Effect of xylitol varnishes on enamel remineralization of immature teeth: *in vitro* and *in situ* studies

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Abstract: We evaluated the efficacy of xylitol varnishes on the remineralization of newly erupted permanent and deciduous teeth *in vitro* and *in situ*. Human enamel specimens were randomly allocated to 8 groups (n = 15/group). Artificial caries lesions were produced and enamel alterations were quantified by surface/subsurface hardness and transverse microradiography. The blocks were then treated with the following varnishes: Duraphat™; 20 wt% xylitol (146 μm) varnish; 20 wt% milled xylitol (80 μm) varnish, and placebo varnish, and removed after 6 h of immersion in artificial saliva. *In vitro*: the blocks were subjected to pH-cycles for 8 days. *In situ*: fifteen subjects wore palatal appliances containing four pre-demineralized and treated enamel specimens, for 5 days. Data were statistically analyzed by ANOVA/ Tukey and Kruskal-Wallis/Tukey's test (p < 0.05). The %SHR in both studies was significantly increased by xylitol and Duraphat™ varnishes when compared to placebo. Considering subsurface remineralization, only the xylitol varnishes were able to significantly reduce the enamel lesion. Xylitol varnishes can be promising alternatives to promote enamel remineralization of newly erupted permanent and deciduous teeth.

Keywords: Dental Caries; Xylitol.

Introduction

Dental remineralization is a natural process consisting of minerals from saliva/biofilm being deposited in partially demineralized tooth structures.[1] However, remineralization can be induced or enhanced by numerous therapies. Among the available therapies, fluoride (F)-based treatments have the highest level of supporting evidence.[2] Although there was a reduction of dental caries in the world population promoted by F use along the last century, a small part of the population still has a high prevalence of caries. As an example, more than half of the children in Latin American and Caribbean countries are still affected by the disease in the 21st Century.[3]

Consequently, most new approaches have been designed to enhance the effect of existing F therapies rather than to replace them, but treatments without F might become an alternative for patients not favorable to its use.[4]

Xylitol is a natural sweetener that is as sweet as normal sugar (sucrose) and is used as a sugar substitute. Also, it has other properties that help in the prevention of tooth decay.[5,6] It is suggested that xylitol may alter the mechanism of polysaccharides production that facilitates bacteria adherence to enamel. Thus, it can potentially decrease biofilm formation and acid production.[5,7]

The importance of xylitol in the prevention of dental caries is already established, and it has been used in chewing gums, syrups, lozenges, sprays, mouthwashes, gels, toothpastes, and candies.[6] The main finding of a recent systematic review was that an F toothpaste containing 10% xylitol used for 2.5 to 3 years may reduce caries by 13% when compared to an F-only toothpaste. However, the authors reported a low-quality evidence that the F-xylitol toothpaste may be more effective than the F-only toothpaste for preventing caries in the permanent teeth of children, although there were no associated adverse-effects from such toothpaste.[8]

Another recent systematic review with meta-analysis favored the use of xylitol in comparison with other caries prevention strategies. Xylitol was found to be effective as a self-applied caries prevention agent or as an over-the-counter (OTC) sweetener, but the studies included in the systematic review had an unclear risk of bias.[9] An important reduction in the salivary levels of *Streptococcus mutans* can be achieved with longstanding and frequent exposure to chewing gums containing xylitol.[5,7] Despite the aforementioned systematic reviews showing that xylitol added to the existing F regimes may help to prevent caries,[8,9] there is still uncertainty about the clinical relevance of oral microorganisms reduction.[10]

A promising alternative to vehicles such as chewing gums is the dental varnish. While chewing gums require a high frequency of use per day to reach a satisfactory salivary concentration of xylitol and depend on the patient's discipline, varnishes maintain prolonged contact with the enamel surface and must be applied by a professional. Two recent studies conducted by our group showed that a 20% xylitol varnish combined or not to F was able to promote enamel surface remineralization with no difference to commercial F varnishes. Considering subsurface

remineralization, the combination of xylitol and F led to worse results. One possible explanation is that F may have blocked superficial enamel pores, preventing access to the deeper areas of the lesion.[11,12] This remineralizing effect of xylitol, even in the absence of bacteria as reported in a previous *in vitro* study,[12] has been demonstrated, and its probable mechanism is by facilitating the access of calcium into the pores and deeper layers of the lesion.[6]

Makinen and Soderling[13] have suggested that sorbitol and xylitol at very high concentrations in a saturated calcium sulfate solution form Ca^{2+}-polyol complexes through the formation of cis-cis-triol coordination complexes. Based on these findings, the authors proposed that these polyols may influence calcium bioavailability in saliva and thereby may directly promote remineralization of enamel subsurface lesions. Similarly, in an *in vitro* study, Miake et al.[14] suggested that a solution containing 20% w/w xylitol produced less remineralization in the outer layers of the lesion but greater remineralization in the deeper layers compared with the solution without xylitol. The authors proposed that xylitol could influence the remineralization of deeper layers of demineralized enamel by facilitating Ca^{2+} movement into the lesion.

Clinically, it is well known that recently erupted teeth are at a higher caries risk than mature teeth.[15] The higher caries susceptibility of younger teeth has been confirmed by experiments in which artificial enamel caries lesions were produced in extracted teeth at different post-eruptive ages.[16] Post-eruptive maturation of enamel involves both chemical and physical changes of the outer layers of enamel. Chemically, considerable amounts of F are incorporated into the enamel surfaces, along with other metal ions associated with enamel solubility, such as zinc.[15,16,17] Besides, the demineralization/remineralization dynamics of primary and permanent enamel in acidic media showed significant differences, with primary enamel having a greater susceptibility to de/remineralization.[18,19]

Since the best results obtained in the previous studies were related to the 20% xylitol varnish, and considering these studies were conducted using bovine teeth, this study aimed to analyze the remineralizing

effect of experimental varnishes containing 20% of xylitol with different particle sizes (146 and 80 µm), on the enamel of human deciduous and newly erupted permanent teeth *in vitro* and *in situ*. The null hypothesis was that 20% xylitol varnishes with different particle sizes have no effect on human enamel remineralization compared to the commercial varnish Duraphat™.

