Original Article

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Effect of fluoride-containing resin sealant on the subsurface enamel microhardness of artificial incipient caries lesions: an *in vitro* study

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Objective: To evaluate the subsurface enamel microhardness of artificial incipient enamel caries treated with fluoride-releasing resin sealants at different depths.

Materials and Methods: Artificial enamel caries were created at the buccal surface of thirty-six extracted human premolars and randomly divided into three groups (n=12): Group 1: untreated group (control), Group 2: non-fluoride releasing resin sealant (NFRS, Concise™), and Group 3: fluoride-releasing resin sealant (FRS, Clinpro™). The sealant was placed on the buccal window (2x2x1 mm^3) of the teeth in groups 2 and 3; and the groups underwent pH cycling for ten days. The teeth were sectioned, and the Knoop hardness number (KHN) was measured at 30-, 60-, 90-, 120-, and 150-µm deep from the enamel surface. The data were analyzed using one-way repeated ANOVA and pairwise comparisons with the Bonferroni test at a significance level of $p<0.05$.

Results: At 30-µm deep, the enamel microhardness in the FRS group was the highest (159.62 ± 30.66 KHN) and significantly higher than the NFRS (120.64 ± 38.07 KHN) and control (31.52 ± 14.75 KHN) groups ($p<0.05$). At 60-µm deep, the FRS group's microhardness was also the highest (234.16 ± 19.42 KHN) and was significantly higher than the control group (206.83 ± 26.40 KHN) ($p<0.05$), but not significantly different from the NFRS group (212.80 ± 15.57 KHN) ($p>0.05$).

Conclusion: The fluoride-releasing resin sealant significantly increased the enamel microhardness at the outer enamel (30-µm deep), which could benefit patients with initial caries.

Keywords: dental caries, enamel microhardness, fluoride, remineralization, resin sealant

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Introduction

Dental caries is a major health problem globally. Pit and fissure caries most commonly occur because the tooth morphology provides an environment for plaque retention [1]. Although earlier dental sealants were introduced to prevent caries in occlusal grooves [2, 3], they are now used for managing initial caries, and some sealants contain fluoride due to its anti-cariogenic effect [4], which inhibits enamel demineralization and promotes its remineralization [5].

Although glass ionomer sealants (GIC) release more fluoride than fluoride-releasing resin sealants (FRS) [6], the significantly poorer retention of GIC compared with resin sealant has been reported in a systematic review. The benefits that dental sealants provide by protecting the pits and fissures are based on good retention and integrity. Moreover,

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GIC's mechanical properties are inferior to those of FRS, thus, resin-based sealants are the most commonly used materials to prevent caries initiation/progression [7]. However, because placing resin sealants is technically sensitive, and they are not permanently retained on the tooth, hence, secondary caries can develop if the sealant is inadequate [2]. Therefore, their caries-preventive effect could be enhanced by incorporating them with fluoride [4].

The remineralization assessment of a fluoride-releasing material consists of direct and indirect techniques, and the one most widely used indirect method is measuring the enamel microhardness [8] which was used in previous studies [3, 9, 10]. The enamel microhardness is positively correlated with its mineral content [11].

Many studies revealed the effect of an FRS on enamel microhardness at the adjacent enamel surface [3, 9, 12,13]. However, only three studies evaluated the effect throughout the lesion depth. Lobo *et al*. [9] and Vatanatham *et al*. [10] found no significant difference in enamel microhardness between FRS and NFRS. In contrast, Kantovitz *et al.* demonstrated that FRS reduced the decrease in enamel hardness after acidic challenge more than NFRS, however, they did not compare the hardness at different enamel depths. They reported only a mineral loss at 10–180 μm from the enamel surface [3]. Therefore, the effect of FRS on enamel microhardness throughout the lesion depth is unresolved, and there is no comparison of the remineralization effect between different enamel depths.

Therefore, the aim of this study was to assess the remineralization effect of an FRS on the subsurface enamel of artificial initial carious lesions at 30-, 60-, 90-, 120-, and 150-μm deep from the enamel surface.

Material and Methods

This study was approved by the Institutional Review Board, Ethics Committee of the Faculty of Dentistry and Faculty of Pharmacy, Mahidol University, Thailand (MU-DT/PY-IRB 2021/DT068).

