

***In situ* effect of a dentifrice with low fluoride concentration and low pH on enamel remineralization and fluoride uptake**

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Abstract: Since the anticaries effect of a dentifrice with low fluoride concentration and low pH is unknown, the aim of the present study was to evaluate *in situ* the enamel remineralizing ability of this type of formulation. A double-blind crossover design employing 3 phases of 45 days was conducted. Six adult volunteers wore palatal devices containing 6 previously demineralized human dental enamel slabs, which were subjected 3 times a day to one of the following treatments: non-fluoridated dentifrice (negative control); dentifrice containing 1, 100 µg F/g, pH 7.0 (positive control); dentifrice containing 550 µg F/g, pH 5.5 (experimental). At the end of each phase, enamel remineralization was assessed in terms of cross-sectional microhardness, and loosely as well as firmly bound fluoride formation was determined on the enamel surface. Fluoridated dentifrices were more effective than the negative control in forming loosely and firmly bound fluoride on enamel ($P < 0.05$). However, the positive control formed more loosely bound fluoride than the other treatments ($P < 0.05$). Microhardness analysis showed that the fluoridated dentifrices were more effective than the negative control ($P < 0.05$) in remineralizing dental enamel, although no statistically significant difference was observed between them. Thus, the experimental dentifrice was shown to be effective in remineralizing dental enamel, and this may be attributable to its ability to form firmly bound fluoride on enamel. (J. Oral Sci. 49, 147-154, 2007)

Keywords: low fluoride dentifrice; caries; calcium fluoride; remineralization, firmly bound fluoride.

Introduction

The use of fluoridated dentifrices has been considered an important factor explaining the decline in dental caries observed in both developed and developing countries during the 20th century (1-4). However, fluoridated dentifrices also carry a risk of dental fluorosis (5), because young children swallow a large amount of toothpaste during brushing (6).

In order to reduce the risk of fluorosis from dentifrice use, the following recommendations have been made for young children: (i) Non-use of a dentifrice, or use of a non-fluoridated dentifrice (7). However the presence of fluoride during toothbrushing is considered indispensable for caries prevention (8). (ii) Use of a small amount of dentifrice (9). However, since unsupervised children put more dentifrice on their brushes than their parents, and the amount of ingested fluoride is negatively associated with parental assistance with brushing, constant supervision is required to decrease the risk of fluoride ingestion (10). (iii) Use of a dentifrice with low fluoride concentration (11). However, the anti-caries efficiency of a dentifrice with less than 1,000 ppm F is not well established (12-14).

Among these recommendations, use of a dentifrice with a low fluoride concentration may be an alternative for decreasing the risk of dental fluorosis, provided that its anticaries efficiency is not jeopardized; this could be achieved if the ability of fluoride to interfere with the dental caries process were improved. At present, there is a consensus that the product responsible for the anti-caries effect of topical fluoride is loosely bound fluoride (LBF) (15),

although the effect of firmly bound fluoride (FBF) may not be negligible (16). It is also recognized that the formation of fluoride products on and in enamel is pH-dependent (17). A clinical trial using a dentifrice with a low pH and fluoride concentration was conducted by Gerdin (18). However, the results were not accepted because of the small number of subjects and because the fluoride source used in the test paste was potassium manganese fluoride salt, whereas the positive control paste contained sodium fluoride (12,13).

In Brazil, dentifrice formulations with a low pH and low fluoride concentration were evaluated *in vitro* by Negri and Cury (19) and Brighenti et al. (20). In terms of LBF formed, the first study showed that the low pH and low fluoride concentration dentifrice had the same efficacy as the conventional one. The second study demonstrated that a 550 µg F/g acidified dentifrice had the same anticariogenic action as a 1,100 µg F/g neutral dentifrice.

Therefore, the aim of this study was to evaluate *in situ* the enamel remineralizing ability of a formulation dentifrice with a low fluoride concentration and low pH.

