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In situ protocol for the determination of dose-response effect of low-fluoride dentifrices on enamel remineralization

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ABSTRACT

No *in situ* protocol has assessed the dose-response effects of fluoride dentifrices involving low-fluoride formulations. Objective: To assess the ability of an *in situ* remineralization model in determining dose-response effects of dentifrices containing low fluoride concentrations ([F]) on bovine enamel. Material and Methods: Volunteers wore palatal appliances containing demineralized enamel blocks and brushed their teeth and devices with the dentifrices supplied (double-blind, crossover protocol) separately for 3 and 7 days. Surface hardness (SH), integrated subsurface hardness (ΔKHN) and [F] in enamel were determined. Data were analyzed by ANOVA, Tukey's test and Pearson's correlation ($p<0.05$). Results: Dose-response relationships were verified between [F] in dentifrices and SH, ΔKHN and enamel [F]. Higher correlation coefficients between enamel [F] and SH and ΔKHN were obtained for the 3-day period. Significant differences in SH and ΔKHN were observed among all groups for the 3-day period, but not between 0-275, 275-550, and 550-1,100 µg F/g dentifrices for the 7-day period, nor between 3- and 7-day periods for the 1,100 µg F/g groups. Conclusions: Considering that the peak remineralization capacity of the conventional dentifrice (1,100 µg F/g) was achieved in 3 days, this experimental period could be used in future studies assessing new dentifrice formulations, especially at low-fluoride concentrations.

Key words: Dentifrices. Dental enamel. Fluorides. Tooth remineralization.

INTRODUCTION

Throughout the 20th century, fluoride was the main agent used for the control of dental caries worldwide, being available to the population through the use of vehicles of varying concentrations and modes of use. Studies carried out in different countries indicate the use of fluoride-containing dentifrices as the main responsible for both the decrease in caries prevalence and the increase in dental fluorosis[24,29]. Among the strategies to decrease fluoride intake from this source, and therefore minimizing the risk to develop dental fluorosis, the reduction of fluoride concentration in dentifrices used by young children has been proposed[15]. Considering the controversies surrounding the efficacy of low-fluoride dentifrices when compared to conventional formulations[3,27], alternatives to improve their efficacy have been tested *in vitro*, which include low-pH formulations[2] and the supplementation with phosphate salts[25]. In order to further confirm these findings, however, *in situ* tests should be conducted to verify the dentifrice capacity to reduce mineral loss and/or enhance remineralization prior to clinical trials, which are more costly and time demanding.

A variety of *in situ* protocols assessing the remineralizing capacity of fluoride dentifrices, solutions and gels is described in the literature. Most of the studies, however, evaluated products containing high fluoride concentrations[1,16], while others compared low-fluoride products with different remineralizing agents[8,19,22,25] or only

with placebo treatment[4,9]. According to recent recommendations, *in situ* protocol designs must determine dose-response effects in order to attest the efficacy of already existing formulations, as well as to validate new formulations[3]. Nevertheless, when developing and evaluating low-fluoride dentifrices, it is difficult to select a protocol that fulfills the above-mentioned recommendations, as existing protocols vary considerably regarding the length of experimental period (7 to 60 days), substrate (human or bovine enamel), treatment frequency (2 to 4 times/day), and whether the application is *in vivo* or *ex vivo*. Moreover, no protocol has assessed differences between groups regarding variation in fluoride concentration.

Since no *in situ* protocol has assessed the dose-response effects of fluoride dentifrices, especially involving low-fluoride dentifrices, the aim of this study was to evaluate an *in situ* remineralization model in order to determine dose-response effects with dentifrices containing low fluoride concentrations using artificially demineralized bovine enamel.

