Elemental analysis of the fluoride varnish effects on root caries initiation

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ABSTRACT

Objectives: The usage of fluoride varnish for a moderate to low caries-risk group has not been well validated. This study aimed to evaluate the preventive and therapeutic efficacies of fluoride varnish on the initiated root caries.

Materials and Methods: Ten premolars were sectioned into quarters, further divided into two windows, one of which was painted with Fluor Protector (1,000 ppm fluoride, Ivoclar Vivadent). An initial lesion with a well-preserved surface layer was produced by pH cycling. Scanned line analysis using energy dispersive spectrometry determined the weight percentages of Ca and P in the demineralized layer. Scanning Electron microscopy and confocal laser scanning microscopy (CLSM) evaluated the varnish-applied root surfaces.

Results: The mean lesion depth (SD) was 12.3 (2.6) μm (single cycling) and 19.6 (3.8) μm (double cycling). Double cycling extended the lesion depth, but induced no more mineral loss than single cycling ($p < 0.05$). The mean weight percentages of Ca and P between groups with and without varnish were not significantly different ($p < 0.05$). A CLSM showed varnish remained within 15 μm of the surface layer.

Conclusions: When a mild acid challenge initiated root tissue demineralization, the application of low-concentration fluoride varnish did not influence the lesion depth or the mineral composition of the subsurface lesion. [J Kor Acad Cons Dent 2011;36(4):290-299.]

INTRODUCTION

Patients with recently exposed root surfaces may become susceptible to acidic environments, even when other pertinent environmental factors remain unchanged.[1] The inorganic chemical events of the root caries are similar to that of the enamel, however, the permeability and reactivity of the root surface allows the demineralization process to occur at a higher pH than in enamel.[2] Therefore, clinicians

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observe that many patients may appear resistant to enamel caries but prone to root caries, after root surfaces become exposed by soft tissue recession, traumatic tooth brushing habits, periodontal treatment, and etc. For exposed root surfaces, fluoride varnish has been advocated both by serving as a desensitizing agent and an anticariogenic agent.[3] Fluor Protector (1,000 ppm F, Ivoclar Vivadent, Schaan, Lichtenstein), 0.1% silane fluoride containing varnish is known not only to supplement additional fluoride ions but also to block a porous structure by building a polymerized barrier.[4] Several studies have demonstrated the prolonged caries inhibition effect of Fluor Protector, even though it has a low fluoride concentration (1,000 ppm F) relative to other varnishes containing 5% NaF (22,600 ppm F, Duraphat, Colgate, New York, NY, USA; Duraflor, Medicom, Tonawanda, NY, USA; CavityShield, 3M ESPE, St Paul, MN, USA).[3,5,6] However, it was shown that dentin required higher levels of fluoride to reach a level of inhibition comparable to that of enamel.[7,8] Ettinger *et al.* suggested that the depth of artificial lesions on root surfaces are dose responsive to the concentration of fluoride used, and that 10,000 ppm F was beneficial.[9] For root dentin, polymer-based varnishes may penetrate into dentinal tubules and act as a type of sealant. But, Hong *et al.* evaluated the efficacy of varnish with the consideration of abrasive contact in the daily circumstance and showed that preventive effect of varnish was diminished by tooth brushing.[10]

Most studies on the efficacy of fluoride varnish have focused on high caries risk groups in clinical situations or induced severe acidic challenges in *in vitro* experiments. It has been rarely addressed about caries inhibition activity of varnish for a normal population who do not additionally require a preventive intervention. However, a previously low to moderate risk group, who practice regular tooth brushing and preclude any drastic cariogenic challenges, may become newly susceptible to the disease due to their recently exposed root surfaces. And, the fluoride varnish can be applied in a preventive measure, even before any clinical signs of caries development are perceived.

