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ABSTRACT

Objectives: This randomized, cross-over in situ study investigated the impact of sodium fluoride dose and concentration in milk on caries lesion rehardening, fluoridation and acid resistance.

Methods: Twenty-eight subjects wore two gauze-covered enamel specimens with preformed lesions placed buccally on their mandibular partial dentures for three weeks. Participants used fluoride-free dentifrice throughout the study and consumed once daily one of the five study treatments: no fluoride in 200ml milk (0F-200), 1.5 or 3mg fluoride in either 100 (1.5F-100; 3F-100) or 200ml milk (1.5F-200; 3F-200). After three weeks, specimens were retrieved. Knoop hardness was used to determine rehardening and resistance to a secondary acid challenge. Enamel fluoride uptake (EFU) was determined using a microbiopsy technique.

Results: A linear fluoride dose-response was observed for all study variables which exhibited similar overall patterns. All treatments resulted in rehardening, with 0F-200 inducing the least and 3F-100 the most. Apart from 1.5F-200, all treatments resulted in statistically significantly more rehardening compared to 0F-200. The fluoride doses delivered in 100ml provided directionally although not statistically significantly more rehardening than those delivered in 200ml milk. EFU data exhibited better differentiation between treatments: all fluoridated milk treatments delivered more fluoride to lesions than 0F-200; fluoride in 100ml demonstrated statistically significantly higher EFU than fluoride in 200ml milk. Findings for acid resistance were also more discerning than rehardening data.

Conclusions: The present study has provided further evidence for the anti-caries benefits of fluoridated milk. Both fluoride dose and concentration appear to impact the cariostatic properties of fluoride in milk.

1. Introduction

The delivery of cariostatic amounts of fluoride through milk is not novel by any means as the first caries studies in children were conducted in the 1960s.[1] Despite its prolific history, a Cochrane Systematic Review[2] concluded 'that there is insufficient evidence to show the effectiveness of fluoridated milk in preventing tooth decay' and highlighted the need for further randomized clinical trials. Yet, milk fluoridation has been shown to be an effective public health measure and more recent studies have provided further supporting evidence.[1,3] Milk fluoridation has been recommended where fluoride concentration in the drinking water is suboptimal, for target groups with high caries prevalence and poor compliance for oral hygiene, in areas without or suboptimal water fluoridation and where school- or kindergarten-based programs to provide milk to children are already in place.[3,4]

Recent research has focused on determining if fluoride in milk follows a dose-response pattern and if an optimum fluoride concentration exists. However, results of laboratory[5-7] and in situ studies[8,9] into the anti-caries effects of different milk fluoride concentrations have been somewhat equivocal – benefits of fluoridated vs. non-fluoridated milk have been reported unanimously in vitro and in situ; although a clear fluoride dose-response relationship has yet to be established in situ. Likewise, there appears to be some disagreement with regards to an optimum fluoride concentration in milk as results obtained using a range of laboratory models led authors to different conclusions.[5-7]

Furthermore, the cariostatic properties of fluoride do not only depend on dose but also on concentration.[10] While drawing parallels to conventional fluoride delivery vehicles, such as dentifrices and rinses, is not straightforward, especially considering their much higher fluoride concentrations in comparison to milk, a study on rinses demonstrated that, for a given fluoride dose, the main driving force for efficacy was fluoride concentration; i.e. rinsing with a smaller volume but higher fluoride concentration was more beneficial than rinsing with a larger volume but lower fluoride concentration.

Therefore, the aims of the present in situ study were three-fold: a) the primary objective was to determine if a higher dose of fluoride in milk would provide a greater caries preventive effect as determined by measuring early caries lesion rehardening, fluoridation and acid resistance; and b) the secondary objectives were to determine if a higher concentration of fluoride in milk would

provide a greater caries preventive effect and if the caries preventive effect of fluoridated milk follows a dose-response pattern.

2. Materials and Methods

2.1. Ethical Aspects

The study protocol was reviewed and approved by the IUPUI Institutional Review Board, #1206008830. It was conducted at the Oral Health Research Institute of the Indiana University School of Dentistry. All subjects signed a written informed consent prior to screening and received oral soft and hard tissue examinations throughout the study.