MATERIAL AND METHODS

Study design

This study followed a double-blind, crossover protocol, assessing the effect of 4 different treatments (dentifrices containing 0, 275, 550 and 1,100 µg F/g, as NaF), tested separately for 3 and 7 days. Bovine enamel blocks (3×3 mm, *n*=330) selected by surface hardness (SH) were artificially demineralized, and subsequently submitted to post demineralization hardness test (SH_1). Ten blocks were also analyzed by polarized light microscopy prior to the *in situ* phases. Sample size was established at 9 volunteers *per* group to obtain a power of 80% (α=0.05), based on a previous study conducted with similar research protocol[11]. Considering the possibility of dropouts, 12 volunteers were included in the study. During each experimental phase, volunteers wore palatal appliances containing 4 demineralized enamel blocks and were randomly assigned to 1 of the 8 possible treatments. They were instructed to brush their teeth and the palatal devices with the dentifrices supplied (3 times/day); a fluoride-free dentifrice was used by the volunteers for 7 days between each experimental phase. Surface (SH_2) and cross-sectional hardness were assessed to calculate the percentage of surface hardness recovery (%SH) and integrated subsurface hardness (ΔKHN), respectively. Fluoride concentration in enamel was also determined. Dentifrices and experimental periods were considered as variation factors, and surface hardness (SH, SH_1 and SH_2), integrated subsurface hardness (ΔKHN) and fluoride concentration in enamel (µg F/mm^3) were considered as the response variables. The project was reviewed and approved by the Research Ethics Committee of Univ. Estadual Paulista (UNESP), Brazil (#2007/02270).

Dentifrice formulation and fluoride assessment

Dentifrices used in the study were produced in the laboratory of Pediatric Dentistry of Araçatuba Dental School (UNESP, Brazil) contained the following components: titanium dioxide, carboxymethylcellulose, methyl-p-hydroxybenzoate, sodium saccharin, peppermint oil, glycerol, hydrated silica, sodium lauryl sulfate, and water. Sodium fluoride (NaF, Merck®, Darmstadt, Germany) was added to the formulations in order to obtain the concentrations of 275, 550 and 1,100 µg F/g. A dentifrice without fluoride (placebo) was also prepared.

Total fluoride (TF) and ionic fluoride (IF) were assessed according to Delbem, et al.[10] (2002), using an F ion-specific electrode (Orion 9609 BN; Orion Research Inc, Beverly, MA, USA) coupled to an ion analyzer (Orion 720 A$^+$; Orion Research Inc, Beverly, MA, USA), previously calibrated with five standard solutions (0.125, 0.25, 0.5, 1.0, and 2.0 µg F/mL).

Volunteer selection

Twelve young adults, aged 20-30 years, living in an area with fluoridated drinking water (0.7 mg F/L) were randomly selected, with no gender distinction. As inclusion criteria, volunteers should present good general and oral health and normal salivary flow rate[11]. Exclusion criteria included the use of any medicine likely to interfere with salivary secretion, use of fixed or removable orthodontic appliances, pregnancy or breastfeeding, smoking, or presence of any systemic illness. Volunteers who showed average mineral gain higher than 20% using placebo dentifrice (3-day experimental regimen) were also excluded (*n*=2).

Enamel blocks preparation and subsurface demineralization

Enamel blocks (3×3 mm, *n*=330) were obtained from bovine incisors, sequentially polished (200 µm) and selected by surface hardness (SH). Artificial subsurface demineralization was produced according to Queiroz, et al.[21] (2008) as modified by Spiguel, et al.[23] (2009). Selected blocks were isolated with acid-resistant varnish, except for the enamel surface (9 mm^2), and immersed individually in demineralizing solution (1.3 mmol.L^{-1} Ca, 0.78 mmol.L^{-1} P in 0.05 mol.L^{-1} acetate buffer [pH 5.0]; 0.03 ppm F; 18 mL/block) for 16 hours, at 37°C. Blocks were then submitted to post demineralization

surface hardness (SH_1).

In situ study protocol and instructions to volunteers

Volunteers were given information on the study protocol and signed a written, informed consent prior to the beginning of the study. Palatal appliances should be used all the time during each experimental phase (including during sleep), and should be removed only during the main meals. The participants were also instructed not to use any fluoride products (except fluoridated drinking water) and/or antibacterial mouthwashes.