Materials and Methods

Ethical aspects

Six adult volunteers took part in this study after signing an informed, written consent form (Resolution No. 196 of the National Health Council, Health Ministry, Brasília, DF, 10/03/1996). In addition, the donors of the third molars used in this study signed a donation form before tooth donation.

Experimental design

This crossover *in situ* study was performed in three phases of 45 days each, during which 6 volunteers wore acrylic palatal appliances containing six demineralized human enamel slabs. In each phase, the slabs were subjected to one of the following treatments: non-fluoride dentifrice with synthetic silica as an abrasive (negative control); synthetic silica-based dentifrice containing 1,100 ppm F as sodium fluoride (w/w; NaF), pH 7.0 (positive control); synthetic silica-based dentifrice containing 550 ppm F as sodium fluoride (w/w; NaF), pH 5.5 (experimental) (Fig. 1). This study was a triple-blind one, since the examiner,

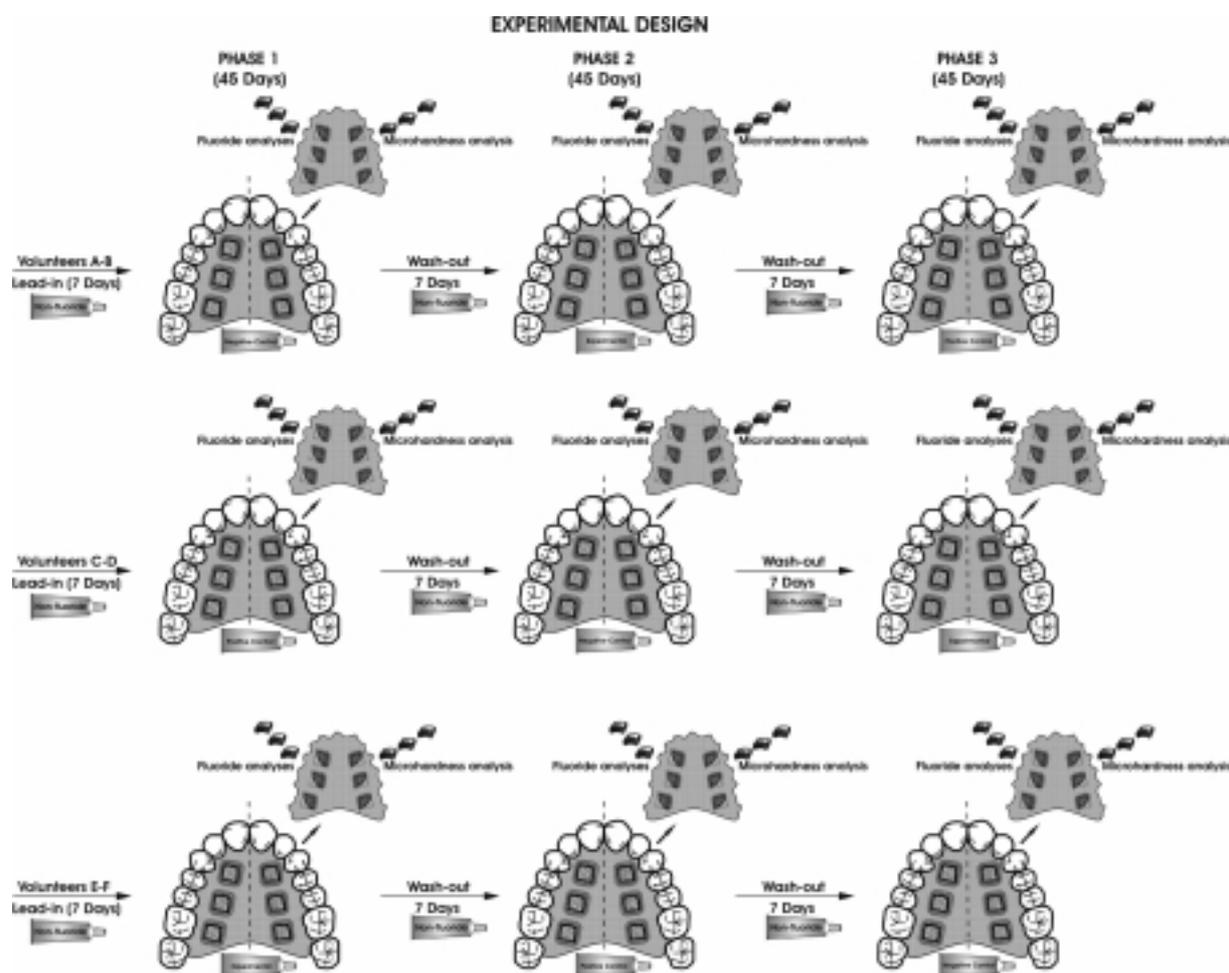


Fig. 1 Experimental design used in the study.

the volunteers, as well as the technician who performed the analyses were unable to identify the dentifrice type used in each phase. In order to test the remineralizing efficacy of the dentifrice, the Knoop hardness number (KHN) at 5 depths and the integrated area of the mineral content were evaluated as response variables. In addition, formation of loosely and firmly bound fluoride on and in the enamel was evaluated.

Sample preparation

The enamel slabs were obtained from impacted human third molars and sterilized by storage in 10% buffered formalin solution, pH 7, for 7 days (21). One hundred and forty-four enamel slabs were obtained from buccal and lingual surfaces. Enamel slabs measuring $4 \times 4 \times 2$ mm were sectioned using a double-faced diamond disc and screened by stereoscopic microscopy to reject those with white spots, cracks or crevices. Eighteen slabs of sound enamel were used to determine the sound enamel microhardness before the experiment, and artificial caries lesions were induced in the other 126 enamel slabs. Cross-sectional microhardness analysis was performed on 18 enamel slabs with artificial caries lesions to evaluate the enamel mineral loss before the experiment. The effect of the investigated dentifrices on enhancement of enamel remineralization was determined *in situ* in the other 108 enamel slabs.

