Effect of Fluoride Concentration on Reduction of Enamel Demineralization According to the Cariogenic Challenge

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Fluoride present in toothpaste at 1,100 µg/g is considered effective on caries control. However, under high cariogenic challenge due to increasing sugar exposure, higher fluoride concentration (5,000 µg/g) could be necessary to compensate the unbalance on caries process. This was tested in a pH-cycling regimen, which evaluated the effect of fluoride concentration relative to toothpaste on reduction of enamel demineralization under conditions of two levels of cariogenic challenge. Enamel slabs (n=20) were subjected to two pH-cycling regimens, simulating 8x and 16x/day sugar exposure and were treated with solutions containing: 0 (no fluoride), 275 or 1,250 µg F/mL, resulting in 6 treatment groups: 4-h/0-F; 8-h/0-F; 4-h/275-F; 8-h/275-F; 4-h/1,250-F and 8-h/1,250-F. The 275 and 1,250 µg F/mL concentrations simulate mouth salivary dilution when 1,100 and 5,000 µg/g toothpastes are used. Enamel demineralization was assessed by surface (%SHL) and cross-sectional hardness. Fluoride taken up by enamel was also evaluated. Data were analyzed by ANOVA one-way and Tukey's test. The treatment with 1,250 µg F/mL significantly reduced %SHL compared with 275 µg F/mL (p<0.05), irrespective the level of cariogenic challenge (4-h/1,250-F vs. 4-h/275-F and 8-h/1,250-F vs 8-h/275-F comparisons, respectively). These data were supported by fluoride concentration found in enamel. These findings suggest that higher fluoride concentrations could partly compensate the greater caries risk under higher cariogenic challenge due to increasing sugar exposure.

Key Words: dental caries, dental enamel, fluorides, tooth demineralization, toothpaste.

Introduction

Dental caries is a biofilm and sugar-dependent disease that can lead to a progressive loss of tooth mineral (1). Biofilm accumulation is a necessary but not a sufficient factor for the development of caries lesions, while the frequent exposure to sugars is the determinant factor (2). Indeed, sugar has been considered the only cause of caries (3).

One way to try to balance the detrimental effect of sugar on caries is the fluoride. Working on caries process, reducing demineralization and enhancing remineralization, fluoride is clearly effective to slow down the progression of caries lesions but it does not avoid the disease (4,5). Epidemiological surveys have shown in children and adults the effect of sugars on caries and the limited effect of fluoride on its control (6,7).

The relationship between sugar exposure and fluoride effect on caries has been evaluated in well-controlled *in situ* studies (8-11). Duggal et al. (9) and Ccahuana-Vasquez et al. (10) showed that if the daily frequency of sugar were not above 7 to 8 times/day, fluoride from 1,100 µg/g toothpaste would be effective to control enamel demineralization. Cury et al. (11) evaluated the relationship between fluoride concentration in toothpaste and sugar exposure on human enamel demineralization. They showed that a toothpaste containing 1,100 µg F/g was more effective than one with half this concentration to slow down caries progression when sugar exposure was above 2x/day.

Considering that it is based on evidence that sugar exposure is the cause of caries (3) and that the fluoride effect on enamel caries is concentration-dependent (12,13), it is plausible to hypothesize that an increase in the concentration of fluoride could compensate for caries increment caused by increasing sugar exposure. To test this hypothesis the effect of fluoride concentration in toothpaste on the reduction of enamel demineralization, using a pH-cycling model and simulating conditions of higher cariogenic challenge (sugar exposure of 8x and 16x/day), was evaluated.