Custom-made acrylic palatal appliances were provided to the volunteers[11], which included 4 demineralized enamel blocks (two on each side), fixed on the same level of the appliance in order to minimize plaque accumulation. Each experimental dentifrice was tested separately for 3 and 7 days, totaling 8 experimental phases. Volunteers were instructed to brush their natural teeth and the palatal appliances (including the enamel blocks), three times *per* day, for 1 minute each time, and to rinse their mouth with tap water. One week before the beginning of the experiment and between treatments, volunteers used a fluoride-free dentifrice (wash-out).

Analysis of enamel hardness

Surface and cross-sectional enamel hardness was measured using a Shimadzu HMV-2000 hardness tester (Knoop diamond, 25 g, 10 s, HMV-2000; Shimadzu Corporation, Tokyo, Japan) coupled to CAMS-WIN software (Newage Testing Instruments, Southampton, PA, USA). Five impressions were performed in the central region of each block, 100 µm distant from each other (SH). After artificial enamel demineralization (SH_1) and after the *in situ* experimental remineralization (SH_2), five impressions 100 µm distant from each other and from the initial impressions (SH) were performed[26].

Blocks were then longitudinally sectioned into two halves through the central part of the exposed enamel. One half was embedded in acrylic resin and sequentially polished. For cross-sectional hardness measurements, 3 sequences of 8 impressions were performed at distances 10, 30, 50, 70, 90, 110, 220, and 330 µm from the external surface of enamel; one sequence was performed in the central region of the block and the other two sequences were done on each side (100 µm) of the first sequence. The mean of the three measurements was calculated for each distance. The integrated area under the curve (cross-sectional hardness profiles into the enamel), using hardness values (KHN), was calculated by the trapezoidal rule (GraphPad Prism, version 3.02, San Diego, CA, USA) for each depth (µm) from the lesion to sound enamel. This value was subtracted from the integrated area of sound enamel to obtain the integrated area of subsurface regions in enamel, which was named integrated loss of subsurface hardness (DKHN)[23].

Lesion depth analysis

Sections of 600 µm thick were obtained from demineralized blocks not submitted to the *in situ* protocol (n=10, control) using a diamond disk, and subsequently ground and polished (Phoenix Beta-Vector; Buehler, Lake Bluff, IL, USA) resulting in slices 100 µm thick. Enamel sections were mounted on slides in deionized/distilled water, covered with a coverslip and sealed with ethelan resin. Sections were then examined by polarized light microscopy (Axiophot; Zeiss DSM-940 A, Oberkochen, Germany) at 400x magnification. Three areas in the central region of the slices were analyzed to verify the presence and thickness of the superficial enamel layer and to assess demineralization depth (µm), using Axiovision Software Rel. 4.3. The same procedure was done for each group (3 blocks/group), on the embedded enamel halves used for the cross-sectional hardness analysis.

Determination of fluoride in enamel

Enamel biopsy was performed based on the study by Weatherell, et al.[28] (1985) as modified by Alves, et al.[2] (2007). Enamel blocks (1.5×1.5 mm) were obtained from the halves not embedded for cross-sectional analysis, fixed to a handpiece mandrel, coupled to a microscope attached to a digital micrometer (Pantec, São Paulo, SP, Brazil) so that biopsy depth could be measured. Self-adhesive polishing discs (13 mm in diameter), 400 grit silicon carbide paper (Buehler Ltd, Lake Bluff, IL, USA) were fixed to the bottom of polystyrene crystal tubes (J-10, Injeplast, São Paulo, SP, Brazil), to remove a 50 µm enamel layer. To each tube, 0.5 $mol.L^{-1}$ HCl (0.5 mL) was added, which were constantly shaken for 30 minutes. Following, 0.5 $mol.L^{-1}$ NaOH (0.5 mL) was added[2].

Samples were buffered with TISAB III (Orion Research Inc, Beverly, MA, USA) and fluoride was analyzed using a fluoride ion-selective electrode (9409, Orion Research Inc, Beverly, MA, USA) and a reference microelectrode (Analyser, São Paulo, SP, Brazil), both connected to an ion analyzer (Orion $720A^+$). Five standard solutions (0.045 to 0.720 µg F/mL), containing HCl, NaOH and TISAB III at the same ratios as the samples were used to calibrate the electrode. The results are expressed as $\mu g/mm^3$.