Production of caries-like lesions

A 3-mm-diameter circular area of enamel was exposed in the center of each enamel slab using circular strips of plastic tape and acid-resistant nail varnish. To create caries-like lesions, 126 enamel slabs were partially demineralized by immersion in 25 ml of lactate buffer for 72 h at 37°C. The buffer consisted of 0.1 M lactic acid solution containing 500 mg/l hydroxyapatite and 0.02% sodium azide, pH 4.6 (22), which induced a typical subsurface caries-like lesion about 30-40 µm in depth.

Palatal device preparation

Acrylic palatal devices with 6 cavities ($6 \times 6 \times 3$ mm) prepared on the left and right sides were made, and one slab was inserted into each of them. In order to allow accumulation of plaque, and protect it from mechanical disturbance, a piece of gauze was fixed to the acrylic resin, leaving a 1-mm space from the surface of the specimen (23,24).

Subjects and intra-oral phase

Six healthy dentate volunteers (average = 22.8 yr, range 21-26 yr) that had no currently active caries took part in

this study. The volunteers were allocated at random within each leg of the cross-over design to one of the treatments groups. During a 7-day pre-experimental and wash-out period, the volunteers brushed their natural teeth with a non-fluoride silica-based formulation. All dentifrices used in this study were specially prepared by Kolynos do Brasil Ltd. (São Paulo, SP, Brazil). The subjects were residents of Piracicaba, SP, Brazil, which has a controlled water fluoridation program (annual range 0.63 ± 0.03 mg F/L), and received written instructions as described previously by Cury et al. (24). The volunteers were asked to brush their teeth and their appliances three times per day, using only the dentifrice provided by the researchers. At the end of each phase, the intraoral device of each volunteer was removed and the enamel slabs were examined for fluoride concentration and cross-sectional enamel microhardness.