2.2. Experimental Design

The study was randomized, investigator-blind, observer-blind, laboratory analyst-blind, and utilized a 5-way cross-over design. Subjects were partially blind to the treatments (labeled A to E) as they consumed two different milk volumes throughout the study. Two to three days following a dental cleaning, two partially demineralized specimens were placed in the buccal flange area of the subject's mandibular partial denture. Specimens were individually wrapped in Dacron gauze to facilitate plaque growth. Wrapped specimens were mounted in close proximity to each other and flush with the denture surface. Subjects were instructed on the milk preparation (see below), consumed the first treatment under supervision at the study site and received a diary for home use which they returned at the end of each treatment period. During each of the five, three-week test periods, subjects drank their assigned milk test product after dinner (in the evening), once per day for either five (100 ml dose) or ten (200 ml dose) timed minutes, wearing their mandibular partial dentures 24 hours a day during the test period including during meals. Dinnertime was chosen to avoid interfering with the subjects' lifestyles and to maximize the cariostatic benefits of fluoride.[11] Subjects used fluoride-free dentifrice (Natural Tea Tree Oil Toothpaste, Desert Essence, NY, USA) two to three days before and continuously during each treatment period. This choice was made to mimic high-risk populations, although subjects were exposed to fluoridated water (approx. 1.0 ppm) during the study. At the end of each three-week test period, subjects returned to the study site, specimens were removed and analyzed. All subjects received a professional fluoride treatment (APF Gel, PediaGel, Preventech, NC, USA) at the end of the study. The main response variables were percent surface microhardness

recovery (%SMHr), enamel fluoride uptake (EFU), and percent acid resistance (%AR) measured on the enamel experimental specimens. Each subject served as his or her own control.

2.3. Power Calculation

Based on prior studies using a variety of conventional oral care products in this model [unpublished data], the within-product standard deviation of %SMHr is estimated to be 13% and the correlation between products is expected to be approximately 0.5. With a sample size of 28 subjects in a 5-way cross-over study, the study had 80% power to detect a %SMHr difference of 8.6%, assuming two-sided tests each conducted at a 5% significance level.

2.4. Enamel Specimens and Lesion Creation

Specimens obtained from human permanent teeth were used as the hard tissue test substrate. The teeth were collected and transported to OHRI in a saturated thymol solution. Upon receipt, the teeth were sorted, cleaned and the root tips removed. The teeth were then stored in saturated thymol solution during sample preparation procedures. Teeth were selected based on the following criteria: free of caries and major restorations; no discoloration and no markings, such as cracks, when viewed under a microscope at 20× magnification; sufficient tooth surface to provide a large size specimen to meet study requirements.

Up to two specimens were obtained from the buccal and/or lingual smooth surface of each tooth. Longitudinal sections approximately 3 mm in thickness were made parallel to the selected tooth surfaces. The tooth sections were then cut into 4 × 4 mm specimens using a Buehler Isomet low-speed saw. Specimens were ground and polished to create planar parallel dentin and enamel surfaces. The dentin side was ground flat using 500 grit silicon carbide paper, followed by grinding and polishing of the enamel side. A small orientation cut was placed on each block (fig. 1). The enamel surface of each specimen was ground using 1200 grit silicon carbide paper followed by 2400 and then 4000 grit silicon carbide paper. The polishing step involved the use of a 1 μm diamond suspension on a polishing cloth. Resulting specimens had a thickness range of 1.7 to 2.2 mm. The enamel surface had a minimum polished surface of 2.5 × 2.5 mm in the center of the enamel surface.

Early caries lesions were created in the enamel specimens using a modification of the method described by White.[12] Sound enamel specimens were each immersed into 40 ml demineralization

solution containing 0.05 M lactic acid, 0.2 % Carbopol 907 (BF Goodrich Co., Cleveland, OH, USA) that was 50% saturated with respect to hydroxyapatite at pH 5.0 for 24 h. Before clinical use, all enamel specimens were sterilized by ethylene oxide gas.

2.5. Study Population

Twenty-eight subjects between the ages of 47 and 80 (mean 63, standard deviation 10; median 64; 11 male; 17 female) undertook the study, with 22 completing all treatments (the anticipation was to finish with 20). Three subjects withdrew from the study due to antibiotic usage, one moved out of town, one could not wear the partial denture 24 h a day, and one withdrew due to an adverse event that was not treatment related. Inclusion criteria were: subjects had to be between the ages of 18 and 80 years at screening and in general good health, exhibit no evidence of active caries or periodontal disease, have stimulated and unstimulated salivary flow rates equal or greater than the minimum requirement of 0.8 and 0.2 ml/min, respectively. Potential subjects reporting they were pregnant, intending to become pregnant during the study period or lactating were excluded from participation. They also had to be able to tolerate the taste and room temperature of the mixed milk product as demonstrated by drinking it at screening. Subjects had to stop using topical or systemic fluoride products for the duration of the study.