Sample size calculation

The sample size calculation was based on Lobo et al., 2005. The significance level (α) was set at 0.05, and the power (1-β) was 0.9. The number of calculated samples was at least seven teeth per group. In the present study, the sample size was twelve teeth in each group to allow for greater precision, accuracy, and a higher confidence level.

Specimens preparation

Thirty-six human premolar teeth extracted from patients for orthodontic reasons were stored in 0.1% Thymol. The root of each tooth was removed using a carborundum disc and the disto-buccal surface of the occlusal ⅓ of the crown was embedded in acrylic resin. The enamel surface was polished with 600-, 1,200-, 2,500-, and 4,000-grit silicon carbide sandpaper (Buehler, Lake Bluff, IL) for 5 sec per grit number using a polishing machine (RotoPol-21, Struers, Copenhagen, Denmark). The polished enamel surface was outlined using a ruler and pencil in a square window measuring 2x2 mm^2, and applied with acid-resistant nail varnish, except for the window (Figure 1).

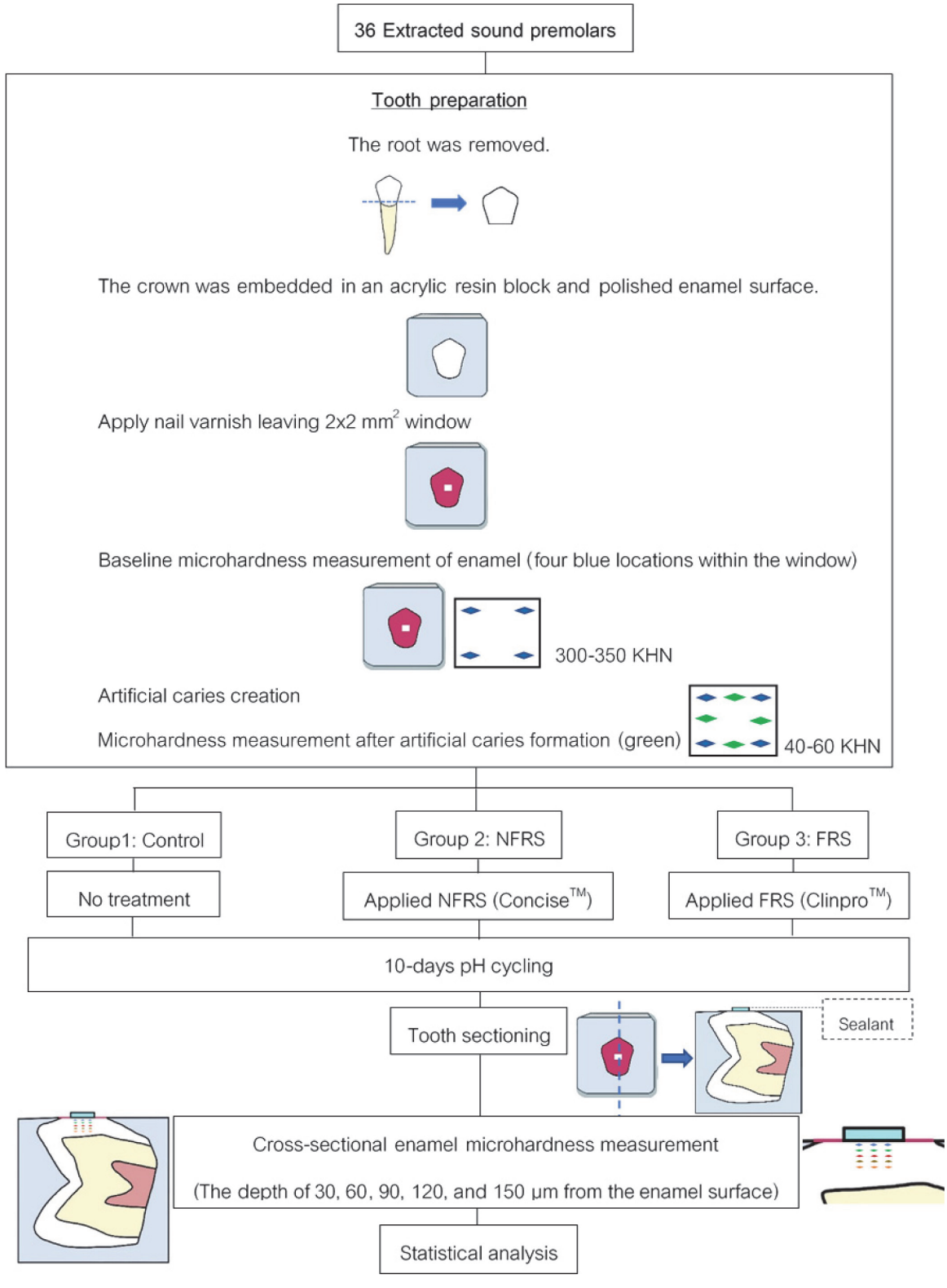

Figure 1 Schematic of the methodology and experimental design

Baseline microhardness measurement

The surface enamel microhardness (SMH) was measured with a Knoop indentation instrument (FM-ARS 9000, Future-Tech, Kanagawa, Japan) using a 50-g load for 10 sec at 4 locations 1000-μm apart within the window (Figure 1). The average of the four readings was calculated for the surface microhardness value at baseline. Only teeth with a mean microhardness of 300–350 KHN were used [14, 15].

Artificial caries creation

Each tooth was immersed in a mixture of 0.1 M lactic acid, 0.2% Carbopol C907, 4.1 mM $CaCl_2$ $2H_2O$, and 8.0 mM KH_2PO_4 adjusted to pH 5.0 (using KOH) for 6 d at 37°C [16], each sample was then rinsed with deionized water, and wiped dry with tissue paper.