Statistical analysis

Statistical analysis was performed using GMC software version 2002[6], with a significance level of 5%. Experimental dentifrices and regimen periods

(3 and 7 days) were considered as variation factors, and values of SH, SH_1, SH_2, ΔKHN, and enamel fluoride concentration were considered as variables. Data passed normality (Kolmogorov-Smirnov) and homocedasticity (Cochran) tests, and were submitted to 2-way ANOVA, followed by Tukey's test. The relationships between enamel fluoride concentrations and SH_2 and ΔKHN were verified by Pearson's correlation coefficient. Post hoc power analysis was calculated for SH_2 and ΔKHN between groups that did not reach significant differences.

RESULTS

Total and ionic fluoride concentrations (SD, *n*=3) in the experimental dentifrices were 8.2 (0.7) and 5.5 (0.2); 263.9 (3.6) and 280.1 (3.6); 544.3 (8.2) and 567.2 (7.6); 1,129.6 (15.4) and 1,150.4 (6.9) µg F/g, respectively for the placebo, 275, 550, and 1,100 µg F/g dentifrices. Mean (SD) initial surface hardness (SH) for all blocks was 362.5 (4.2) Kg/mm², ranging from 359.7 (3.2) to 366.2 (4.2) Kg/mm² in the experimental groups (p=0.492). Mean (SD) post demineralization surface hardness (SH_1) was 74.8 (13.4) Kg/mm² for all blocks, ranging from 66.0 (12.5) to 82.5 (9.0) Kg/mm² in the experimental groups (p=0.344).

Enamel blocks analyzed by polarized light microscopy presented a superficial enamel layer followed by an area of extreme demineralization, as shown in Figure 1. Mean demineralization depth (SD) was 47.7 (12.6) µm, ranging from 36.5 to 66.5 µm. The superficial enamel layer thickness (SD) was 9.8 (0.9) µm, ranging from 8.3 to 10.8 µm. Figure 1 also shows decreased demineralized areas with increased fluoride concentration in the dentifrices and increased duration of the experimental protocol.

Ten volunteers completed the study; two subjects were excluded from the experiment, as they presented average mineral gain higher than 20% using placebo dentifrice. A dose-response relationship was observed between fluoride concentrations in the dentifrice and surface hardness (SH_2), as shown in Figure 2A. For the 3-day experimental period, all experimental groups showed statistically significant differences among each other. For the 7-day period, no significant differences were observed between groups treated with dentifrices containing 0 and 275 (power=0.93, p=0.164); 275 and 550 (power=0.86, p=0.06); and 550 and 1,100 µg F/g (power=0.88, p=0.413). When both treatment periods were compared, only the 3-day placebo group was statistically different from all other groups (p=0.004). No significant differences were observed among the 7-day placebo group and 3- and 7-day 275 µg F/g groups (p=0.08); or 7-day 275 µg F/g, 3-day 550 µg F/g and 7-day 550 µg F/g groups (p=0.137). Blocks treated with the 1,100 µg F/g dentifrices were not significantly different for the 3- and 7-day periods (p=0.821), which showed statistically similar values to the 7-day 550 µg F/g group (p=0.257, p=0.226, respectively).