2.6. Study Treatments – Milk Preparation and Usage

The five study treatments are shown in Table 1. Excluding the fluoride-free placebo, the study followed a 2 × 2 factorial design with the factors fluoride dose (1.5 or 3 mg) and milk volume (100 or 200 ml). The investigational drug product (fluoridated milk) was prepared by a licensed pharmacy in Indianapolis, IN (USA) using powdered milk (Nonfat Dry Milk, Kroger, Indianapolis, IN, US) and pharmaceutical grade sodium fluoride (PCCA Sodium Fluoride USP 51927-1038-00). Milk powder and sodium fluoride powder were homogenized and weighed into 100 or 200 ml bottles which contained a fill line indicating either a 100 or 200 ml volume once reconstituted using bottled water (Ice Mountain, Nestle, CT, USA). Subjects received 23 of the 24 bottles prepared for each subject (i.e. one bottle per day plus two extra bottles, one bottle was retained for analysis – see 2.7.) of their assigned study treatment per test period. They were instructed to fill the bottle to the line with the provided bottled water (to be stored at room temperature) immediately before use and shake the bottle for a minimum of 30 s to allow the

milk powder and sodium fluoride to dissolve (determined to be sufficient prior to study start). Subjects were instructed to sip their assigned study treatment after dinner during either a five- (100 ml dose) or ten-minute period (200 ml dose) by gently moving every sip around their mouth before swallowing. Timers and product use diaries were provided to subjects. Subjects had to refrain from eating and drinking for 30 min after milk consumption.

2.7. Milk Analysis

Prior to study start and throughout the study, analyses of the reconstituted powdered milk were conducted at the present authors' laboratories by a third party who was not otherwise involved in the study, to verify fluoride content of the milk and to retain blindness. One bottle per subject per treatment period was randomly chosen from each subject's allotment and processed for fluoride analysis as described below (prior to dispensing to subjects). In addition, extra bottles returned by the subjects after treatment period 1 were also analyzed to confirm fluoride stability.

For treatment period 1, milk samples were analyzed for total fluoride using a modification of the micro diffusion method of Taves[13] as modified by Martinez Mier et al.[14] One ml of each reconstituted milk sample was dispensed into Petri dishes (60 × 15 mm disposable Petri dishes, Fisher Scientific, PA, USA) along with 2 ml of deionized water; a sodium hydroxide (Thermo Fisher Scientific, PA, USA) trap solution was loaded onto the Petri dish lid and, after adding sulfuric acid (Thermo Fisher Scientific, PA, USA) saturated with hexamethyldisiloxane (Sigma-Aldrich, MO, USA), each dish was immediately tightly sealed. As the diffusion process occurred overnight, fluoride was released by acid hydrolysis and captured in the NaOH trap. The fluoride-containing trap was then removed and buffered to pH 5.2 with 0.1 M acetic acid (Thermo Fisher Scientific, PA, USA). The resulting solution was adjusted to a final volume of 100 µl with deionized water. Fluoride levels of each sample were determined by comparing the millivolt reading of each sample to standard curves prepared from a 0.1 M sodium fluoride solution (Thermo Fisher Scientific, PA, USA), covering the range of the samples' values and prepared from the data for standard solutions of diffused fluoride determined at the time the samples were analyzed. For comparison, selected samples were also analyzed directly under the electrode (see below).

For treatment periods 2-5, milk samples were analyzed directly as this method was found to be considerably faster and more reliable for this type of matrix. One ml of each reconstituted milk

sample was mixed with 1 ml of total ionic strength adjustment buffer (TISAB II, Thermo Fisher Scientific, PA, USA). Fluoride levels of each sample were determined by comparing the millivolt reading of each sample to standard curves prepared from a 0.1 M sodium fluoride solution (Thermo Fisher Scientific, PA, USA), covering the range of the samples' values and prepared in a similar manner compared to the milk samples.

For both analyses, a fluoride ion-selective electrode (Thermo Fisher Scientific, PA, USA) and a pH/ion meter (Thermo Fisher Scientific, PA, USA) were used.

2.8. Surface Microhardness Recovery

The surface microhardness (SMH) test was used to assess changes in the mineral status of partially demineralized enamel specimens. SMH was measured using a designated microhardness tester (2100 HT; Wilson Instruments, Norwood, MA, USA). Each enamel specimen was secured on a one-inch square acrylic block with sticky wax and then placed on the microhardness tester. Five baseline indentations spaced 100 µm apart were placed with a Knoop diamond under a 50 g load in the center of a flattened, polished sound enamel specimen (fig. 1). SMH was determined by measuring the length of the indentations using Clemex CMT HD version 6.0.011 image analysis software. For enamel specimens to be acceptable for use in the study, the mean of the 5 baseline indentation lengths had to be 43 ± 3 µm with a standard deviation of < 3. After in vitro demineralization, the enamel specimens were again SMH tested by placing five indentations 100 µm to the left of the baseline indentations. To qualify for the study, the mean (n = 5) indentation lengths of the partially demineralized specimens had to be 120 ± 20 µm with a standard deviation of < 10. After 21 days of intra-oral exposure the enamel specimens were again SMH-tested by placing five indentations 100 µm to the right of the baseline indentations. The extent of rehardening (%SMHr) was calculated based on the method of Gelhard et al.:[15]

$$\%\mathrm{SMHr} = \frac{\mathrm{D1} - \mathrm{R}}{\mathrm{D1} - \mathrm{B}} \times 100$$

B = indentation length (µm) of sound enamel specimen at baseline

D1 = indentation length (µm) after first in vitro demineralization

R = indentation length (µm) after intra-oral exposure (rehardening).