Material and Methods

Experimental Design

An *in vitro* experimental, randomized and blind study was conducted to evaluate the effect of fluoride concentration on the reduction of enamel demineralization under conditions of higher cariogenic challenges. Enamel slabs obtained from bovine incisor teeth and selected according to surface hardness (SH), were subjected to

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pH-cycling regimens, simulating two different levels of cariogenic challenge (4- and 8-h immersion in demineralizing solution, simulating respectively 8 and 16x/day sugar exposure). The slabs (n=20/group) were exposed to the levels of cariogenic challenge described above and subjected 2x/day to treatments with solutions containing: 0 (negative control), 275 or 1,250 μg F/mL. This resulted in 6 treatment groups: 4-h/0-F; 8-h/0-F; 4-h/275-F; 8-h/275-F; 4-h/1,250-F and 8-h/1,250-F. The pH-cycling regimen lasted 8 days and, at the end of the 5th day, de- and remineralizing solutions were replaced by fresh ones. At the end of the experiment, percentage of surface hardness loss (%SHL), carious lesion area (ΔS) and concentration of F in enamel were assessed in the slabs. In de- and remineralizing solutions collected at the 5th and last day of pH cycling, fluoride concentration was determined. The null hypothesis tested was that higher fluoride concentration would compensate a greater risk to demineralization by a higher cariogenic challenge. The data were analyzed by one-way ANOVA and Tukey's test. Significance level was set at 5%.

Enamel Slabs Preparation

Bovine incisor teeth, stored in 2% formaldehyde solution for at least 30 days (14), were used to prepare flattened and polished enamel slabs (4x4x2 mm). The baseline surface hardness (SH) of the dental slabs was determined by a hardness tester (Future-Tech Corp., Tokyo, Japan), with Knoop diamond under a 50 g load for 10 s. One hundred and twenty enamel slabs with baseline SH of 332.3 ± 14.9 kg/mm^2 were selected. These slabs were randomly distributed into six groups of treatment (n=20) described in the experimental design section. A piece of adhesive paper (2x4 mm) was placed over the polished central surface of the enamel slabs, which were then coated with acid-resistant varnish (nail polish, Risqué®, São Paulo, SP, Brazil). After drying, the adhesive paper was removed to expose a surface area of 8.0 mm^2 to the treatments.

Demineralizing pH-Cycling Regimen and Treatments

The slabs were subjected to two conditions of cariogenic challenge, using the validated pH-cycling model described by Queiroz et al. (15). They were daily immersed during 4 h or 8 h in demineralizing solutions, representing sugar exposures of 8x and 16x/day, respectively (considering that biofilm pH remains 30 min below the critical value for enamel demineralization every time that sugar is ingested). Both pH-cycling regimens lasted for 8 days. The slabs were individually immersed in a demineralizing solution containing 0.1 M acetate buffer, pH 5.0, 1.28 mM Ca, 0.74 mM P and 0.03 μg F/mL (16) for 4 h and for 8 h at 37 °C. For the remainder of the daily cycle, slabs were

individually immersed in remineralizing solution (Tris buffer 20 mM, pH 7.0, 1.5 mM Ca, 0.9 mM P, 150 mM KCl, 0.05 μg F/mL). The duration of immersion in the demineralizing solution was pre-determined and standardized in a pilot study to create two levels of cariogenic challenge. The volume of demineralizing and remineralizing solution per area of exposed enamel surface was 6.25 and 3.12 mL/mm^2, respectively. Twice a day (before and after the immersion in demineralizing solution), the slabs were washed with purified water and exposed to treatments with purified water (negative control), 275 or 1,250 μg F/mL (from NaF) under agitation in a shaker for 5 min. These fluoride concentrations match the dilution (1:3 w/w) that occurs in the oral cavity (17) with toothpastes containing 1,100 and 5,000 μg F/g, respectively. After the fifth day of pH cycling, de- and remineralizing solutions were replaced by fresh ones. At the end of the 8th cycle, the slabs were immersed in remineralizing solution for additional 24 h before analysis (18).