An inverse relationship was observed between

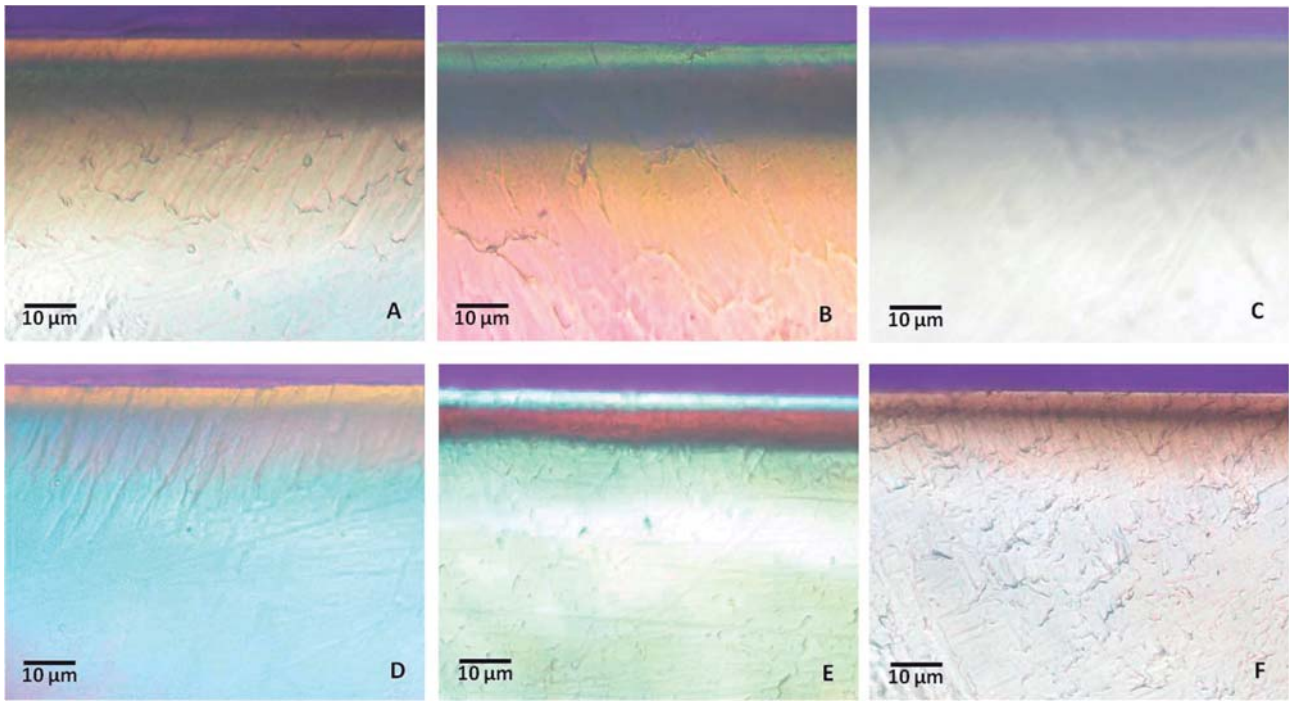

Figure 1- Polarized light photomicrographs of lesions formed after subsurface demineralization prior to the beginning of the experiment (A), and after *in situ* treatment with the following dentifrices and experimental periods: placebo, 3-day (B); placebo, 7-day (C); 275 µg F/g 7-day (D); 500 µg F/g 7-day (E); and 1,100 µg F/g 7-day (F). Images at 400x magnification