Methodology

The study was performed following the guidelines of good clinical practice and conformed to the Declaration of Helsinki. Ethical approval for the study involving permanent/deciduous teeth and the volunteers from the *in situ* arm was granted by the local Ethics Committee (Protocol CAAE: 64748517.2.0000.8084 and 64748917.9.0000.8084).

The company FGM-Dentscare (Joinville, SC, Brazil) manufactured the placebo varnishes. The varnishes contained colophonium, synthetic resin, thickening polymer, essence, and ethanol (as informed by the manufacturer). The xylitol was supplied by Danisco (Xylitab™ 300, Danisco Brasil Ltd., Cotia, Brazil). Xylitol particles of the "20% milled xylitol" group were subjected to a ball mill for 60 minutes at a frequency of 10 Hz to decrease the decantation of the particles in the varnish.

Preparation of enamel specimens

In vitro arm

One hundred and fifty enamel specimens were obtained from freshly extracted primary teeth and recently erupted third molars that were disinfected by storing in 2% formaldehyde solution (pH 7.0) for 30 days at room temperature. After visual inspection, spotted and/or damaged teeth were excluded. In addition, the attached soft tissues were removed from the coronal and root surfaces using a periodontal curette (Duflex™; S S White, Rio de Janeiro, Brazil). The specimens (3 x 3 x 2.5 mm – primary teeth; 4 x 4 x 2.5 mm – third molars) were obtained from two double sections of the widest portion of the dental crowns, and polished as described by Magalhães et al.[20]

One hundred and twenty (60 from primary teeth and 60 from third molars) enamel specimens were selected based on the baseline surface hardness (mean KHN, 230.53 ± 52.6 – primary teeth; 291.42 ± 62.6 – third molars). One-third of the surface was protected by nail varnish (control area) and specimens were further subjected to the formation of artificial caries lesion by immersion in 30 mL of buffer containing 50 mM lactic acid, 3 mM $CaCl_2.2H_2O$, 3 mM KH_2PO_4, 6 mM tetraethyl methyl diphosphonate and traces of thymol (KOH to adjust the pH to 5.0) for 11 days[21]. After demineralization, the other 1/3 of the surface was protected with nail varnish (demineralized control area).

Treatment and pH-cycling

The enamel specimens were randomly allocated to four different groups (n = 15 primary and 15 third molars/group), according to the type of varnish that would be applied: a) 20% xylitol (pH 5.0, FGM-Dentscare); b) 20% milled xylitol (pH 5.0, FGM-Dentscare); c) Duraphat™ (5% NaF, 2.26% F, pH 5.0; Colgate, São Bernardo, Brazil) d) Placebo, no xylitol or fluoride (pH 5.0, control; FGM-Dentscare).

A thin layer of the varnishes was applied (≈ 0.2 mL of varnish/specimen) onto the enamel using a microbrush, after which samples were immediately immersed in artificial saliva (0.2 mM glucose, 9.9 mM NaCl, 1.5 mM $CaCl_2.2H_2O$, 3 mM NH_4Cl, 17 mM KCl, 2 mM NaSCN, 2.4 mM K_2HPO_4, 3.3 mM urea, 2.4 mM NaH_2PO_4, and traces of ascorbic acid; pH 6.8; 30 mL per sample)[22] at 25°C for 6 h.[20] Then, the varnishes were removed using a surgical blade and cotton swabs soaked in a 50% acetone solution.[23]

The specimens were then subjected to pH-cycling for 8 days, according to the method suggested by Queiroz et al.[24] The blocks were kept for 2 h in the demineralizing solution (0.05 mol/L acetate buffer, pH 5.0; containing 1.28 mmol/L Ca, 0.74 mmol/L P, and 0.03 µg F/mL) and for 22 h in the remineralizing solution (1.5 mmol/L Ca, 0.9 mmol/L P, 150 mmol/L KCl, 0.05 µg F/mL in 0.1 mol/L Tris buffer, pH 7.0) at 37°C. On the fourth day, the de- and remineralizing solutions were replaced by fresh ones. After another 4-day cycle, the enamel remineralization was evaluated.

In situ arm

Fifteen healthy adults (13 women and 2 men, 18–40 years of age) took part in this double-blind study performed in one phase of 5 days. Inclusion criteria were normal stimulated physiological salivary flow rate (> 1 mL/min) and good oral health (i.e. no frank cavities or significant gingivitis/periodontitis). Exclusion criteria were systemic illness, pregnancy or breastfeeding, use of fixed or removable orthodontic appliances, use of fluoride mouth rinse or professional fluoride application in the last 2 months, and hyposalivation. A sample size of 11 subjects was previously calculated considering an a-error of 5% and a b-error of 20% (www.ddsresearch.com) according to the results of a previous *in situ* study.[11] Due to the possibility of a subject dropout, 15 volunteers were recruited. Informed consent was obtained from all subjects before starting the study.