Traditionally, the initial stages of carious lesions are characterized by partial dissolution of the tissue, leaving a 20 - 50 μm thick, rather well-mineralized surface layer and a subsurface body of the lesion with a mineral loss of up to 30 - 50%.[11] Such a detectable amount of disintegration is resulted from the preceding continuum of reactive chemical events. To quantify the beneficial effects of fluoride for the initiation of the pathologic change, it may be useful to trace the elemental redistribution with a fine resolution.[12] In this study a scanning electron microscope (SEM, JEOL JSM-6610LV, JEOL, Tokyo, Japan) and an energy dispersive spectrometer (EDS, Oxford instruments, Bucks, UK) were employed to measure quantitative changes in mineral composition and to locate the demineralized layer. A confocal laser scanning microscope (CLSM, Zeiss LSM-Pascal, Carl Zeiss, Göttingen, Germany) was used to determine the depth of varnish penetration into root tissue. We hypothesized that low concentration fluoride varnish applied to the root surface would influence the early profile of subsurface demineralization under a mild acid challenge.

MATERIALS AND METHODS

Specimen preparation

This study was approved by the Institutional Review Board of Seoul National University Dental Hospital. Ten human lower premolars extracted for orthodontic treatment were used within 6 months of extraction and disinfected in 0.5% chloramines-T and stored in distilled water at 4℃. The teeth were inspected to ensure that they were free of fractures or other defects, and the periodontal ligament was removed using a Gracey curette under microscopy. The crowns of the teeth were removed at the cementoenamel junction with a low-speed diamond saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) and the apical roots were removed leaving 4-mm-long root segments. The segments were sectioned mesiodistally and buccolingually into four parts (Figure 1). Each quarter root was covered with a thin layer of acid-resistant nail varnish, creating two 1 mm × 4 mm windows on the outer root surface and randomly assigned into one of four experimental groups (Table 1).

Table 1. Classification of experimental groups

Group (treatment regimen)	Subgroup	Experimental Sequence		
		pH cycling I	Varnish application	pH cycling II
A (no acid challenge)	Ao (no varnish)	–	–	–
	Av (varnish)	–	+	–
B (acid challenge before varnish application)	Bo (no varnish)	+	–	–
	Bv (varnish)	+	+	–
C (acid challenge after varnish application)	Co (no varnish)	–	–	+
	Cv (varnish)	–	+	+
D (acid challenge before and after varnish application)	Do (no varnish)	+	–	+
	Dv (varnish)	+	+	+

Group A: no pH cycling
Group B: pH cycling before varnish application
Group C: pH cycling after varnish application
Group D: pH cycling before and after varnish application

Each group was divided into two subgroups; v (varnish application) or o (no varnish application).

Fluoride varnish application and pH cycling

Fluor Protector (1,000 ppm F) was applied on one of the two windows of each specimen according to the manufacturer's directions and dried for 5 minutes. After storage in 100% humidity for 24 hours at 37℃, the thin varnish layer was pulled off using a No.15 surgical blade. The specimens were immersed in 2 mL of demineralizing solution (1.4 mM Ca, 0.9 mM P, 0.05 M acetate buffer, 0.03 ppm F, pH 5.0) for 3 hours, followed by immersion in 2 mL of remineralizing solution (1.5 mM Ca, 0.9 mM P, 0.1 M Tris buffer, 0.05 ppm F, pH 7.0) for 20 hours.[13,14] After 5 days of cycling, specimens were immersed in remineralizing solution for 2 days. Solutions were changed for each immersion and placed in a 37℃ incubator without agitation.

SEM/EDS analysis

Specimens were embedded in epoxy resin (Epofix, Struers, Glasgow, UK) and horizontally cross-sectioned along the midline (Figure 1). The exposed cut surfaces were serially polished with 500, 1,300, and 2,400 grit Al_2O_3 abrasive papers, followed by 1 and 0.25 μm diamond and 0.1 and 0.05 μm alumina polishing suspensions (Struers, Copenhagen, Denmark). The specimens were ultrasonically cleaned in deionized water for 10 minutes, dried for 72 hours in a desiccator and carbon sputter-coated. On the cross-sectioned surfaces, the demineralization bands were identified at ×600 magnifications in a phase contrast of backscattered electron imaging (BSI) mode of SEM. To identify variations in the amounts of elements from the outer surface throughout the inner dentin, two line analyses with a 120-μm span were performed perpendicular to the outer root surface (Figure 2). The spot measurements at 1-μm-pixel intervals were obtained from the observation areas (surface layer, demineralized layer, and sound dentin) which were identified in SEM images. Each measurement within the observation area was summed and averaged into a single value for analysis. The unit of value was the intensity of the characteristic x-ray per 200 msec, which indicated the relative weight of each element. A fluorapatite crystal (3.38% fluoride) was used as a standard for Ca, P, and F. Operating conditions for both the image and elemental analyses of EDS were 15 kV of accelerating voltage and 50 nA of beam current.