Microhardness measurement after artificial caries creation

The SMH of each tooth was measured again in the same manner as at baseline (Figures 1-2). Only specimens with a mean microhardness ranging from 40–60 KHN were included in this study [15, 16]. The teeth were randomly divided into three groups.

Control and Experimental groups

The three groups (n = 12) were: Group 1: no treatment (Control), Group 2: treated with NFRS (Concise™, 3M ESPE), and Group 3: treated with FRS (Clinpro™, 3M ESPE).

The 2x2 mm^2 windows of tooth groups 2 and 3 were etched with 35% phosphoric acid (3M ESPE) for 15 sec and washed. After air-drying, the sealant was applied with a micro-brush inside a window 2x2x1 mm^3 of the custom-made silicone mold to ensure the same sealant amount and thickness. The sealant was polymerized using an LED curing light wavelength 420–480 nm for 20 sec per the manufacturer's instructions.

The specimens were subjected to pH cycling, simulating the dynamics of the mineral loss and gain of carious lesions. The teeth were individually immersed in 3 ml demineralization solution (2.2 mM CaCl2, 2.2 mM KH2PO4, and 0.05 M Acetic acid, pH 4.4) for 6 h at 37°C. They were then washed in deionized water for 10 sec, dried with absorbent paper, and individually immersed in 3 ml remineralization solution (1.5 mM CaCl2, 0.9 mM NaH2PO4, and 0.15 M KCl, pH 7.0) for 18 h, at 37°C. The solutions were changed daily for 10 d [17].

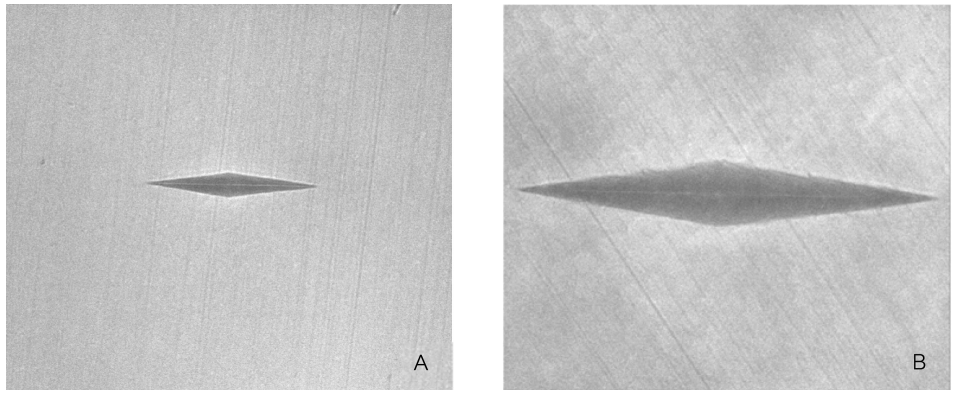

Figure 2 Knoop indentation on the sound enamel surface (magnification 50X) (A), the indentation after artificial caries creation (magnification 50X) (B)