Enamel Demineralization Assessment

Surface hardness (SH) of the 20 slabs of each treatment group was measured before and after the pH cycling. The mean values from the three indentations, spaced 100 μm from each other, and the baseline values were averaged and the percentage of surface hardness loss [%SHL = 100 (SH after pH cycling - baseline SH)/baseline SH] was calculated. Subsequently, 15 enamel slabs from each group were randomly selected and longitudinally sectioned through the center for cross sectional hardness (CSH) determination. Three rows of 13 indentations each were made at 10 up to 60 μm (10 μm between each indentation) and from 60 to 200 μm (20 μm between each other) depths from the outer surface of the enamel slabs. The mean values for the 3 measuring points at each distance from the surface was then averaged and expressed as Knoop hardness number (kg/mm^2). The area of carious lesion (ΔS) was calculated by difference between the area under the curve (kg/mm^2 x μm) of the sound enamel and the area of the demineralized one (11). For these analysis, a hardness tester (Future-Tech FM Corp., Tokyo, Japan) was used with a FM-ARS software, and a Knoop indenter with a 50 g for 10 s and 25 g for 5 s, respectively, for SH and CSH. SH and CSH were used as indicators of enamel demineralization because they are considered reliable methodologies to estimate enamel demineralization (19,20). There is also an association between enamel SH and CSH with white spot caries lesions (21).

Fluoride Concentration in Enamel

Total fluoride formed in enamel was determined in the 5 slabs from each group not used for CSH assessment. Two

enamel layers were sequentially removed by immersion of each slab in 0.4 mL of 0.5 M HCl for 15 and 30 s under agitation. The extract containing the dissolved enamel layer was buffered with the same volume of TISAB II, pH 5.0, modified with 20 g of NaOH/L (22). Fluoride was determined using an ion-selective electrode (Orion 96-09; Orion Research Inc., Boston, MA, USA) and an ion analyzer (Orion EA-940; Orion), previously calibrated with standard fluoridated solutions ranging from 0.031 to 8 µg F/mL. The concentration of fluoride in each layer of removed enamel was summed up and the results were expressed in µg F/cm^2 (23).

Fluoride Determination in De- and Remineralizing Solutions

The concentrations of fluoride were evaluated in de- and remineralizing solutions after the fifth and last day of pH-cycling regimen. For fluoride analysis, an ion-selective electrode (Orion 96-09; Orion) and an ion analyzer (Orion EA-940, Orion), previously calibrated with standard fluoridated solutions ranging from 0.023 to 0.364 µg F/mL, were used.

Statistical Analysis

The assumptions of equality of variances and normal distribution of errors were checked by Shapiro-Wilks test for all the tested response variables (24) and the data were analyzed by ANOVA one-way and Tukey's test. The significance level was 5%. The analyses were performed using the statistical software R 3.0.1 (www.r-project.org).

Results

Table 1 shows that the pH-cycling model used was able to simulate two different levels of cariogenic challenge, because the treatment group 8-h/0-F presented higher

Table 1. Means and SD of percentage of surface hardness loss (%SHL), area of carious lesion (∆S; Kg/mm^2 x µm) and fluoride in enamel (µg F/cm^2), according to the level of cariogenic challenge (4- or 8-h in de- solution) and treatments with fluoride (0 to 1,250 µg F/mL)

Cariogenic challenge/ Fluoride treatment	%SHL (n=20)	∆S (n=15)	Fluoride in enamel (n=5)
4-h/0-F	51.9±7.3 a	5259.2±1872.7 a	1.5±0.2 a
8-h/0-F	80.2±10.2 b	9246.7±3244.9 b	1.4±0.4 a
4-h/275-F	30.0±7.2 c	3098.9±1646.1 c	5.2±1.9 a
8-h/275-F	38.6±6.5 d	3665.1±997.9 ac	10.4±2.1 b
4-h/1,250-F	21.7±6.1 e	2501.8±1347.8 c	5.8±3.1 a
8-h/1,250-F	29.7±5.4 c	2545.1±1579.7 c	16.3±3.8 c

Means followed by distinct lowercase letters in columns differ statistically at p<0.05.

demineralization in terms of %SHL and ∆S than 4-h/0-F (p<0.05). Regarding the effect of fluoride concentration, the treatment with 1,250 µg F/mL significantly reduced %SHL compared with 275 µg F/mL (p<0.05), irrespective the level of cariogenic challenge (4-h/1,250-F vs. 4-h/275-F and 8-h/1,250-F vs 8-h/275-F comparisons, respectively), but for ∆S the differences were not significant (p>0.05).