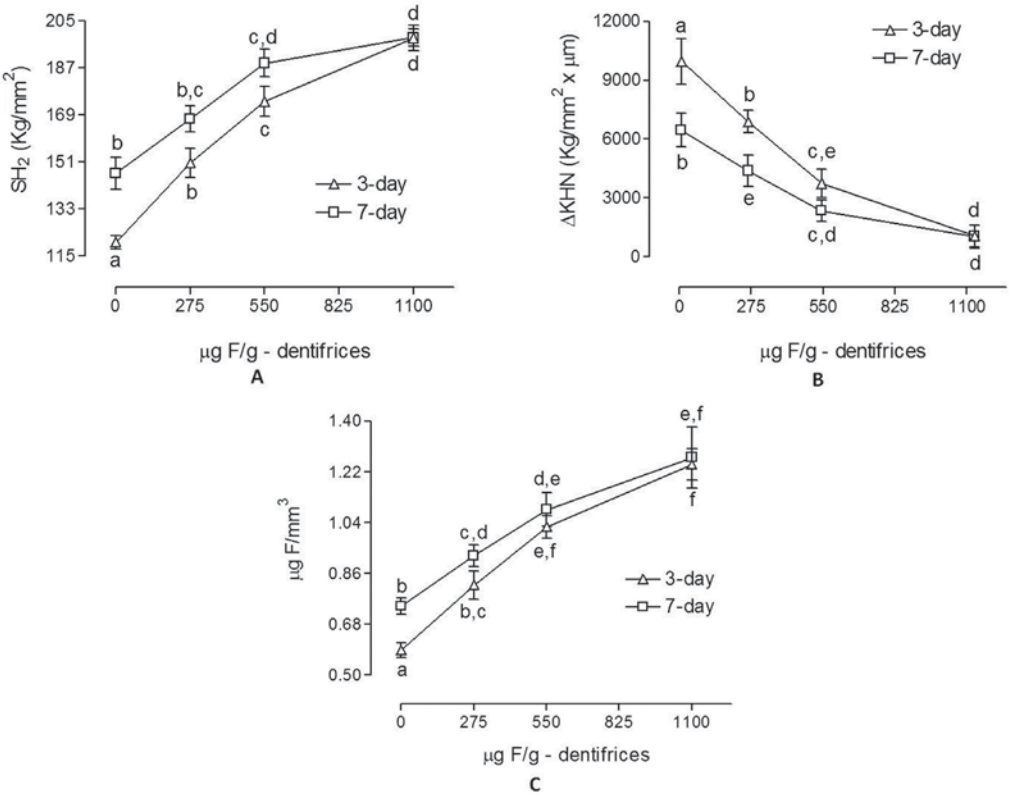

Figure 2- Mean surface hardness (A), integrated subsurface hardness (B) and enamel fluoride concentrations (C) according to fluoride concentration in dentifrices and treatment regimens (3 and 7 days). Values within parentheses in A indicate percentage of surface hardness recovery. Different letters indicate statistically significant differences (2-way ANOVA, followed by Tukey's test, $p<0.05$). Bars indicate standard errors (n=10)