CLSM analysis

Six premolars were de-coronated, and the root segments were mesiodistally sectioned into halves. Each buccal segment had a window of 3 mm × 4 mm pro-
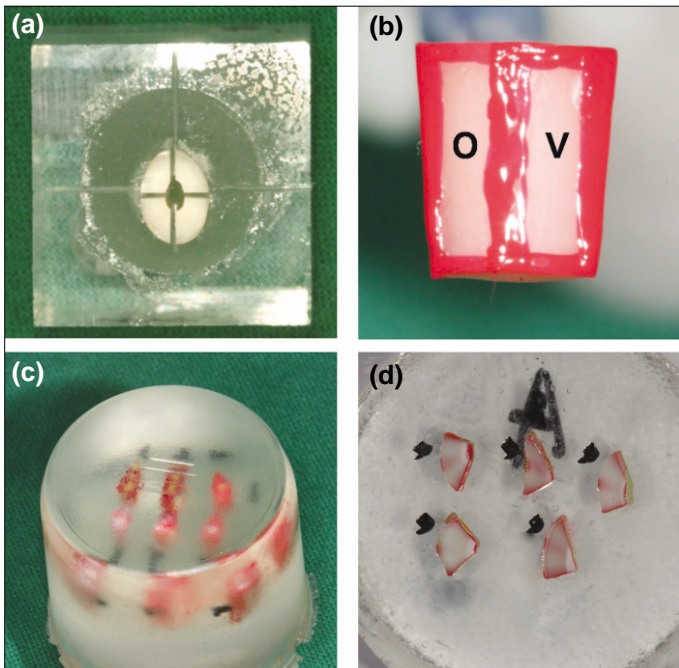
Figure 1. Specimen preparation. (a) The tooth was embedded in acrylic resin followed by crown removal. The coronal root was sectioned into quarters. (b) Two 4 mm × 1 mm windows were created by nail varnish coating. Fluoride varnish was applied to one window (v), while the other window (o) had no varnish application. (c) Specimens were embedded in epoxy resin. (d) The epoxy resin block was horizontally cross-sectioned in the midline of specimens and polished to expose the observation surface. The dark arrows were drawn on the surface of the specimen to distinguish the varnish applied windows (v) from the no-varnish applied windows (o).
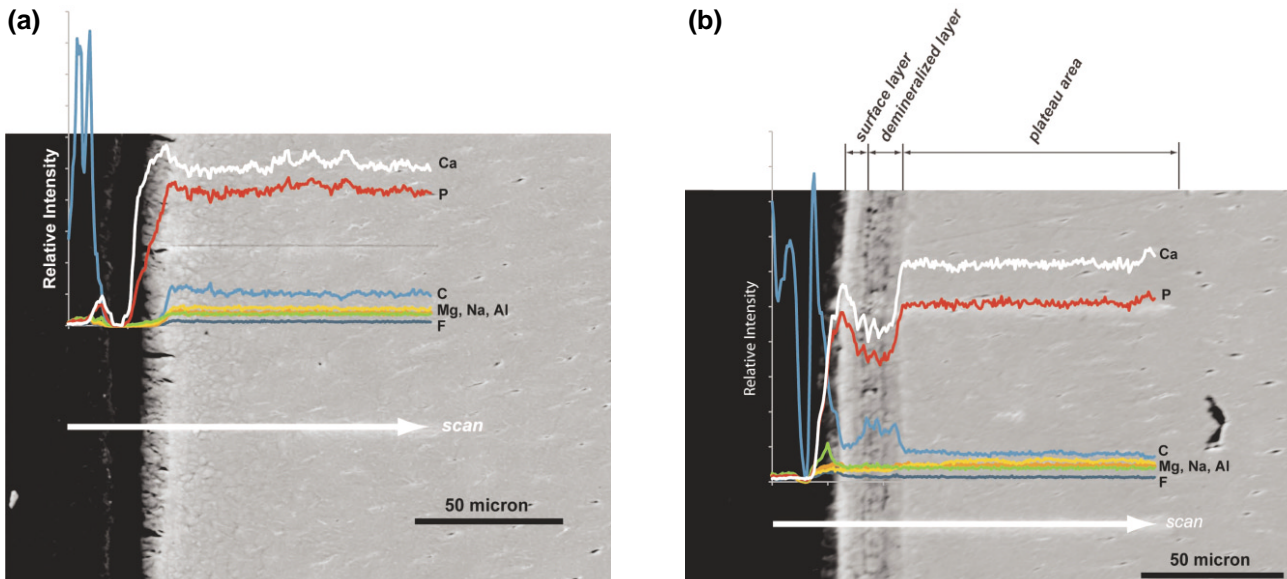