Statistically higher F concentration in enamel (p<0.05) was found in the slabs treated with 8-h/275-F and 8-h/1,250-F in comparison with those in the negative control group (Table 1).

De- and remineralizing solutions showed significantly higher fluoride concentrations in the groups 4-h/1250-F and 8-h/1250-F (p<0.05) than the negative control and the groups 4-h/275-F and 8-h/275-F, either in the solutions collected on the 5th or the last day of the pH cycling.

Discussion

The pH-cycling model used was adapted from Queiroz et al. (15) and allowed the simulation of two different levels of cariogenic challenge. Considering both %SHL and ∆S (Table 1), 8-h immersion in demineralizing solution significantly resulted in higher mineral loss than immersion for 4 h. The duration of immersion of the enamel slabs in the demineralizing solution was defined in a pilot study and represents the exposure to cariogenic challenges at two different levels, simulating *in vivo* periods of lowering of biofilm pH (immersion in demineralizing solution) and return to baseline values (immersion in remineralizing solution). For practical reasons, only one period of demineralization, representing all episodes, was conducted in the pH-cycling regimen per day. *In vivo,* every time sugar is ingested, there is a pH drop in dental biofilm, falling below the critical pH for enamel, which may last about 30 min (25). Thus, two clinical conditions were simulated in the present study: daily sugar consumption of 8 times (4-h period) and of 16 times (8-h period) for a higher cariogenic challenge.

The effect of 1,250-F in 8-h challenge was equivalent to that of 275-F in 4-h challenge, since both groups did not differ statistically with regards to %SHL (p>0.05). In higher cariogenic challenge, a greater fluoride concentration seemed to be necessary to compensate the high risk of caries and decreased %SHL, as could be seen in this study. The effect of high fluoride concentration, which simulates the use of high F toothpaste, was more pronounced in reducing mineral loss under conditions of higher cariogenic challenges. These findings are in agreement with a clinical study in which the effect of fluoride in higher concentrations was evaluated in high caries risk

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adolescents, who do not use toothpaste regularly (26). The use of higher concentrations of fluoride in dentifrice (5,000 µg F/g) produced lower progression of caries when compared with the 1,450 µg F/g after 2 years, according to clinical and radiographic examinations. Therefore, one must consider changes in exposure to fluoride based on the individual risk of caries and the present findings suggest that high fluoride toothpaste would be required for higher cariogenic challenges in order to achieve the same effect as a conventional toothpaste at the initiation of mineral loss. Nevertheless, studies with toothpastes on the market are necessary to validate this pH-cycling regimen of high

cariogenic challenge.