Subsurface enamel microhardness measurement

After pH-cycling, each specimen was washed in deionized water, dried, and cut with a carborundum disc through the center of the square window on the specimen. One of the tooth halves was randomly selected and polished with silicon carbide sandpaper. The KHN at five distances from the tooth surface, i.e., 30, 60, 90, 120, and 150 μm, depending on the least distance that a distinct Knoop indentation could be performed, was measured in 3 areas at least 150 μm apart (Figure 3), and the average of the three readings was calculated.

Statistical analysis

The data were recorded and analyzed using the Statistical Package for the Social Sciences (SPSS), version 21. The mean microhardness values at each distance from the enamel surface for each group were calculated. The KHN data had its normal distribution evaluated by the Shapiro-Wilk test. The statistical tests employed a 95% level of confidence and 0.05 significance level. One-way analysis of variance (ANOVA) was used to determine whether there were significant inter-group differences at baseline and after artificial caries. The Paired T-test was used to

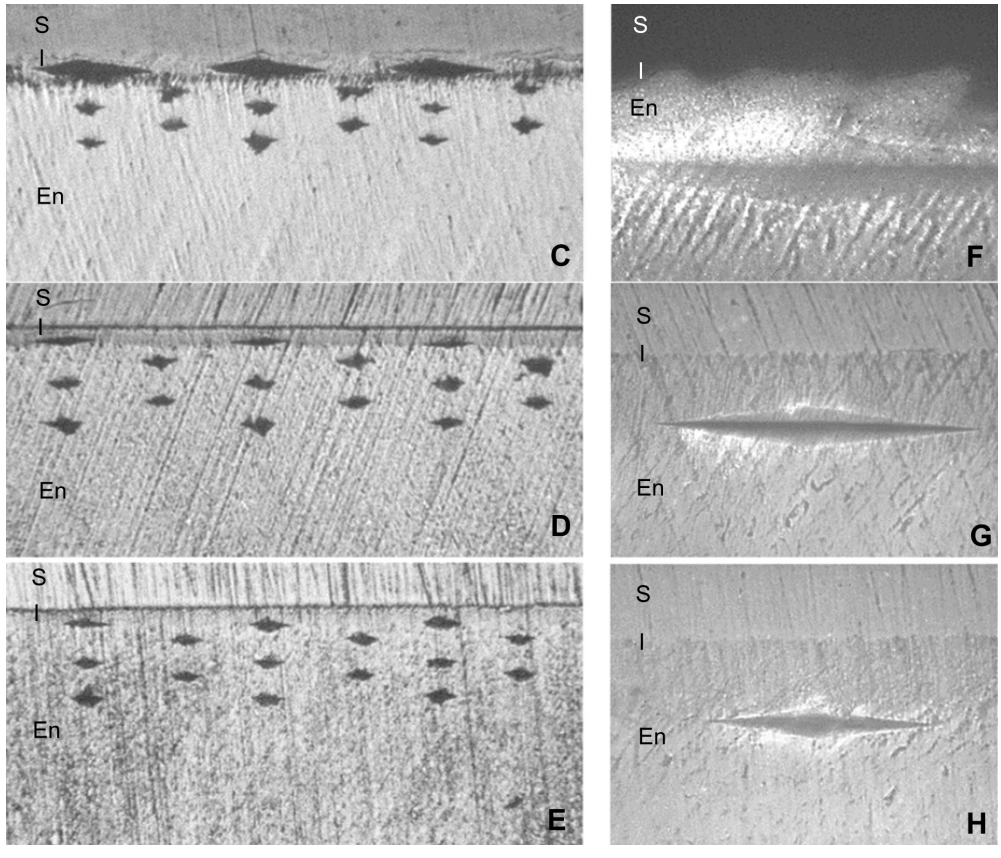

Figure 3 Knoop indentations were made 30-, 60-, 90-, 120-, and 150-μm deep from the outer enamel surface (magnification 5X) in the Control (C), NFRS (D), and FRS (E) groups. Knoop indentation at 30 μm deep (magnification 50X) in the Control (F), NFRS (G), and FRS (H) groups. S, Sealant; I, Interface; En, Enamel.

determine whether there were significant intra-group differences between baseline and after artificial caries. One-way ANOVA and repeated ANOVA with Pairwise comparisons using the Bonferroni test were performed to demonstrate significant intra-group and inter-group differences.