Human enamel blocks from third molars (4x4 mm, n = 60) were selected, cut, polished, demineralized, and randomly allocated to the same 4 groups of varnish described for the *in vitro* study (n = 15/ group). Each specimen was treated with one of the experimental varnishes, then immersed in artificial saliva for 6 h (30 mL per specimen) and after this period the varnishes were removed using a surgical blade and cotton swabs soaked in 50% acetone solution. Two cavities, 5-mm long x 5-mm wide x 4-mm deep, were made on the left and right sides (*i.e.* four cavities in total) of each acrylic palatal appliance and the specimens were fixed with wax in these cavities. All the volunteers used the intraoral appliance with 4 specimens treated with one of the experimental varnishes.

Seven days before and throughout the 5-day phase, the subjects brushed their teeth with a 1500 ppm fluoride toothpaste (Sorriso Fresh, Colgate-Palmolive), in order to standardize the amount of F in the oral reservoirs. During the *in situ* phases, the appliance was only removed when the subject was eating main meals (four times a day, maximum 1 h duration each, with interval between meals of 2–3 h). Immediately after meals, before replacing the appliance into the mouth, the subjects were advised to perform oral hygiene using a soft end-rounded bristle toothbrush (Eco™, Johnson&Johnson) with a small portion of fluoridated dentifrice (Sorriso Fresh, Colgate-Palmolive™) and dental floss (Hillo™ Ind. e Com., Aperibé, Brazil). The subjects were also advised not to eat or to drink while the appliances were in place and not to use antiplaque agents.

The subjects received written instructions including the schedule and were extensively trained for all procedures required during the study.

Assessment of surface/subsurface hardness

The enamel alterations from both study arms were quantified by surface and cross-sectional hardness to calculate the percentage of surface hardness recovery (%SHR) and integrated loss of subsurface hardness (ΔKHN).

Surface hardness (SH) was measured at baseline, after demineralization, and after treatments in the *in vitro* or *in situ* protocols by measuring three indentations at distances of 100 μm from each other (Knoop diamond, 25 g, 10 s, HMV- 2; Shimadzu Corporation, Tokyo, Japan). At the end of the studies, the percentage of SHR was calculated as follows:

$$\%SHR = \frac{(SH\ final - SH\ lesion)}{(SH\ baseline - SH\ lesion)} \times 100$$

To perform the cross-sectional hardness (CSH) tests, the blocks were longitudinally sectioned through the center, embedded, and polished. Two rows of eight indentations each were made — one in the central region of the exposed dental enamel and the other in the control area (1/3 of the surface was protected with nail varnish), using a 25 g load for 10 s. The indentations were made at 10, 30, 50, 70, 90, 110, and 220 μm from the outer enamel surface in two sequences. The mean values of the two measuring points at each 100 μm distance from the surface were then averaged. The integrated area under the curve (cross-sectional hardness profiles into the enamel), using hardness values (KHN), was calculated by the trapezoidal rule for each depth (μm) from the lesion to the sound enamel. This value was subtracted from the integrated area of the sound enamel to obtain the integrated area of subsurface regions in enamel; this was named integrated loss of subsurface hardness (ΔKHN).[25]

Transverse Microradiography (TMR)

The enamel blocks of the *in situ* study arm were sectioned perpendicularly to the central area. One half was analyzed through cross-sectional hardness (CSH) tests and the other half was analyzed using transverse microradiography (TMR). However, due to the difficult technique, only 11 specimens per group could be analyzed. The preparation of the enamel blocks and methodology for acquiring microradiographs for each specimen obtained were performed as previously described by Cardoso et al.[11] The mineral content was calculated from one picture of each enamel specimen (demineralized and remineralized areas), and the step-wedge grey levels were obtained using the formula from Angmar et al.[26] Sound enamel mineral content was assumed to be 87 vol%. The lesion depth (LD) was obtained using a 95% threshold of the mineral content of sound enamel (82.7%). For the comparison between the demineralized and remineralized enamel areas (integrated mineral loss - ΔZ), the differences were calculated as follows: $\Delta\Delta Z = \Delta Z$ initial lesion – ΔZ final lesion; $\Delta LD = LD$ initial lesion – LD final lesion.

A flowchart of the experimental design of the study is shown in Figure 1.
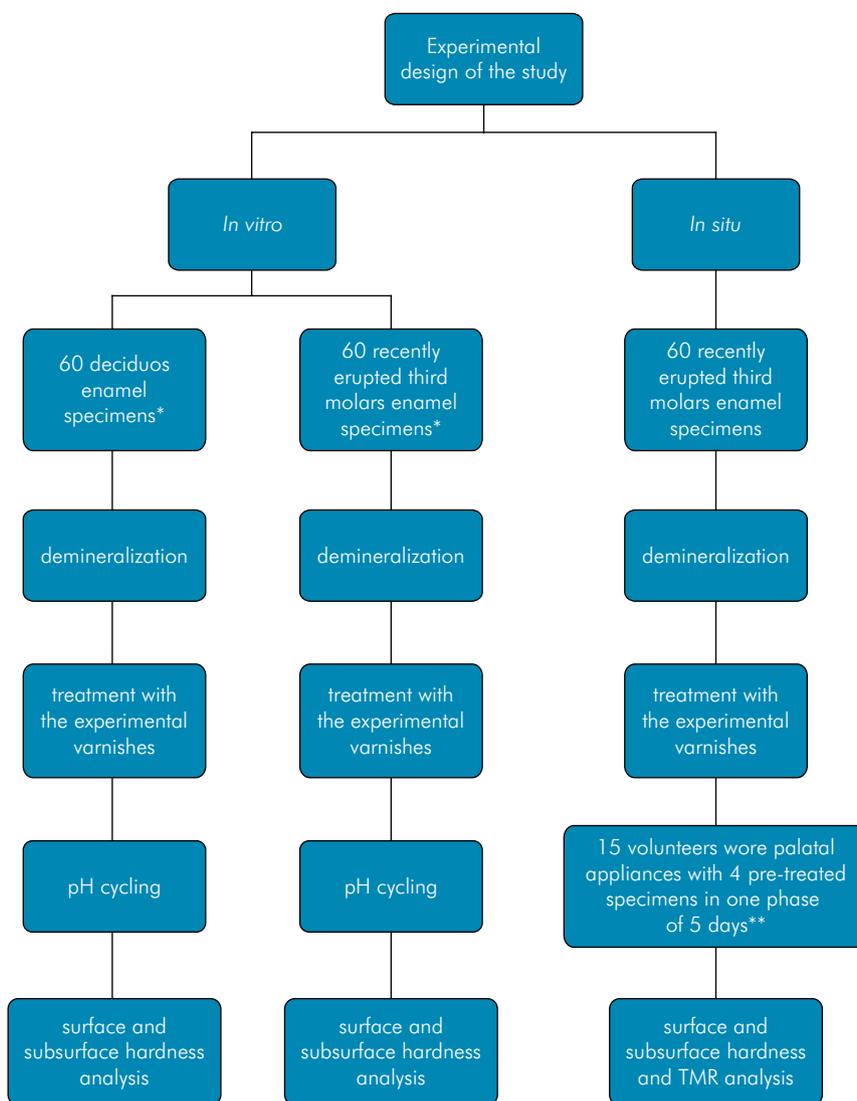
Figure 1. Experimental design of the study.