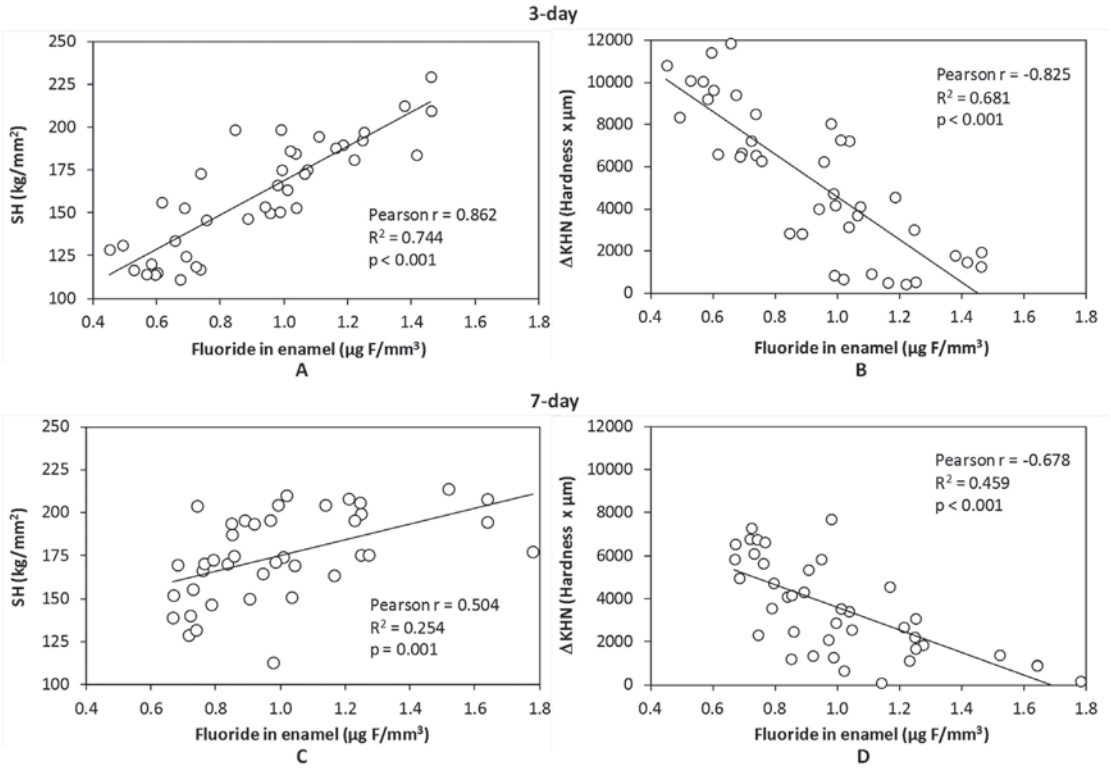

Figure 3- Relationships between enamel fluoride concentration and surface hardness (A and C) and integrated subsurface hardness (B and D), respectively for the 3- and 7-day experimental protocols. Pearson's correlation coefficient ($p<0.05$)

fluoride concentrations in the dentifrices and the integrated subsurface hardness (ΔKHN), as shown in Figure 2B. As for SH_2, significant differences were observed among all groups for the 3-day period ($p<0.001$). For the 7-day period, no significant differences were observed for the 550 and 1,100 µg F/g groups (power=1.00, p=0.181). Comparing both treatment periods, statistically similar values were seen between 7-day placebo and 7-day 275 µg F/g groups (p=0.128), 7-day 275 µg F/g and 3-day 550 µg F/g groups (p=0.593), as well as among 3- and 7-day 1,100 µg F/g and 7-day 550-µg F/g groups (p=0.184, p=0.181, respectively).

A dose-response relationship was verified between fluoride concentrations in the dentifrices and in enamel (Figure 2C). For the 3-day treatment groups, significant differences were observed among all groups (p=0.03), except between groups treated with 550 and 1,100 µg F/g dentifrices (p=0.127). For the 7-day period, no significant differences were observed between the 275 and the 550 µg F/g groups (p=0.132), or between the 550 and the 1,100 µg F/g groups (p=0.613). When comparing both treatment periods, similar results were observed between the 7-day placebo and 3-day 275 µg F/g groups (p=0.430), as well as between 3- and 7-day 275 µg F/g groups (p=0.303). No significant differences were observed among 3- and 7-day groups treated with 550 and 1,100 µg F/g dentifrices (p=0.101).

Enamel fluoride concentration was positive and significantly correlated with SH_2 and ΔKHN (Figure 3). Higher correlation coefficients were observed for the 3-day experimental protocol (A and B) when compared to the 7-day protocol (C and D), for both variables.

DISCUSSION

The supersaturation of saliva with respect to the dental mineral indicates that enamel remineralization naturally occurs when salivary pH is above 5.5, and even traces of fluoride make saliva highly supersaturated with respect to fluorhydroxyapatite[5]. Therefore, *in situ* protocols assessing the remineralizing effects of fluoridated products should allow for the remineralization process to occur by the action of saliva when associated to a placebo treatment, indicating that it primarily occurs through the effects of intraoral calcium and phosphate ions. Some *in situ* models have shown mineral loss after a placebo treatment, thereby hindering observation of the effects of salivary ions in the process[8]. Other protocols have not included a placebo group, making it impossible to verify whether the resulting effects were due to the salivary action or the treatment itself[16]. The present 3-day experimental model, on the other hand, promoted a mineral gain of 18.4% in the placebo group when compared to baseline values. This result was 1.5 times higher (28.2%) when the same dentifrice was used for 7 days, indicating that the protocol fulfilled the above-mentioned requirement.

In the present study, only volunteers with mineral gain lower than 20% after using a placebo dentifrice for a 3-day period were selected. This selection criterion was adopted so that the capacity of fluoride products in enhancing remineralization in difficult situations (by enrolling less responsive subjects) could be evaluated, besides minimizing variability among volunteers. When a remineralizing agent is used in the clinical situation, it is expected that its action occurs within the shortest length of time possible. However, as *in vivo* demineralization and remineralization processes depend on multiple factors[5], the accurate determination of the length of time for *in situ* protocols becomes a difficult task. In this sense, some important variables should be considered before determining the experimental period of an *in situ* study. Among these factors, the type of substrate and the depth of the artificial caries lesion seem to be the most important.