Results

There were no differences in the microhardness values between the groups at baseline or after artificial caries formation (p>0.05) (Table 1).

Comparing between the groups, the enamel microhardness in the FRS group was the highest and significantly higher than the NFRS and control groups at 30 µm deep (p<0.05). At 60 µm deep, the FRS group also had the highest microhardness and was significantly higher than the control (p<0.05), but not significantly different from the NFRS group (p>0.05). At 90, 120, and 150 µm deep, there were no significant differences between groups (p>0.05) (Table 2).

Compared within each group, the enamel microhardness at 30 µm in each group was the lowest and was significantly lower than the other depths (p< 0.05). At 60-µm deep, there was no difference in enamel microhardness compared with 90-, 120-, and 150-µm deep (p> 0.05), except in the control group, where the value at 60-µm deep was significantly lower than at 90-µm deep (p< 0.05) (Table 2).

Table 1 Surface enamel microhardness in KHN (Mean ± SD)

Groups	Baseline	After artificial caries formation
Control	331.87 ± 13.70$^{A, a}$	54.78 ± 2.77$^{B, b}$
Concise™ (NFRS)	330.76 ± 15.16$^{A, a}$	54.42 ± 1.97$^{B, b}$
Clinpro™ (FRS)	331.94 ± 15.67$^{A, a}$	53.25 ± 4.71$^{B, b}$

n = 12 per group

Different upper case letters indicate significant differences between groups at baseline and after artificial caries (One-way ANOVA at p<0.05)

Different lower case letters indicate significant differences between baseline and after artificial caries within each group (Paired T-test at p<0.05)

Table 2 The KHN of enamel after sealant treatment and pH cycling (Mean ± SD)

Groups	Distance from the enamel surface				
	30 µm	60 µm	90 µm	120 µm	150 µm
Control	31.52 ± 14.75 ^{A,a}	206.83 ± 26.40 ^{A,b}	238.20 ± 21.72 ^{A,c}	229.96 ± 26.58 ^{A,c}	233.16 ± 24.16 ^{A,c}
Concise™ (NFRS)	120.64 ± 38.07 ^{B,a}	212.80 ± 15.57 ^{AB,b}	227.88 ± 12.60 ^{A,b}	224.87 ± 14.69 ^{A,b}	229.55 ± 14.20 ^{A,b}
Clinpro™ (FRS)	159.62 ± 30.66 ^{C,a}	234.16 ± 19.42 ^{B,b}	231.89 ± 19.46 ^{A,b}	235.16 ± 21.88 ^{A,b}	229.89 ± 17.15 ^{A,b}

n = 12 per group

Different upper case letters indicate significant differences inter-groups at the same distance
(One-way ANOVA and Pairwise comparisons with the Bonferroni test at p<0.05)

Different lower case letters indicate significant differences between distances intra-group
(One-way repeated ANOVA and Pairwise comparisons with Bonferroni test at p<0.05)

Discussion

The present study evaluated the effect of the FRS on subsurface enamel under artificial initial carious lesions at different enamel depths. The baseline microhardness of the enamel in this study ranged from 302–348 KHN. This value was similar to those of previous studies that demonstrated normal enamel microhardness ranging from 315.7–354.1 KHN [14, 18]. Creating incipient carious lesions in this experiment caused an approximately 84% decrease in enamel microhardness (40–59 KHN), similar to Lippert *et al.* who reported a microhardness ranging from 49–49.8 VHN (Vickers hardness number) [16].