Statistical analysis

The software SigmaPlot 11.0 (SigmaPlot Inc., La Jolla, CA) was used for statistical analysis. The assumptions of equality of variances and normal distribution of errors were checked for all data. Surface and subsurface hardness data were statistically analyzed by ANOVA/Tukey and Kruskal-Wallis/ Tukey's test with a significance level of 5%.

Results

In vitro arm

Tables 1 and 2 show the mean and standard deviation of initial, post-demineralization, and final SH, %SHR (surface hardness recovery), and integrated mineral loss (ΔKHN) values for primary and permanent teeth. Kruskal-Wallis and Tukey test for individual comparisons showed a significant difference between the treatments regarding the percentage of surface microhardness recovery (%SHR) (p < 0.05). The Duraphat™ varnish and the 20% xylitol and 20% milled xylitol experimental varnishes differed significantly from the placebo varnish, showing their ability to remineralize and prevent enamel demineralization *in vitro,* both for the primary and permanent teeth. There was no significant difference between 20% xylitol, 20% milled xylitol and Duraphat™ varnishes. Regarding the

Table 1. Surface hardness measurements at baseline (SH baseline), after demineralization (surface hardness loss - %SHL), after treatment and pH cycling (SH final), percentage of surface hardness recovery (%SHR) and integrated mineral loss (ΔKHN) for the primary teeth *in vitro* (n = 15).

Varnishes	SH Baseline	SH Lesion	SH Final	%SHR*	ΔKHN**
	(KHN)	(KHN)	(KHN)	(Median, CI)	
Placebo	216.3 ± 52	108.7 ± 50.3	69 ± 71.7	-36.8[b] (-128.3–12.3)	6044.1 ± 2738.7[b]
20% xylitol	251.2 ± 57.2	141.2 ± 55.2	191.3 ± 65.9	33.5[a] (-16.1–81.5)	2196.4 ± 1257.6[a]
20% milled xylitol	228.1 ± 74.1	137.4 ± 49.6	166.9 ± 55.2	27.9[a] (-0.7–68.5)	2545.2 ± 1181.3[a]
Duraphat™ (Colgate)	222.1 ± 33.7	121.4 ± 54.5	158.9 ± 42.3	36.0[a] (12.4–80.8)	2451.8 ± 745.8[a]

Results of %SHR are given as median (CI, minimum/maximum) and ΔKHN as mean (±SD). Values in the same column that have different superscript letters differ significantly from each other. *Significance was determined using the Kruskal-Wallis test followed by Tukey's test (p < 0.05) and **ANOVA followed by Tukey's test (p < 0.05).

Table 2. Surface hardness measurements at baseline (SH baseline), after demineralization loss - %SHL), after treatment and pH cycling (SH final), percentage of surface hardness recovery (%SHR) and integrated mineral loss (ΔKHN) for the permanent teeth *in vitro* (n = 15).

Varnishes	SH Baseline	SH Lesion	SH Final	%SHR*	ΔKHN*
	(KHN)	(KHN)	(KHN)	(Median, CI)	
Placebo	290 ± 48.8	72 ± 57.8	64 ± 35.9	3.2[b] (-8.7–7.9)	3477,8[b] (1645.4–8273.6)
20% xylitol	276 ± 38.8	65 ± 47.9	80 ± 46.4	8.3[a] (2.4–15.1)	980[a] (430–5250)
20% milled xylitol	293 ± 68.8	66 ± 46.4	88 ± 38.9	10.4[a] (-10.4–23.9)	1184.4[a] (710–2960)
Duraphat™ (Colgate)	293 ± 51.4	67 ± 50.0	84 ± 40.2	8.1[a] (-1.2–29.7)	1754,3[a] (-480–1900)

*Results of %SHR and ΔKHN are given as median (CI, minimum/maximum). Values in the same column that have different superscript letters differ significantly from each other. Significance was determined using the Kruskal-Wallis test followed by Tukey's test (p < 0.05).

integrated mineral loss (ΔKHN), Kruskal-Wallis and Tukey's test for individual comparisons showed the same trend of results between treatments (p < 0.05). The varnish Duraphat™ and the xylitol 20% and milled xylitol 20% experimental varnishes differed significantly from the placebo varnish, showing their remineralization capacity in deep enamel *in vitro*. There were no significant differences between 20% xylitol, 20% milled xylitol and Duraphat™ varnishes.