Although the effect of fluoride on reduction of demineralization has been found in both treatment groups according to %SHL and ΔS values (Table 1), the findings of this study showed that the magnitude of its effect at the same concentration seems to be different, depending on the cariogenic challenge. Regarding %SHL, treatment with 275-F resulted in 43% and 52% inhibition, respectively, for the 4-h and 8-h cariogenic challenge, whereas in the treatment with high F concentration (1,250-F) an inhibition of 58% and 63% was found. ΔS data corroborate these findings: inhibition of 41% and 60% for treatment
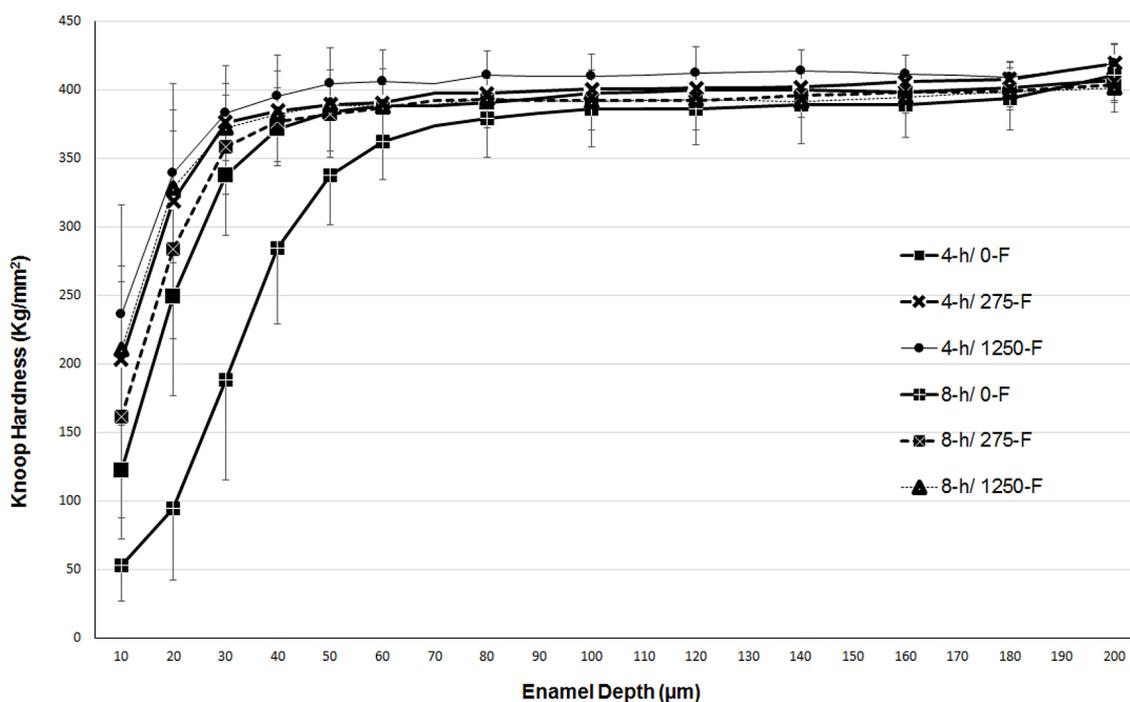

Figure 1. Enamel hardness (kg/mm²) according to the distance (µm) from the surface and the treatment used (n=15 per group). Bars denote standard deviations (SD).

Table 2. Means and SD of fluoride (µg/mL) concentration in de- and remineralizing solutions after the 5th and last day of pH cycling, according to the level of cariogenic challenge (4- or 8-h in de- solution) and treatments with fluoride (0 to 1,250 µg F/mL) (n=20)

Cariogenic challenge/ Fluoride treatment	F Demineralizing Solution (5th day)	F Demineralizing Solution (last day)	F Remineralizing Solution (5th day)	F Remineralizing Solution (last day)
4-h/0-F	0.032±0.002 a	0.030±0.001 a	0.036±0.009 a	0.039±0.002 a
8-h/0-F	0.030±0.004 a	0.030±0.001 a	0.040±0.002 a	0.041±0.001 b
4-h/275-F	0.037±0.002 b	0.033±0.001 b	0.052±0.004 b	0.047±0.002 c
8-h/ 275-F	0.039±0.003 b	0.035±0.002 c	0.058±0.005 b	0.051±0.005 d
4-h/1,250-F	0.046±0.007 c	0.049±0.006 d	0.066±0.008 c	0.067±0.012 e
8-h/ 1,250-F	0.049±0.004 c	0.043±0.003 e	0.079±0.007 d	0.062±0.005 e

Means followed by distinct lowercase letters in columns differ statistically at p<0.05. Initial F concentration (fresh solution). Demineralizing solution: 0.032 µg F/mL; Remineralizing Solution: 0.047 µg F/mL.

with 275-F, respectively, for the 4-h and 8-h cariogenic challenge, while 52% and 72% were found for the treatment with 1,250-F concentration. Considering caries lesions as indicator of caries risk, the data are in agreement with clinical trials showing that the anti-caries effect of fluoride from toothpaste increased with higher baseline levels of D(M)FS (27).

The profile of hardness according to the distance from the surface (Fig. 1) confirms the %SHL and ΔS data, showing that the pH-regimens of 4-h and 8-h in demineralizing solution were able to produce caries lesions of different depths; lesions about 50- and 80-μm deep, respectively, were found. It was previously shown (15) by polarized microscopy that the lesions produced by the daily 4-h immersion in demineralizing solution were subsurface without erosion on the surface. The original model was modified, increasing this time for 8-h to simulate a greater cariogenic challenge. Even in this condition, no erosion was found on the surface, because it was possible to accurately measure the indentations made on the enamel surface.