Based on the design of the present study, we included only samples with similar tooth characteristics. The inclusion criteria were the tooth type, age, and baseline enamel microhardness value. Only premolar teeth from 15–20-year-old patients were included because the KHN is significantly different among age groups [19]. For the baseline enamel microhardness, we selected only teeth in the range of 300–350 KHN. For testing the fluoride effect of resin sealant, this study used two types of sealant (NFRS and FRS) from the same manufacturer (3M ESPE, USA) which have similar compositions (TEGDMA and Bis-GMA), except that FRS contains Tetrabutylammoniumtetrafluoroborate [20]. Therefore, the significantly higher KHN could be affected by the fluoride-releasing effect. Our experiment investigated the effect of FRS during the constant level of fluoride release phase, which occurs at least seven days after sealant placement [21]. We measured the KHN ten days after FRS treatment. The KHN of the indentation was measured at the first 30-µm layer because of the fluoride-remineralizing effect from the previous study initially presented at 30 µm deep from the enamel surface, and a significant difference at 60 µm [22]. Furthermore, the 30-µm interval also was calculated from the minimum spacing between indentations without interfering to have the accuracy of testing [23].

Comparing the groups, the KHN in the FRS group was significantly higher than the NFS group at 30 µm deep. These results corresponded to those of Kantovitz *et al.*, who found that the FRS inhibited the loss of enamel hardness significantly more than the NFRS. However, only a mean value of depths from 10–180 µm without the KHN at each depth was reported [3]. In contrast, Vatanatham *et al.* revealed that the mineral loss in incipient caries sealed with FRS was not significantly different from NFRS, however, the FRS and NFRS were placed on the adjacent area of the same tooth specimen [10], thus fluoride ion exchange from the FRS to the NFRS areas might have occurred and affected the remineralization outcome. Lobo *et al.* also found no significant differences in KHN between FRS and NFRS, however, the study was done on sound tooth enamel [9], which presented less remineralization than carious enamel. Because demineralized enamel is highly porous, higher fluoride distribution occurred in the carious enamel compared with the sound enamel, affecting the remineralization results [24]. Comparing the FRS and the control groups, the remineralization in the FRS group found at 30–60 µm is likely due to the high fluoride content in the form of calcium fluoride precipitation that is limited to the outermost 50-µm layer [25].

Comparing between depths, the enamel microhardness at 30 µm in all three groups was the lowest and was significantly lower than the other depths, suggesting that the outermost layer contacted with acid more than the other layers. However, there was no sealant barrier in the control group to protect the enamel from acid during pH cycling. Thus, the enamel would be demineralized deeper than in the sealant groups [26]. Furthermore, there were no significant differences between the 90-, 120-, or 150-µm depths in the three groups (230.85 ± 19.35 KHN). These values were significantly lower than the KHN value on sound enamel (379 ± 18 KHN) found in a previous study [19]. However, the artificial caries formed following Lippert *et al.*'s regimen [16] were limited to the enamel with a chalky white appearance. This type of initial enamel caries can be treated with a dental sealant or topical fluoride treatment, following the concept of minimally invasive dentistry [27].

Other fluoridated products, such as resin-modified glass ionomer cement (RMGIC) and fluoride varnish (FV), have a limited remineralization effect on the superficial enamel surface [15, 22]. The effect of RMGIC (Fuji II LC®) occurred in the first 150-µm layer of the enamel surface [15], and FV's (Clinpro™, Duraphat®, Enamelast™) effect occurred at the outer 80-µm depth [22]. Their anticaries effects occurred deeper than those of FRS, which is likely due to their higher fluoride content. The FRS has a lower fluoride concentration (0.85 ± 0.26 ppm) [28] than FV (1.83 ± 0.5 ppm) [29] and glass ionomer cement (2.54 ± 0.68 ppm) [28]. Therefore, the FRS results in this study suggest that remineralization occurs at a shallower depth than the previous study.

The present study has some limitations. The in-vitro nature of the study simulated the dynamics of mineral loss and gain in the oral cavity using a pH-cycling model for ten days [17]. However, this model could not imitate the complex intraoral condition, such as bacterial biofilm [30].

In conclusion, the FRS significantly increased the KHN at 30-µm deep. However, there was no significant difference in KHN between the FRS and NFRS from 60–150-µm deep. Our results indicate that the FRS provides a cariostatic effect on the outer enamel surface, which could benefit patients with initial enamel caries.

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