However, when considering the enamel depths separately (10, 30, 50, 70, 90, 110, and 220 μm), the remineralizing capacity differed not only between the experimental varnishes and commercial varnish in relation to the placebo varnish, but also between the experimental varnishes and the Duraphat™ varnish. For the permanent teeth, at 10 μm depth, all varnishes differed significantly from the placebo. However, experimental varnishes containing xylitol differed significantly from Duraphat™ varnish and did not differ significantly from each other (p < 0.05). At depths of 30 and 50 μm, only experimental varnishes containing xylitol had significantly higher remineralization than the placebo varnish. Duraphat varnish did not differ significantly from placebo

varnish at both depths. In other depths (70–220 μm), none of the trial varnishes, as well as the commercial varnish Duraphat™ were able to significantly differ from placebo varnish.

For the deciduous teeth, again, at 10 μm depth, all varnishes differed significantly from the placebo varnish and did not differ from each other. At a depth of 30 μm, only the experimental varnish containing 20% milled xylitol (80 μm) and the Duraphat® varnish provided significantly greater remineralization compared to the placebo varnish. At 50 μm depth, only the varnish containing 20% milled xylitol (80 μm) differed significantly from the placebo varnish, however, there was no significant difference in relation to the other varnishes. At the other depths (70 to 220 μm), none of the experimental varnishes, as well as the commercial Duraphat® varnish were able to differ significantly from the placebo varnish (Figures 2 and 3).

In situ arm

All fifteen subjects were able to finish the experimental periods. No participants reported adverse events or side effects. ANOVA and Tukey's test for individual comparisons showed a significant
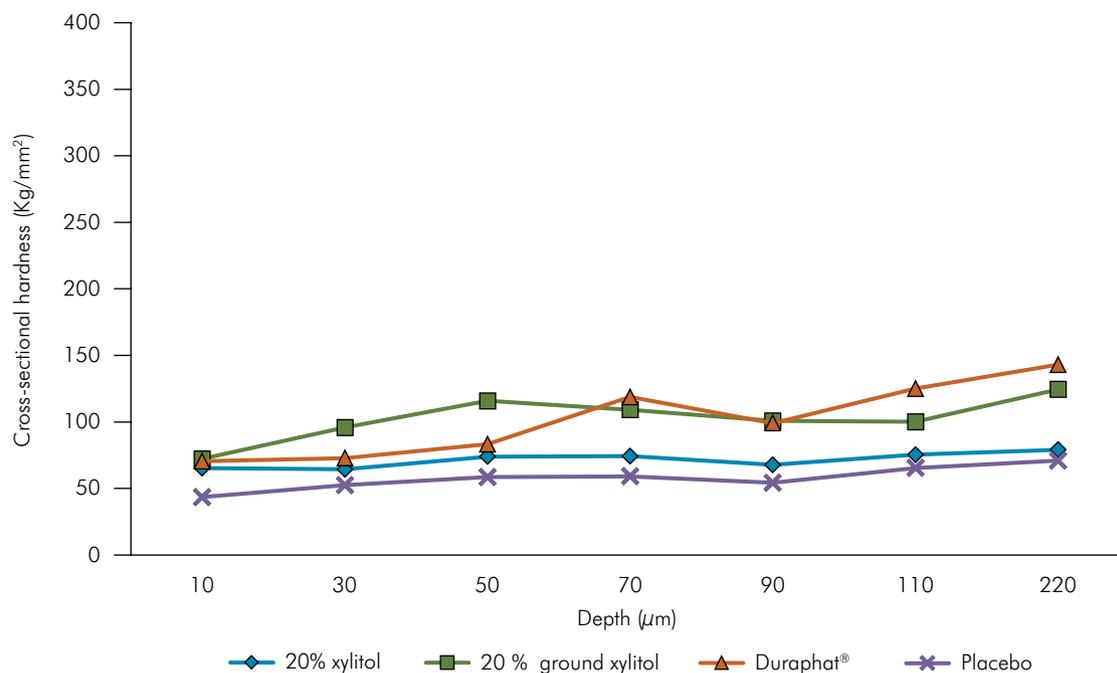
Figure 2. Subsurface hardness (kg/mm²) at the different depths of the experimental groups for the deciduous teeth *in vitro*.

difference among the treatments regarding the percentage of surface hardness recovery (%SHR) (p < 0.05). Duraphat™ varnish and 20% xylitol and 20% milled xylitol experimental varnishes differed significantly from the placebo varnish. There was no significant difference between 20% xylitol, 20% milled xylitol, and Duraphat™ varnishes.