Determination of loosely bound fluoride

Three enamel slabs from each volunteer were used to measure the loosely bound fluoride on dental enamel. A new circular area 2 mm in diameter was isolated from the former 3-mm-diameter artificial lesion. Each enamel slab was immersed in 0.2 ml of 1 M KOH for 24 h under shaking. After this period, an equal volume of TISAB II (acetate buffer 1.0 M, pH 5.0, containing 1.0 M NaCl and 0.4% 1,2-cyclohexanediamine-tetraacetic acid) modified with 1.0 M HCl was added to each solution before analysis of loosely bound fluoride. The LBF was determined with the use of an Orion EA-940 ion analyzer (Thermo Orion, Beverly, MA, USA) calibrated with fluoride standards from 0.10 to 1.0 µg F/ml, equipped with an ion-specific electrode (Orion 96-09). The results were expressed as µg F/cm² of enamel area.

Determination of firmly bound fluoride

After loosely bound fluoride had been determined, the same three enamel slabs were used to determine FBF in enamel. The fluoride concentration was measured by analyzing a layer of enamel removed from the surface of each enamel slab by immersion in 0.5 ml of 0.5 M HCl for 2 min. Fluoride in the dissolved enamel was determined using an ion-specific electrode (as described above) after addition of 0.5 ml TISAB II modified with 20 g/l NaOH. In order to be able to determine the depth of each etch, it was necessary to determine the amount of phosphorus in each etched sample using the colorimetric method of Fiske and Subarrow (25). The phosphorus concentration was compared between the groups, and differences at $P > 0.05$ were considered not statistically significant. The results were expressed as µg F/cm².

Microhardness analysis

The three remaining enamel slabs were used to perform cross-sectional microhardness (CSMH) tests. They were longitudinally sectioned through the center of the artificial lesion and embedded in Polylight T 208 resin. The test specimens obtained were consecutively polished with 200, 320, 420 and 600 grade silicon carbide paper, followed by 3 and 1 μ m diamond abrasive slurry (Buehler Ltd. Lake Bluff, Illinois, USA). The CSMH analyses were performed by a HMV-2000 (Shimadzu Corporation, Kyoto, Japan) microhardness tester with a Knoop diamond and a 20-g static load that was applied for 30 sec. Five indentations were made on the enamel. The first impression was located 10 μ m from the outer enamel surface, and the others were placed at 20, 30, 50, 70 μ m from the enamel surface. The KHN was obtained from the automatic tester. The cross-sectional microhardness was determined in the dental slabs without induction of artificial caries (baseline), after induction of artificial caries (cariou enamel), and after the intra-oral treatments (post-treatment). After obtaining the KHN values for each depth evaluated, mineral profiles and the integrated area of mineral content (Z parameter) were obtained for all slabs, volunteers and treatments. The KHN values were plotted against depth for each slab and the integrated mineral content of the treated enamel (Z parameter) was calculated (26). Next, based on these data, the percentage of enamel remineralization was calculated according to Cury et al. (27).

Enamel Remineralization Percentage = $[100 (Z \text{ Post-treatment} - Z \text{ Caries}) / (Z \text{ Baseline} - Z \text{ Caries})]$.

The percentage of remineralization was calculated individually and the mean was then calculated for the groups.

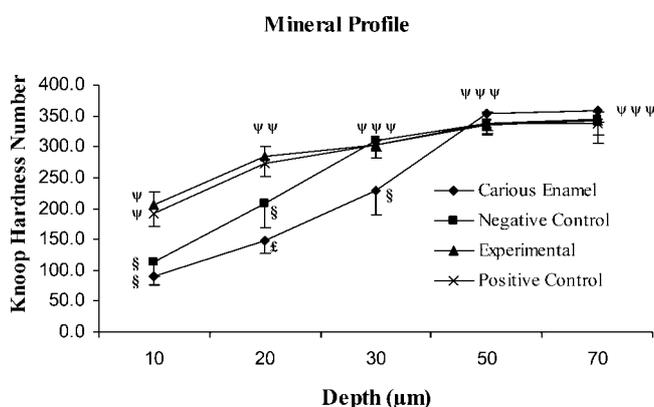


Fig. 2 Knoop Hardness Number of enamel according to the treatments and the distance from the surface. Different symbols show statistical significance ($P < 0.0001$) among treatments at each distance from the enamel surface and bars denote standard deviations.