2.9. Enamel Fluoride Uptake

Microdrill analysis of the enamel specimens was carried out after 21 days of intra-oral exposure for each of the treatments, after the SMH procedure had been completed. The microdrill enamel biopsy technique as described by Sakkab et al.[16] was used to analyze the fluoride content of the partially demineralized enamel specimens. Each enamel specimen was mounted perpendicular to the long axis of a drill bit attached to a specially designed microdrill, and drilled to a depth of approx. 100 μm through the entire lesion (four cores per specimen, fig. 1). Drilling and sample collection were performed in a static-controlled atmosphere to prevent loss of enamel powder due to charging effects. The enamel powder from the drill hole was collected, dissolved (20 μl of HClO4 + 40 μl citrate/EDTA buffer + 40 μl deionized water) and analyzed for fluoride by comparison to a similarly prepared standard curve using an ion-specific electrode. The diameter of the drill hole was determined using a calibrated microscope interfaced with an image analysis system. The amount of fluoride uptake by enamel was calculated based on the amount of fluoride divided by the area of the enamel cores and expressed as μg F/cm^2.

2.10. Acid Resistance Test

To test whether the treatments imparted acid resistance to the enamel after the 21-day intra-oral exposure, a second in vitro demineralization was conducted. Specimens were demineralized by immersing them each in 40 ml of the lesion creation solution at 37° C for 5 h. SMH was then evaluated by placing 5 indentations 100 μm to the left of the indentations placed after the first demineralization challenge. The % acid resistance (%AR) was calculated by applying the method of Corpron et al.:[17]

$$\%\ \text{Acid Resistance} = \frac{\text{D1} - \text{D2}}{\text{D1} - \text{B}} \times 100$$

B = indentation length (μm) of sound enamel specimen at baseline

D1 = indentation length (μm) after first in vitro demineralization

D2 = indentation length (μm) after second in vitro demineralization

Acid resistance is indicative of any protection that the treatments and intra-oral exposure may afford the enamel specimens. The net loss of enamel due to clinical demineralization is the result of multiple cycles of demineralization and repair (rehardening). It is well established that repaired enamel is more resistant to subsequent acid challenges.

2.11. Statistical Analysis

For %SMHr, EFU and %AR the two specimens for each subject and study period were averaged. The effect treatment on %SMHr, EFU and %AR was performed using an ANOVA model suitable for a cross-over study. The ANOVA included terms for treatment and study period, and a random effect to account for the within-subject correlations among the five study periods. Additional contrasts were tested to compare the two 100ml milk groups combined against the two 200ml milk groups with fluoride combined, to compare the two 1.5 mg F groups combined against the two 3 mg F groups combined, and to compare the placebo F group against the two 1.5 mg F groups combined and against the two 3 mg F groups combined. The milk group comparisons and the fluoride group comparisons are similar to a 'main effects' test for the milk and fluoride levels of a 2 × 2 ANOVA, which needed to be performed as contrasts in this 5-group cross-over study design. All comparisons employed two-sided tests at a 5% significance level.

3. Results

Results of the milk analyses are shown in Table 2. All but four of the analyzed samples were within 10% of the target fluoride concentration; the four samples were within 16%. The fluoride content of the bottled water was below the limit of detection (< 0.01 ppm).

All data for one subject were removed prior to statistical analysis due to unusually high and unrepresentative SMH data, likely due to calcified plaque buildup requiring the specimens to be cleaned with a scalpel. Subjects who did not complete all the treatments were not omitted from the per protocol analysis: data from unaffected periods were included. No missing data were replaced with substituted values for the statistical analysis.

No differences were found between treatment regimens for sound enamel or lesion baseline SMH values. Table 3 presents the least square means, standard errors of the mean and results of the statistical analyses for all variables. For better visualization of the results and fluoride dose-response, figure 2 presents the %SMHr data. Table 4 presents *p*-values for fluoride dose and milk volume comparisons for the study variables.