Table 3 shows the mean and standard deviation of initial, post-demineralization, and final SH, %SHR (surface hardness recovery), and integrated mineral loss (ΔKHN) values for permanent teeth *in situ*. The results had a smiliar trend of the *in vitro* arm. Kruskal-Wallis and Tukey's test for individual comparisons showed a significant difference between
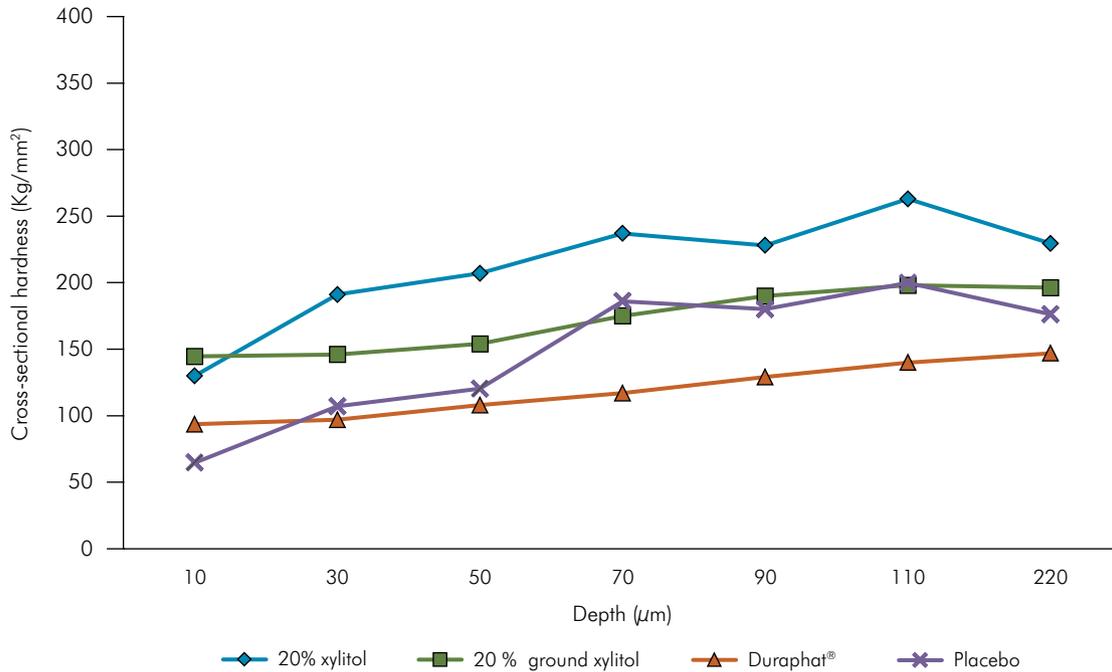
Significance was determined using ANOVA followed by Tukey's test for each depth (p<0.05).

Figure 3. Subsurface hardness (kg/mm²) at the different depths of the experimental groups for the permanent teeth *in vitro*.

Table 3. Surface hardness measurements at baseline (SH baseline), after demineralization (surface hardness loss - %SHL), after treatment and *in situ* phase (SH final), percentage of surface hardness recovery (%SHR) and integrated mineral loss (ΔKHN) for the permanent teeth *in situ* (n = 15).

Varnishes	SH Baseline	SH Lesion	SH Final	%SHR*	ΔKHN**
	(KHN)	(KHN)	(KHN)		
Placebo	279 ± 74.1	58 ± 37.3	56 ± 43.3	-3.64 ± 7.28[b]	2590[b] (140–6100)
20% xylitol	285 ± 80.1	59 ± 38.7	77 ± 37	7.02 ± 6.81[a]	940[a] (340–5270)
20% milled xylitol	306 ± 75.2	62 ± 43.6	82 ± 64.3	7.24 ± 7.48[a]	930[a] (130–3610)
Duraphat® (Colgate)	306 ± 61.3	60 ± 43.3	66 ± 41.4	5.33 ± 6.91[a]	1520[a] (200–1720)

Results of %SHR are given as mean (±SD) and ΔKHN as median (CI, minimum/maximum). Values in the same column that have different superscript letters differ significantly from each other. *Significance was determined using ANOVA followed by Tukey's test (p<0.05) and **Kruskal-Wallis followed by Tukey's test (p < 0.05).

treatments regarding integrated mineral loss (ΔKHN) (p < 0.05). The Duraphat and experimental varnishes differed significantly from the placebo varnish. There was no significant difference between 20% xylitol, 20% milled xylitol and Duraphat™ varnishes.

However, when considering the enamel depths separately (10, 30, 50, 70, 90, 110 and 220 μm), remineralization capacity differed between all the varnishes. At the 10 μm depth, all varnishes differed significantly from the placebo. Experimental varnishes containing xylitol did not differ significantly from Duraphat™ varnish, but the varnish containing 20% of milled xylitol had a significantly greater remineralizing action than the varnish containing 20% xylitol only (p < 0.05). At depths of 30 and 50 μm, only the experimental varnishes containing xylitol promoted significantly higher remineralization than the placebo varnish. Duraphat™ varnish did not differ significantly from placebo varnish at both depths, as seen for the *in vitro* study. Moreover, at 30 μm, the varnish containing 20% milled xylitol had the best remineralizing action, this time differing significantly from the Duraphat™ varnish and not differing significantly from the varnish containing

only xylitol. In other depths (70-220 μm), none of the trial varnishes, as well as the commercial varnish Duraphat™ were able to significantly differ from placebo varnish (Figure 4).

Regarding TMR analysis, Kruskal-Wallis followed by Dunn's test showed that the experimental varnishes and Duraphat™ were able to induce a significant reduction in the integrated mineral loss compared to placebo. However, when the parameter "lesion depth" was considered, only 20% xylitol and 20% milled xylitol varnishes significantly differed from placebo (Table 4). Figure 5 shows a microradiograph image of a demineralized specimen (subsurface lesion) and the microradiograph image of a re-mineralized specimen, after treatment with 20% xylitol and 20% milled xylitol varnishes.