Statistical analysis

The LBF data were log₁₀-transformed. After assuming equality of variances and that a normal distribution of error was satisfied for LBF, FBF, KHN at 5 depths and remineralization percentage, an analysis of variance (ANOVA) model for block design in the split-plot scheme was constructed. The Tukey test was chosen to evaluate the significance of all pairwise comparisons. For the variable KHN versus micrometer, the assumptions were not satisfied, and therefore a non-parametric one-way ANOVA and Kruskal-Wallis tests were used for these data. The SAS software package (version 8.02, SAS Institute Inc., Cary, NC, 1999) was used, and the significance limit was set at 5%.

Results

Table 1 shows the analyses of the enamel blocks for variable values of KHN versus micrometer and remineralization percentage. With regard to KHN versus micrometer, the cariou enamel group was significantly different from the negative control group as well as from the positive control and experimental groups. There was also a significant difference between the negative control and fluoridated treatments for both variables. Fig. 2 shows that the treatments with fluoridated dentifrices were significantly more effective than the negative control in terms of enamel remineralization at 10 and 20 μ m depths. However, at 20 μ m depth, although not completely, even the negative control was able to remineralize cariou enamel. The values of KHN also demonstrated that the effect of experimental dentifrice treatment was not significantly different from that obtained by the positive control at these distances.

The results obtained by LBF and FBF determination in human dental enamel are shown in Table 2. In both analyses, the highest fluoride concentrations were found for both dentifrices containing fluoride, and were significantly different from the negative control dentifrice. However, when the two fluoridated dentifrices were compared, no significant difference was observed in terms of FBF formation. On the other hand, for LBF, the highest value was found for the positive control, which differed significantly from the other two treatments.

Discussion

Microhardness tests have been widely used to evaluate enamel remineralization occurring in *in situ* caries models (27-35). However, the concern that microhardness measurements do not allow localization of mineral loss or gain is reasonable if the objective of the research is to study in great detail the process of remineralization in the mouth.

Table 1 Knoop Hardness Number versus micrometer and remineralization percentage (mean \pm SD, n = 6) according to the treatments

Groups/Treatments	Knoop Hardness Number \times Micrometer	Remineralization %
Cariou enamel	18777.8 \pm 1621.6£	—
Negative control	19951.8 \pm 1947.8§	30.6 \pm 30.2§
Positive control	21287.3 \pm 1082.3 ψ	63.8 \pm 19.5 ψ
Experimental	21467.8 \pm 806.6 ψ	70.1 \pm 11.1 ψ

For values designated by the same symbol there is no statistically significant difference ($P < 0.05$).

Table 2 Mean fluoride concentrations in artificial caries lesions treated with non-fluoridated and NaF/silica dentifrices

Groups/Treatments	Fluoride Concentration on Enamel ($\mu\text{g F/cm}^{2*}$)	
	Loosely Bound Fluoride**	Firmly Bound Fluoride**
Negative control	0.86 \pm 0.24£	8.17 \pm 1.71§
Positive control	2.84 \pm 0.80 ψ	13.57 \pm 3.83 ψ
Experimental dentifrice	1.75 \pm 0.36§	13.83 \pm 2.63 ψ

* Mean \pm S.D.

** For values designated by the same symbol there is no statistically significant difference ($P < 0.0001$ for alkali-soluble fluoride and $P < 0.0025$ for firmly bound fluoride).

On the other hand, if the intent is to study early changes in the enamel surface or to predict the outcome of an anti-caries treatment, this concern may not be warranted (36). White (1987) (37) has reported that for early carious lesions (25-50 μm depth), the net remineralization as measured by microhardness was highly correlated with remineralization as measured by microradiography ($r^2 = 0.94$; $P < 0.01$). The findings of this study (Table 1) showed that all the treatments were able to increase the hardness of the enamel with early caries lesions. In addition, the data showed that saliva was also efficient in remineralizing the enamel, because even enamel from the negative control group was rehardened (30.6%). These results are in line with those of Dijkman et al. (38) and Paes Leme et al. (39). To explain the remineralization ability of the negative control group, it is important to remember that the volunteers were residents of a city served with fluoridated drinking water, and thus after 12 hours, average plaque [F] brushing with an F or placebo dentifrice is not significantly different (40). Based on this fact, in persons who drink fluoridated water, plaque [F] is not significantly increased throughout much of the day by the use of a fluoridated dentifrice, and therefore fluoridated dentifrices and drinking water may have similar cariostatic effects.