Apart from 1.5F-200, all treatments resulted in statistically significantly more rehardening compared to 0F-200. Larger doses of fluoride and fluoride delivered at higher concentrations but

at the same dose induced directionally more rehardening, which, however, was not of statistical significance. A post-hoc power analysis revealed that 444 subjects would have been needed to observe a significant difference in %SMHr between 1.5 mg fluoride in 100 vs. 200 ml milk and 514 subjects for 3.0 mg fluoride in 100 vs. 200 ml milk. All fluoridated milk treatments delivered more fluoride to lesions than 0F-200. EFU data were more discerning between all treatments. %AR data were somewhat similar to the %SMHr data but more discriminating – the differences being the numerical difference within 1.5 mg F groups and between all groups were larger, rank order within the 3.0 mg F groups was reversed, there was a clearer fluoride dose-response, and there was a significant difference between 1.5F-200 vs. 3F-200.

4. Discussion

The present study investigated the interplay between fluoride dose and concentration in milk utilizing an established in situ caries model,[18] with the key features being the use of gauze-covered specimens mounted flush with the denture surface to facilitate plaque growth and retention, early, surface-softened caries lesions that mimic the onset of caries, and the simulation of a caries-prone stagnation area. Furthermore, no diet restrictions were imposed on the study subjects, and a mandibular partial denture, containing the specimens, was worn by the subjects 24 h a day including during meals. Therefore, this model can be considered of high clinical relevance.

The subject panel consisted of adults with good oral health and no signs of active caries, periodontal disease or xerostomia. While it would have been advantageous to conduct the present study in the target population for milk fluoridation – children, there are several drawbacks that would have ultimately undermined the study aims. As children are not partial denture wearers, a different in situ model design would have to be chosen, with the most common one the intra-oral (mandibular or palatal) appliance model. The comparative drawbacks of such models are speech impairment and discomfort (leading to poor compliance), removal of the appliance during food and drink consumption (elimination of the only cariogenic challenge), appliance removal during the night (impaired remineralization) and specimen placement in case of a palatal appliance model (atypical site for caries occurrence).

Fluoride doses were chosen bearing in mind toxicological aspects and milk doses considering practicality and clinical relevance. The American Academy of Pediatrics proposed a daily

fluoride dose of between 0.05 and 0.07 mg F/kg body weight/day,[19] which is generally accepted as 'a useful upper limit for fluoride intake by children'.[20] Bearing this upper limit in mind, milk fluoride concentrations as high as 8 to 10 ppm (depending on body weight) could be supported for children older than eight years of age. In adults, the present study population, a tolerable upper intake level of 10 mg fluoride per day was recommended by the US Institute of Medicine (IOM) in 1997,[21] thereby allowing additional room for investigation. Powdered milk was chosen for several reasons: it has a considerably longer shelf life than liquid milk, fluoride is more stable in powdered than in liquid milk (table 2),[22] and it has been utilized in a milk fluoridation program in Chile.[23]

The present study has shown that the anti-cariogenic effect of fluoride in milk follows a dose-response pattern (fig. 2) as greater mean %SMHr values were noted with increasing fluoride concentrations. The EFU data showed better discrimination between treatments and a clearer dose-response. Likewise, the %AR data were more discerning than the %SMHr with regards to a fluoride dose-response, highlighting acquired acid resistance of the lesions as a direct result of fluoridated milk exposure. It must be noted though that overall differences between treatments in %SMHr were relatively small (see Zero et al.[18] for comparison) and that only one of the fluoride dose-comparisons reached, whereas the other two approached statistical significance (table 4). In hindsight, it would have been advantageous to increase the power to observe better discrimination between treatments. The power calculation was based on previous studies conducted on conventional topical fluoride products and it appears that the effect of fluoridated milk, at least in the present model, is somewhat muted in comparison to fluoride rinses and dentifrices.

Studying the cariostatic effects of fluoridated milk in situ is not as straightforward as it would seem and cannot be compared to the study of conventional topical fluoride products. Fluoridated milk is often consumed only during school days; i.e. not on weekends or during school holidays, children consume it with food (e.g. bread) or drink it as part of their meal (typically lunch) which increases intra-oral fluoride clearance, fluoride toxicity limits the number of fluoride moments per day as fluoridated milk is swallowed, and the typical fluoride concentration in milk (highest [F] ever employed was 10 ppm[24]) is only a fraction in comparison to over-the-counter fluoride rinses (90-500 ppm) for example. While certain aspects can be mimicked, to fully replicate a milk fluoridation program using an in situ model is challenging, and several compromises were

made presently, such as the consumption time (evening) and duration (5-10 min), to obtain a better mechanistic understanding of fluoride concentration and dose interactions.