Discussion

The higher caries susceptibility of primary and younger permanent teeth[15-17] is the main point that led us to choose these substrates. As a surprise, the extent of enamel demineralization of primary teeth when submitted to acidic media in this study
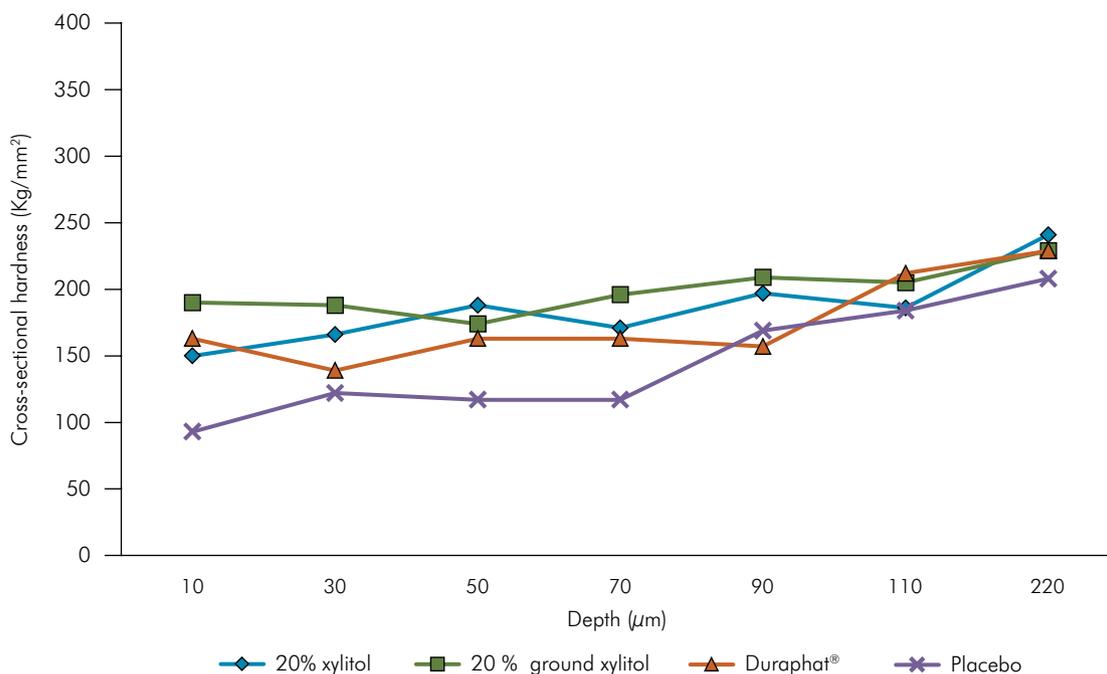
Significance was determined using the Kruskal-Wallis/ANOVA followed by Tukey's test for each depth (p < 0.05).

Figure 4. Subsurface hardness (kg/mm²) at the different depths of the experimental groups for the permanent teeth *in situ*.

Table 4. Median lesion integrated mineral loss (DDZ) (DZ lesion - DZ final effect) and depth (μm) (DL = L lesion - L final effect) of the pre-demineralized enamel specimens treated with different varnishes *in situ* (n = 11).

Groups	$\Delta\Delta Z$ - (%vol.min x μm)	ΔL - Depth (μm)
	(DZ initial–DZ final lesion)	(L initial–L final lesion)
Placebo	221.1 (50–630)[B]	2.3 (-0.5–5.8)[B]
20% xylitol	901.1 (150–2130)[A]	17.8 (11.3–28.2)
20% milled xylitol	880.2 (130–1450)[A]	20.4 (13.4–43.6)[A]
Duraphat®	682.3 (340–1170)[A]	12.0 (2.1–27.7)[B]
p-value*	0.002	0.003

Results are given as median (CI, minimum/maximum). Values in the same column that have different superscript letters differ significantly from each other. Significance was determined using Kruskal-Wallis followed by Dunn's test (p < 0,05).
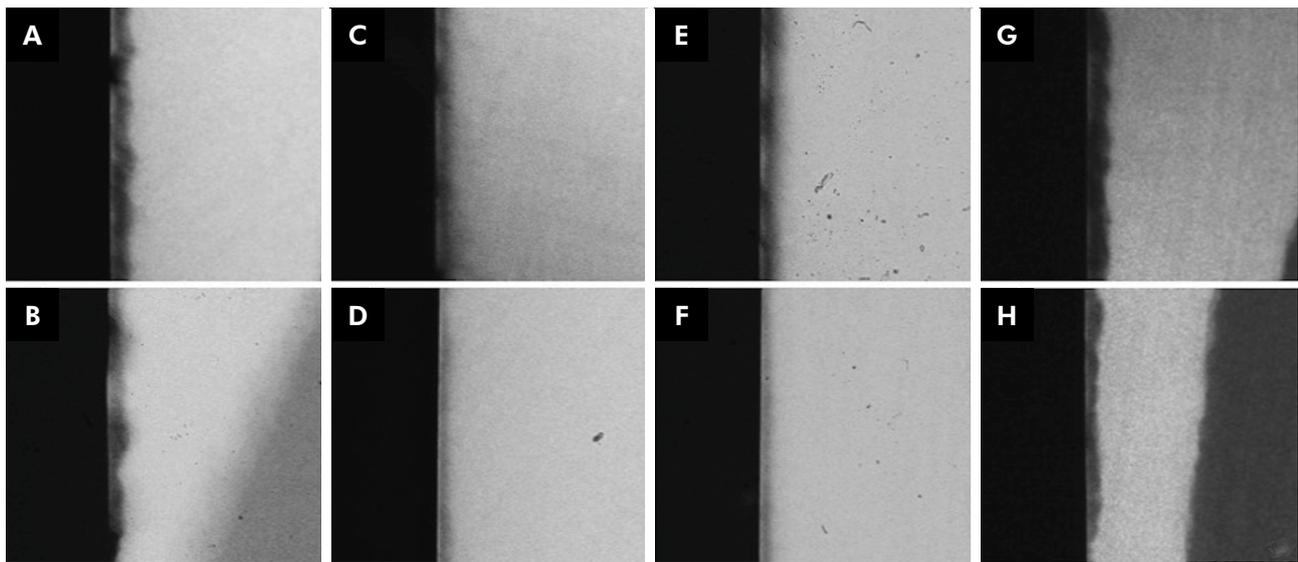
Significance was determined using the Kruskal-Wallis/ANOVA followed by Tukey's test for each depth (p < 0.05).