Figure 2. SEM images of 2 specimens produced from a single tooth (×600). Elemental analysis along the scan line illustrates the differentiated elemental composition. (a) Group Ao. No demineralization band appeared. (b) Group Do. Repeated demineralization bands were produced from a double pH cycling. Note that Ca and P levels dropped in the demineralized layer. C (carbon) level was elevated at the same area. It was assumed that diamond particles of polishing paste were impregnated in a porous structure. Other elements were detected by a small amount, which was at an insignificant level.

duced via application of nail varnish. The windows were coated with 0.4 mL of Fluor Protector mixed with 10 ppm by weight of propidium iodide (Calbiochem, San Diego, CA, USA). After storage in 100% humidity for 24 hours at 37℃, the varnish layer was pulled off. The specimens were mounted, sectioned, and polished in the same way as for the SEM/EDS analysis. The polished surfaces were

examined with CLSM using a C-Apochromal ×40 water lens with an additional ×40 objective lens and ×2 digital zoom (×800 total magnification). A He/Ne G laser was used with an excitation band at 543 nm wavelength and a 560 nm-long pass filter.

Statistical analysis

The Ca and P contents in the sound dentin were set to 100%, and the loss of the contents in the surface layer and in the demineralized layer were calculated as a % (Table 3, Figure 2). The distribution of caries lesion depth and weight loss of Ca and P in percentages were found to be approximately normally distributed using the Shapiro-Wilk test ($p > 0.05$). To compare the mean lesion depth between single and double pH cycling groups, the analysis of variance (ANOVA) with the Tukey's multiple comparison method was used. The mean weight percentages of Ca and P between the with and without varnish groups were compared using the paired t-test. Based on the evaluation of the SEM images of the specimens in the Group Ao, five teeth were assigned to the thick-cementum group and five teeth were assigned to the thin-cementum group. The mean weight percentages of Ca and P between thick- and

Table 2. Means and standard deviations of surface layer and demineralized layer widths (μm)

| pH cycling | Group | N | Width | | | p value |
			Surface layer Mean (SD)	Demineralized layer Mean (SD)	Total lesion Mean (SD)	
None	Ao	10	0 (0)	0 (0)	0 (0)	⟨ 0.01
	Av	10	0 (0)	0 (0)	0 (0)	
Single	Bo	10	4.7 (1.0)	7.6 (2.9)	12.3 (2.6)[a]*	
	Bv	10	4.2 (1.2)	8.5 (2.2)	12.7 (2.1)[a]	
	Co	10	4.7 (1.1)	7.6 (2.8)	12.3 (2.6)[a]	
	Cv	10	6.0 (1.4)	6.9 (2.2)	12.9 (2.9)[a]	
Double	Do	10	6.7 (1.4)	12.9 (3.4)	19.6 (3.8)[b]	
	Dv	10	6.3 (2.3)	12.9 (3.3)	19.2 (3.9)[b]	

*Different superscript letters denote values that are significantly different from one another. Statistical analysis was performed at the significance level of 0.05. More precisely, p-values related to the above figures with different superscript letters were all below 0.01.