The present results are somewhat in agreement with the findings of two previous in situ studies investigating the effects of two fluoride doses and concentrations lower than those studied presently, 0.5 and 1.0 mg or 2.5 and 5.0 ppm, on the prevention of enamel demineralization[9] and enhancement of lesion rehardening.[8] Both fluoride concentrations offered protection against demineralization with the higher dose being directionally more efficacious, whereas there was no difference between fluoride concentrations on the enhancement of rehardening. Models and study designs were inherently different which makes drawing comparisons difficult. Nonetheless, the results of these and the present in situ study have shown that in situ models can be used for the study of fluoridated milk, that it has cariostatic properties and that it follows a fluoride dose-response pattern. The overall results, and especially of the present study, are in agreement with our current knowledge on the anti-cariogenic action of fluoride – its efficacy is dependent on its bioavailability and concentration. It is still debatable though if the cariostatic effects of milk are primarily topical or systemic. The present study, perhaps unintentionally, provided some evidence that fluoridated milk acts mainly topical as the same dose of fluoride delivered at a higher concentration (i.e. at a smaller volume) provided greater EFU (tables 3 and 4), although only directional differences were observed in %SMHr and %AR. A separate study would have to be designed to provide more conclusive evidence by for example providing the same dose of fluoride in an either soluble (e.g. NaF) or insoluble, but digestible form (e.g. CaF_2). Fluoride dose has been shown to be of secondary importance in relation to concentration.[10] The present study was able to demonstrate at least a directional benefit for the same fluoride dose delivered at a higher concentration for caries lesion rehardening (fig. 2). Perhaps differences in fluoride concentration were too low numerically (15 vs. 7.5 ppm and 30 vs. 15 ppm) to observe statistically significant differences in %SMHr in the present model. Furthermore, it should be noted that the study by Duckworth and Stewart[10] investigated salivary fluoride retention which is a surrogate measure for anti-caries efficacy (as is EFU admittedly). Their findings have not yet been supported by clinical or in situ studies investigating the effects of caries lesion surface softening and rehardening, clearly highlighting the need for further research.

The results of the present study need to be seen in the context of milk fluoridation programs and to what extent the present findings can be translated to improve the benefit/risk ratio of

fluoridated milk. While further research into the fluoride dose and concentration interplay is needed to provide more concrete recommendations, it is obvious that a higher dose of fluoride may lead to a greater cariostatic benefit. Likewise, delivering the same dose of fluoride at a lower volume and thus a higher concentration could potentially improve the benefit/risk ratio. Duckworth and Stewart[10] studied volumes as low as 1 ml. While such low volumes can be justified scientifically, they are, however, of little practical value to milk fluoridation programs as the daily milk dose is almost a given constant. Dividing the milk dose into fluoride and non-fluoride 'deliveries' may be cumbersome but could be one route to improve the overall effectiveness and benefit/risk ratio of fluoridated milk. Researchers should therefore undertake every effort to improve fluoride delivery from milk as the present study has shown that there are still many unknowns.

In summary, the present in situ study has provided further evidence for the anti-caries benefits of fluoridated milk. Fluoride dose-responses on enamel caries lesion rehardening, fluoridation and acid resistance have been demonstrated. Higher fluoride concentrations of the same fluoride dose were shown to lead to directionally greater anti-cariogenic benefits.

Declaration of interests

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Table 1 – Study treatments.

Treatment code	Fluoride dose [mg]	Milk volume [ml]	Fluoride concentration [ppm]	Milk consumption time [min]
0F-200	0	200	0	10
1.5F-100	1.5	100	15	5
1.5F-200	1.5	200	7.5	10
3F-100	3	100	30	5
3F-200	3	200	15	10

Table 2 – Fluoride concentrations in reconstituted milk samples for all treatments by treatment periods.

	Treatment period					
Target	1	1 (returned samples)	2	3	4	5
0	< 0.01 (all)	< 0.01 (all)	< 0.01 (all)	< 0.01 (all)	< 0.01 (all)	< 0.01 (all)
7.5	6.5 – 8.2	6.2 – 7.7	6.8 – 7.8	6.9 – 6.9	7.1 – 8.2	6.8 – 7.1
15 (100 ml)	15.8 – 16.6	13.4 – 16.0	13.7 – 14.4	13.9 – 14.7	14.5 – 14.8	14.0 – 15.0
15 (200 ml)	14.6 – 17.1	13.4 – 15.1	13.8 – 14.6	13.6 – 14.8	13.7 – 15.0	13.7 – 14.4
30	31.1 – 32.3	25.9 – 31.2	28.4 – 29.6	28.2 – 30.5	27.7 – 30.0	27.2 – 27.6

All results are ppm values. Results for individual treatment periods are min/max ranges.

Table 3 – Least square means, standard error of the least square means and results of the statistical analyses for all study variables.