No statistically significant differences were found among the fluoridated treatments, in agreement with the *in vitro* study performed by Brighenti et al. (20).

The ability of fluoride dentifrices to control dental caries may rely on the reaction products formed after topical application of F, which can be loosely (calcium fluoride-like; 'CaF₂') and firmly bound F (41). The formation of

products when F reacts with the enamel depends on the F concentration, duration, pH, frequency and the treatment method (42). With regard to acidity, it has been shown that pH considerably influences the formation of fluoride products on enamel (19,43,44) as well as the anticariogenic action of a dentifrice (20).

In this study (Table 2) it was demonstrated that although the experimental dentifrice formed less LBF than the positive control, remineralization was the same for both. One possible explanation is that the enhancement of remineralization is positively, but not linearly related to the concentration of the fluoride products formed (FBF and LBF). The LBF formed by the positive control dentifrice was 1.6 times higher than that formed by the experimental dentifrice. It is possible that this increase was not enough to significantly enhance the remineralization process. In a recent *in situ* study performed by Lagerweij and ten Cate, (45) the difference in LBF formation between two regimes with different fluoride concentrations (daily application of fluoridated topical gel combined with fluoridated toothpaste and fluoridated toothpaste alone) was very evident. However, although LBF formation was about 27 times higher for daily application of fluoridated topical gel combined with fluoridated toothpaste, the mineral gain promoted by this method was significantly higher only in the surface layer. In addition, the highest LBF formation was 46 times higher than that found in the present study. Another possible explanation for the similarity of effects would be that LBF seems to be more effective in inhibiting enamel demineralization instead of enhancing remineralization, since the design of this study was

favorable for remineralization. Moreover, the acidulated dentifrice may have deposited a large amount of LBF on the saliva-coated enamel at the time of the last toothbrushing. During a subsequent acidic attack, the LBF would have been exposed to acid phosphate or phosphate ions, and this may have resulted in calcium fluoride being hydrolyzed to fluoridated hydroxyapatite, which was determined by the fluoride analyses that were performed 12 h after exposure to the dentifrice treatment. A similar mechanism occurs in dental plaque, as reported by Whitford et al. (40).

Another result of this study was that the experimental dentifrice, as well as the positive control dentifrice, significantly increased the FBF content of the enamel. Furthermore, the effect of the experimental dentifrice did not differ from that of the positive control dentifrice. These results are in line with previous investigations showing that the incorporation of FBF in enamel may provide significant protection against caries (16,46). Thus, the remineralization capacity of the experimental dentifrice is probably related to its ability to form FBF and LBF concentrations high enough to cause a similar remineralizing effect when compared with the positive control. In addition, the results of fluoridated treatments might have been influenced by the use of impacted third molars, which are generally accepted to be not fully mature and may be more reactive to fluoride treatments.

According to Ammari et al. (14), some clinical studies have confirmed that toothpastes containing 275 µg F/g are less efficient than those containing 1,100 µg F/g. However, no consistent studies have compared toothpastes containing 550 and 1,100 µg F/g. Our results should not be considered definitive, since this study had some limitations such as a small sample size, shallow lesions that might have been easy to remineralize, and lack of verification of the dose-response relationship of the dentifrices. However, the data can be used as a basis for further clinical studies to examine the benefits of reducing the pH of low fluoride dentifrices in order to decrease caries development. In this way, it may be possible to improve the remineralizing efficacy of dentifrices by reducing their pH, since the acidified low-fluoride dentifrice evaluated appeared to have the same remineralization action as neutral 1,100 ppm F.

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