Table 3. Mean losses of weight percentages of Ca and P in the surface layer and the demineralized layer compared among groups with inner sound dentin levels set to 100%

| Group | N | Surface layer / Ca | | Demineralized layer / Ca | | Surface layer / P | | Demineralized layer / P | |
		Mean (SD)	p value	Mean (SD)	p value	Mean (SD)	p value	Mean (SD)	p value
Ao	10	0 (0)	–	0 (0)	–	0 (0)	–	0 (0)	–
Av	10	0 (0)		0 (0)		0 (0)		0 (0)	
Bo	10	1.1 (6.7)	0.056	12.1 (4.8)	0.465	− 0.4 (7.1)	0.030	13.4 (5.8)	0.227
Bv	10	6.9 (7.6)		13.9 (4.8)		17.3 (18.5)		18.0 (8.3)	
Co	10	1.1 (6.9)	0.351	12.1 (4.6)	0.040	− 0.4 (6.1)	0.049	13.4 (6.8)	0.042
Cv	10	4.4 (9.0)		15.3 (4.8)		21.1 (28.8)		25.2 (17.2)	
Do	10	3.5 (5.0)	0.985	13.8 (6.0)	0.259	4.7 (5.7)	0.654	14.5 (6.2)	0.254
Dv	10	3.4 (8.8)		10.5 (8.0)		5.9 (8.4)		11.6 (7.7)	

thin-cement groups were evaluated using an independent *t*-test. A *p* value of 0.05 was selected as the threshold for statistical significance. Analyses were performed using SPSS version 12.0 statistical software (SPSS Inc., Chicago, IL, USA).

RESULTS

SEM exhibited demineralization band formation underneath root surfaces that had undergone pH cycling (groups B, C and D), while such demineralization was not observed in group A (Figure 2). The average lesion depth (SD) in the single pH cycling groups (groups B and C) was 12.5 (2.5) μm, significantly smaller than the average lesion depth in the double pH cycling group (group D), 19.4 (3.8) μm (*p* 〈 0.05, Table 2). There were no significant differences in lesion depths between subgroups o (no varnish) and v (varnish) within each group (*p* 〈 0.05).

In group A, no changes in the Ca and P contents from the outer surface to the inner dentin depth of 120 μm were distinguishable, and the loss was set to 0%. In the surface layer, between the varnish and no varnish groups (Ao/Av, Bo/Bv, Co/Cv and Do/Dv), there were no significant differences in Ca content (*p* 〈 0.05). But, Bv and Cv had significantly higher P loss, as compared with each counterpart (Bo and Co) (*p* 〈 0.05). In the demineralized layer, the Ca and P contents significantly decreased in all groups except group A (*p* 〈 0.05). These patterns of elemental change were concordant with the microscopic images (Figure 2). The average Ca loss (SD) was 13.4 (4.8)% in the single pH cycling groups (groups B and C) and 12.7 (7.1)% in the double pH cycling group (group D). The average P loss (SD) was 17.5 (11.1)% in the single pH cycling group and 13.0 (7.0) % in the double pH cycling group. There were no significant differences in Ca and P loss between the single pH cycling and the double pH cycling groups (*p* 〈 0.05). Between the varnish and no varnish groups, Ca and P loss were significantly higher in Cv than in Co in the demineralized layer (*p* 〈 0.05).

The mean thickness (SD) of cementum was 38.4 (18.8) μm in the thick-cementum group and 2.8 (4.4) μm in the thin-cementum group. There were no significant differences in lesion depth or in Ca and P contents between the thick-cementum group and the thin-cementum group (*p* 〈 0.05) (Table now shown). The teeth from the thick-cementum group had a demineralized layer within the cementum layer (Figure 3). An image of the varnish applied to the root surface is shown in Figure 4. Varnish impregnation into the root surface is represented by the red fluorescent band. The depth of impregnation did not exceed 15 μm in any specimen.
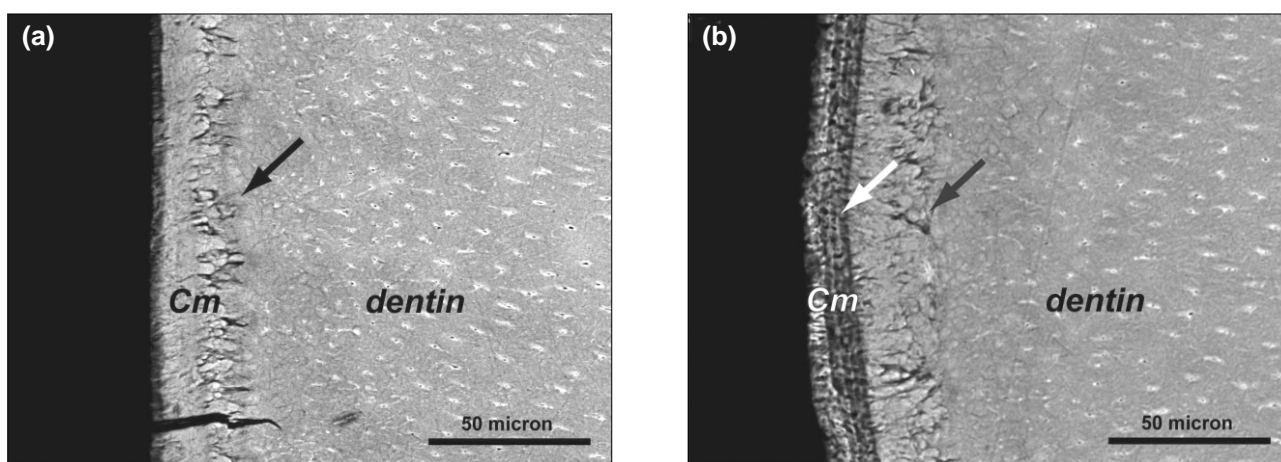