Figure 5. Microradiographs of a demineralized and re-mineralized specimen from all groups of the *in situ* study: A) demineralized specimen of placebo group; B) re-mineralized specimen after treatment with placebo varnish; C) demineralized specimen of 20% xylitol varnish; D) re-mineralized specimen after treatment with 20% xylitol varnish; E) demineralized specimen of 20% milled xylitol varnish; F) re-mineralized specimen after treatment with 20% milled xylitol varnish, and G) demineralized specimen of Duraphat group; H) re-mineralized specimen after treatment with Duraphat varnish.

was lower than that observed for permanent teeth after 11 days of the cariogenic challenge. This may be because the permanent teeth used in this study were unerupted or partially erupted, which means that they had not been exposed to the oral environment yet and consequently not undergone enamel maturation.

However, a greater %SHR was seen for the primary teeth, showing a higher capacity of mineral exchanges between the deciduous enamel and the oral environment, due to the anatomic characteristics of the primary teeth, such as the size of the enamel pores.[18,19] Besides, for the primary enamel, the milled xylitol was the only varnish able to promote subsurface remineralization of deeper layers (50 μm) compared to the other varnishes, which may be due to the capacity of penetration and calcium accessibility of smaller particles (xylitol acts as a carrier of Ca^{2+} ions) into the pores of the lesion.[6] Additionally, the smaller particles increase superficial areas and thus present more interaction with the dental substrate.

The initial idea for milling xylitol was to reduce the decantation of xylitol particles in the varnish bottle, which is one of the challenges for implementing this material in its commercial form. In this study, xylitol particles were subjected to a ball mill for 60 minutes to contribute to the advancement in the clinical application of the material. These particles have dimensions of more than 100 micrometers in its natural form (D50 = 145.8 μm) and a density of 1.57 g/mL (varnish density - 0.84 g/mL). Due to these characteristics, it was observed that with the addition of 20% by mass of xylitol particles, a large part of these particles sediment in the bottom of the bottle. The reduction of the particle size of xylitol by 45% (milled xylitol) decreased its decantation in the experimental varnish; however, it did not influence the remineralizing capacity of the material in general. In an unprecedented way, for the deciduous enamel, only the milled xylitol was able to promote subsurface remineralization in deeper layers (50 μm). These results suggest that further studies should focus on the use of nanoparticulated xylitol, which could be expected to improve its preventive effect.

The concentration of xylitol (20%) employed in the present experimental varnishes was chosen due to its better performance in comparison to other concentrations, as confirmed in previous studies from our group.[11,12,27] Other studies using transverse microradiography (TMR) analysis with bovine teeth (both *in vitro* and *in situ*) have demonstrated the remineralization capacity of 20% xylitol varnish; this capacity was influenced by region of the enamel that was analyzed and the combination or not to fluoride.[11,12] In the *in situ* study using bovine enamel, the experimental 20% xylitol varnish led to a significant decrease in lesion depth (ΔL) compared to the positive control varnish (Duraphat™), and this could also be seen in the present study when considering the enamel depths separately (10, 30, 50, 70, 90, 110 and 220 μm).

For the *in vitro* arm, the experimental varnishes containing xylitol performed significantly better than Duraphat™ at 30 and 50 μm, differing significantly from the placebo varnish, which was only seen for Duraphat™ at 10 μm or when considering the integrated loss of subsurface hardness (ΔKHN). The results from transverse microradiography (TMR) of the *in situ* study corroborate these findings, and again, only the experimental varnishes were able to significantly reduce the enamel lesion in depth. Thus, the mineral gain observed with Duraphat™ is confirmed to occur mostly in the outer surface and intermediate enamel layers, as F may block superficial enamel pores, preventing access and remineralization of deeper areas of the lesion. The experimental varnishes may favor remineralization in deeper layers, either by decreasing the acidogenic potential of plaque or by facilitating the movement of Ca^{2+} ions from the saliva towards enamel.[6,11,28]

The cross-sectional hardness and integrated area under the curve (calculated by the trapezoidal rule) were calculated to show the integrated loss of subsurface hardness (ΔKHN)[25] for both arms of this study and transverse microradiography was also evaluated for the *in situ* arm. Ideally, both methods, hardness and transverse microradiography, should be combined, as hardness does not necessarily measure the mineral content and some studies regarding the conversion of hardness to mineral volume are controversial.[29] However, it gives important information regarding the mechanical properties (physical strength) of the lesions, which is not provided by TMR. Complementary techniques should also be employed to assess the changes in both the physical and chemical characteristics of the lesion.[30] Taking into account the findings from bovine teeth using TMR[11,12] and the findings of the present study, it can be inferred that the same trend is observed using both methods.

The limitations of the present study rely mainly on the difficulty to obtain the deciduous enamel specimens, since children always want to bring their extracted teeth home and, therefore, the sample of primary teeth took almost a year to be complete. In addition, deciduous enamel is very thin, which led to specimen losses during the cutting and polishing steps. Still, due to the small thickness of the deciduous enamel and the difficulty to prepare specimens of 100 μm to perform the TMR analysis, we chose to perform the microradiography only with the permanent teeth samples.

Conclusion

Based on these findings, 20% xylitol or 20% milled xylitol varnishes can be considered as an effective alternative to F for the remineralization of white spot lesions in primary and newly erupted teeth. The present results should be confirmed *in vivo*, with properly designed randomized controlled clinical trials. The beneficial effect of xylitol on the inhibition of bacterial metabolism and growth should also be better investigated as it could improve its remineralizing and preventive effects.

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