representing enamel demineralization (Ca and P_i in the "plaque fluid" after cariogenic challenge and the %SMC) were high and negative (Table 2).

DISCUSSION

Most F formed on enamel in this study was CaF₂, in agreement with the expected effect of the low pH of the acidulated phosphate F solution used (Larsen and Richards, 2001). Also, the aging protocol used was effective in creating enamel with dissimilar concentrations of CaF₂, due to the undersaturation of saliva with respect to CaF₂ (McCann, 1968). FAp dissolution was probably suppressed by the small amount of F being released to the saliva-like solution. Thus, CaF₂ concentration decreased 10 times after 48 hrs in the continuous flow of artificial saliva used, and no change in FAp levels was observed.

Although the experimental model used did not rely on natural biofilm formation, which is the main limitation of the present study, it facilitated the evaluation of the dynamics among F reservoirs on enamel, their dissolution, and the inhibition of enamel demineralization. Thus, F released to the "plaque fluid" was significantly correlated to the CaF₂ concentration created on enamel. Also, a dose-response effect was observed between the concentration of CaF₂ reservoirs on enamel and F released to "plaque fluid" and the subsequent inhibition of enamel demineralization.

In fact, the %SMC was inversely correlated to F concentration in the "plaque fluid" before the cariogenic challenge. The higher Ca and P_i concentrations in the "plaque fluid" 45 min after the challenge appear to be the result of tooth dissolution, since they were significantly correlated to the %SMC (for Ca, $r = 0.71$, $p < 0.0001$; for P_i, $r = 0.65$, $p < 0.0001$). Thus, F release to "plaque fluid" from CaF₂ dissolution significantly inhibited enamel demineralization, at a greater extent for the groups with a higher CaF₂ content. Enamel dissolution would have been impaired by F available in the "plaque fluid", and at the same time that hydroxyapatite from enamel dissolves, there is a concomitant gain of FAp (ten Cate, 1997). Indeed, an increase of FAp in enamel was observed after the cariogenic challenge in enamel blocks from groups not aged and aged for 6 hrs. Considering the high F concentration in "plaque fluid" at the moment of the cariogenic challenge, some inhibition of acid production by test plaque bacteria cannot be overruled as an additional effect decreasing enamel demineralization (Bradshaw *et al.*, 2002).

A continuous dissolution of CaF₂ during the intra-oral test was apparent, since "plaque fluid" F values were not different before and 45 min after the cariogenic challenge. The CaF₂ concentration remaining on the blocks after the *in situ* test was about half of the initial values. It could be observed that, after the 75-minute intra-oral test, a similar CaF₂ concentration remained on enamel treated but not aged, in comparison with that exposed for 6 hrs in the artificial saliva used to age the samples, even considering that the access to the artificial saliva *in vitro* was greater than that to natural saliva in the mouth. This suggests that the low pH environment created by sugar exposure increased the dissolution rate (Rölla and Ögaard, 1986). Although it is not known how long CaF₂ enamel reservoirs would last, a higher F concentration in whole plaque was still found 14 days after topical F application, under a high cariogenic challenge *in situ* (Paes Leme *et al.*, 2004), but such evaluation of F availability is still lacking for plaque fluid.

Since the concentration of FAp in enamel was not altered by the aging protocol, the correlation of FAp with F in the "plaque fluid" or mineral loss observed was weak. However, FAp in enamel increased after the cariogenic test for the groups APF and APF aged for 6 hrs, and the significance of this increase in the inhibition of mineral loss in subsequent demineralization events remains to be tested.

In conclusion, the findings showed a very clear relationship among concentration of CaF₂ on enamel, F release to plaque fluid, and the consequent reduction of demineralization.

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