The results of %SHL and ΔS are also confirmed by fluoride concentration in enamel (Table 2), which is an indicator of fluoride effect in de- and remineralization (28). Decreased mineral loss observed either for %SHL or ΔS in groups treated with fluoridated solutions, especially those exposed to high cariogenic challenge, are supported by the highest fluoride incorporation in enamel found in these groups. Such data are consistent with the *in vitro* findings of Velo et al. (29), Fernández et al. (28) and *in situ* results obtained by Cury et al. (11), who showed that under high exposure to sugars, more F in enamel was observed and with greater fluoride uptake as function of fluoride concentration. During the demineralization, which was conducted at two different levels in the present study, apatitic minerals were dissolved, while minerals containing fluoride, like fluorapatite, were incorporated into enamel. Thus, mineral loss in enamel was reduced, which was found in groups treated with fluoride, especially the slabs treated with high fluoride concentration, which also resulted in an enrichment of enamel by fluoride.

Moreover, higher fluoride concentrations were found in de- and remineralizing solutions in groups treated with 1,250 μg F/mL than in the other groups (p<0.05). These results are supported by the reactivity of fluoride present in the treatment solution with enamel, forming fluoride products that are released into de- and remineralizing solutions. In addition, these findings may support the data found in F concentration in enamel, %SHL and ΔS in treatments with different fluoride concentrations, since more fluoride was found in 1,250-F groups and more fluoride was available to interfere in de- and remineralizing processes.

In conclusion, the outcomes of this study suggest that the effect of fluoride was influenced by the level of cariogenic challenge and higher concentrations of fluoride showed a greater effect on the reduction of the initiation of mineral loss in situations of higher cariogenic challenge. The *in vitro* findings could indicate that under higher cariogenic challenge due to increasing sugar exposure, higher fluoride concentrations could partially compensate the higher caries risk.

Resumo

O fluoreto presente em dentifrícios de 1,100 μg/g é considerado efetivo no controle de cárie dental. No entanto, sob alto desafio cariogênico devido ao aumento da exposição ao açúcar, maior concentração de fluoreto (5.000 μg/g) poderia ser necessária para compensar o desequilíbrio no processo de cárie. Isso foi testado em um modelo de ciclagens de pH, que avaliou o efeito da concentração do fluoreto, relacionada ao dentifrício, na redução da desmineralização do esmalte dental sob condição de dois níveis de desafio cariogênico. Blocos de esmalte (n=20) foram submetidos a dois regimes de ciclagens de pH, simulando 8 e 16 exposições diárias ao açúcar, e foram tratados com soluções contendo: 0 (sem flúor), 275 ou 1.250 μg F/mL, resultando em 6 grupos de tratamento: 4-h/0-F; 8-h/0-F; 4-h/275-F; 8-h/275-F; 4-h/1.250-F e 8-h/1.250-F. As concentrações de 275 e 1.250 μg F/mL simularam uma diluição salivar na cavidade bucal quando dentifrícios de 1100 e 5000 μg F/g são usados. A desmineralização do esmalte foi avaliada na superfície (%PDS) e dureza em corte. Fluoreto incorporado pelo esmalte também foi determinado. Os dados foram analisados por Análise de Variância e teste de Tukey. O tratamento com 1.250 μg F/mL reduziu significativamente %PDS comparado ao de 275 μg F/mL (p<0,05), independente do nível do desafio cariogênico (comparações 4-h/1,250-F x 4-h/275-F e 8-h/1,250-F x 8-h/275-F, respectivamente). Esses resultados foram suportados pela concentração de fluoreto encontrada no esmalte. Os resultados sugerem que maior concentração de fluoreto pode parcialmente compensar um maior risco de cárie sob um maior desafio cariogênico provocado pelo aumento de exposição ao açúcar.

Acknowledgements

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