Treatment	n	%SMHr		EFU [μg F/cm^2]		%AR	
0F-200	22	20.0±2.0	C*	1.20±0.18	D	-3.4±2.0	C
1.5F-100	23	24.0±2.0	AB	2.40±0.17	BC	2.6±2.0	AB
1.5F-200	25	22.5±1.9	BC	2.12±0.17	C	-0.2±1.9	BC
3F-100	22	26.7±2.0	A	3.09±0.18	A	5.4±2.0	A
3F-200	22	25.4±2.0	AB	2.67±0.18	B	6.0±2.0	A

* Statistically significant differences between treatments within variable are highlighted by different letters.

Table 4 – *p*-values for fluoride dose and fluoridated milk volume comparisons for all study variables.

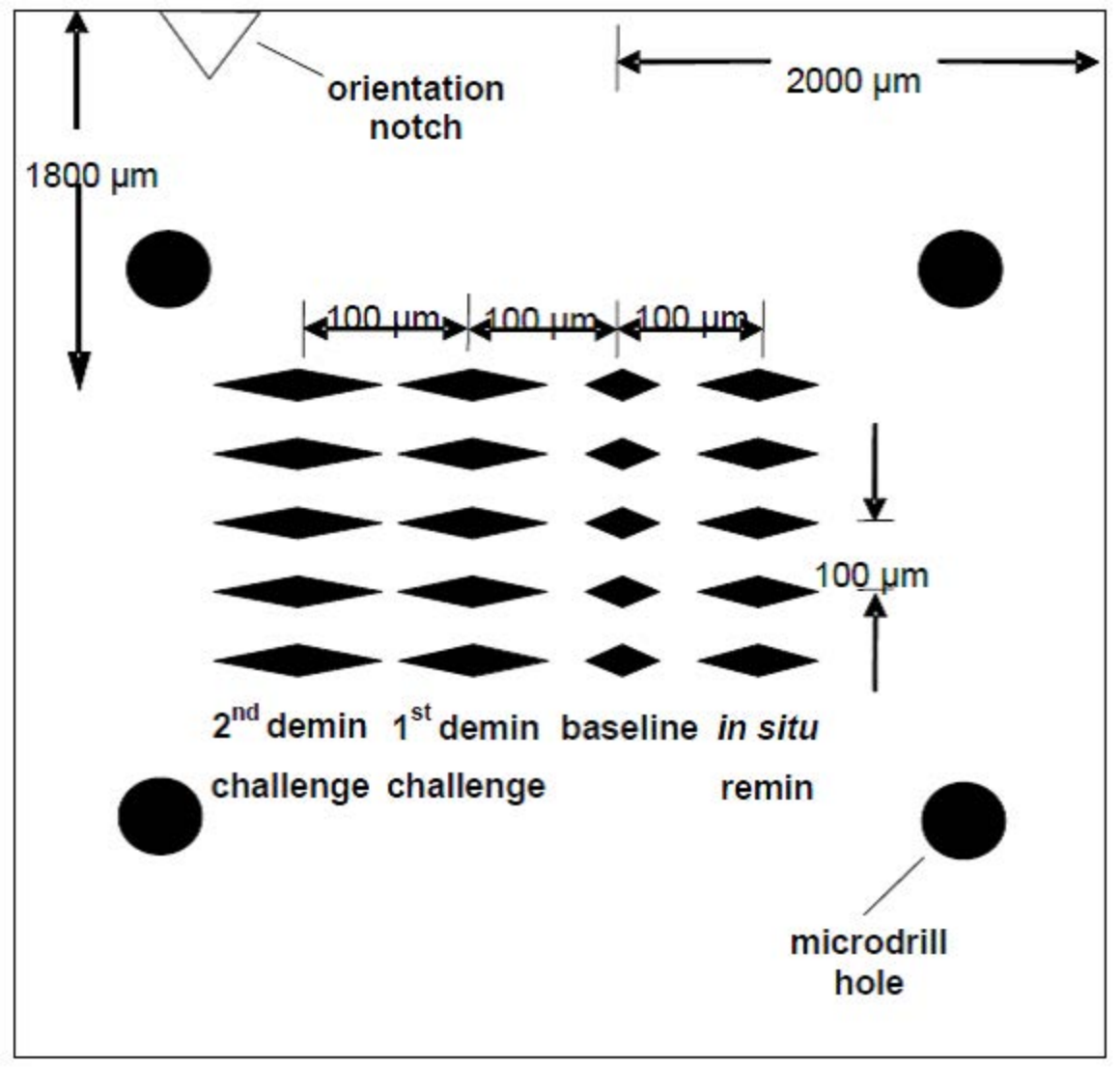
Treatment comparisons	%SMHr	EFU	%AR
0F-200 vs. 1.5 mg F[1]	0.0615	**<0.0001**	**0.0296**
0F-200 vs. 3.0 mg F[1]	**0.0010**	**<0.0001**	**0.0001**
1.5 mg F vs. 3.0 mg F	0.0580	**0.0009**	**0.0149**
100 ml[2] vs. 200 ml[2]	0.34	**0.0150**	0.53

[1] combined data for the same fluoride dose

[2] combined data for the same fluoridated milk dose

Fig. 1 – Enamel specimen with Knoop indentations, orientation notch and microdrill holes.