Bovine enamel has a higher reactivity and porosity, which leads to faster remineralization when compared to human enamel[12,13,18]. It has been shown that human enamel remineralization *in situ* was accomplished in nine days, exhibiting a higher remineralization rate on the first days[14,16]. In the present study, the demineralized areas associated to the use of the 1,100 µg F/g dentifrice were 9.4 and 3.5 times lower than those verified after using the placebo and the 550 µg F/g dentifrices, respectively, in the 3-day experimental model. For the 7-day experimental period, an increase in mineral gain around 30% was observed for the placebo group, whereas no changes were verified after using the 1,100 µg F/g dentifrice, in comparison to groups treated with the same dentifrices for 3 days. Moreover, the remineralization results (SH_2 and ΔKHN) were directly related to enamel fluoride concentrations, but longer exposure to fluoride-containing dentifrice did not increase fluoride concentration in enamel. Also, the correlation coefficients observed between enamel fluoride concentrations and SH_2 and ΔKHN were higher for the 3-day experimental protocol when compared to 7-day one. Taking the considerations above into account, it becomes clear that longer experimental designs may reduce the model capacity to verify fluoride dose-response effects. This is particularly critical in study protocols using 14- to 60-day experimental periods[1,4,7-9,16,19,22,25].

As for the substrate, the depth of enamel demineralization may also interfere with remineralization time; however, few studies have

considered the depth of the demineralized area in their protocols, which ranged from 30.0 to 77.3 μm[4,16]. The mean depth of artificial demineralization in the present study was 47.7 μm, resembling a subsurface lesion (Figure 1A). It is known that the remineralization process is slower in deep lesions (±100 μm) due to a longer distance for ion diffusion when compared to that seen in the present study[20]. The present results indicate that the peak remineralization capacity of the 1,100 μg F/g dentifrice was achieved in only 3 days, with no significant difference between the results obtained 7 days after using the same dentifrice. Considering that the placebo, 275 and 550 μg F/g dentifrices tested for 7 days achieved SH_2 and ΔKHN values similar to those found for the 275, 550 and 1,100 μg F/g dentifrice, respectively, it can be assumed that all treatment groups could achieve the same values as those obtained for the 1,100 μg F/g dentifrice had the experimental protocol been longer. This assumption is also supported by the analysis of the polarized light microscopy images, which show a clear reduction in the demineralized areas influenced by both the fluoride concentration in dentifrices and the time of the experimental protocol. Therefore, according to the present protocol, longer experimental periods would not allow for the assessment of the actual effects of the fluoride concentrations in the dentifrices.

Another factor allowing for fast remineralization in the 3-day protocol, after using the 1,100 μg F/g dentifrice was the type of lesion. Figure 1A shows a demineralized area under a more mineralized surface zone (red) than the lesion body (deeper zone); the surface zone, however, is extremely less mineralized than sound enamel (yellow). This type of lesion presents a faster remineralization rate due to the higher number and diameter of the lesion pores, being suitable for comparing the efficacy of different remineralizing regimens[17].

CONCLUSIONS

In summary, it can be concluded that the present *in situ* remineralization model was able to verify dose-response effects with both 3- and 7-day experimental periods. However, as the 3-day protocol resulted in higher correlation coefficients between fluoride concentration in the dentifrice and SH_2 and ΔKHN, and considering that the peak remineralization capacity of the conventional dentifrice formulation (1,100 μg F/g) was achieved in 3 days, this experimental period could be used in future studies assessing the effects of new dentifrice formulations, especially at low-fluoride concentrations. It is important to highlight that in addition to the ability to determine dose-response effects, this short-term experimental design may lead to better volunteer compliance, which would result in more reliable and standardized results.

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