Fig. 2 – %SMHr as a function of fluoride and milk dose with linear regression for the 200 ml milk dose. Error bars denote standard error of the mean.


orientation notch
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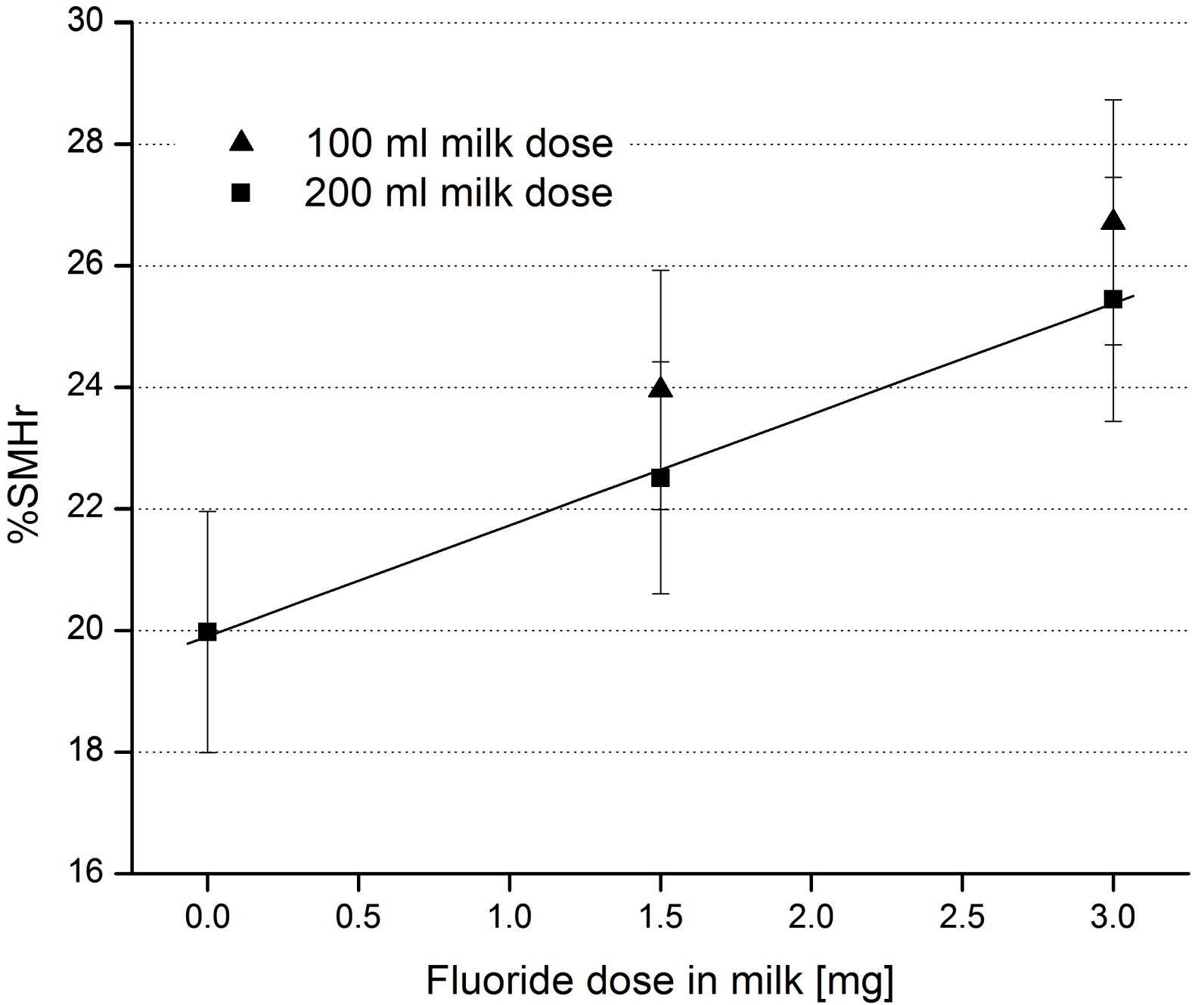
1800 µm
100 µm
100 µm
100 µm
100 µm
2nd demin challenge
1st demin challenge
baseline
in situ remin
microdrill hole


30
28
26
24
22
20
18
16
%SMHr
100 ml milk dose
200 ml milk dose
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Fluoride dose in milk [mg]