Figure 3. Two specimens from a single tooth in the thick cementum group (×600). Dark arrows indicate a junction between cementum (Cm) and dentin. While the sound specimen (group Ao) (a) shows no sign of demineralization, the varnish-applied and pH-cycled specimen (group Cv) (b) has the repeating bands of demineralization and remineralization in the outermost cementum layer (white arrow).
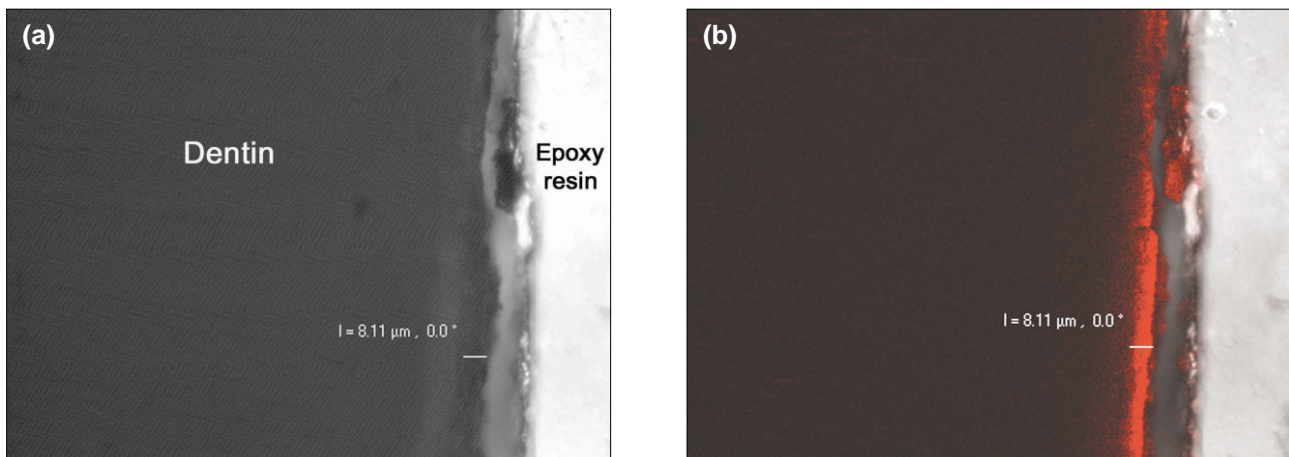
Figure 4. CLSM presents the cross-sectional image of the varnish impregnated root surface (×800). (a) Differential interference contrast image shows the varnish applied root surface without a fluorescent profile. (b) Laser fluorescence image shows the varnish layer as a band of red fluorescence. In the upper middle, free remnant of varnish was detached from the dentin and the superficially infiltrated varnish remained in the surface.

DISCUSSION

In this study, we induced a low level of pH cycling on root surfaces and evaluated the influence of fluoride varnish on the initiation of root caries. Among the three experimental groups that were subjected to pH cycling (groups B, C, and D), in group B, pH cycling initiated demineralization and varnish was used as a potential therapeutic agent. Group C represented clinical situations in which varnish is applied to sound root surfaces as a preventive measure, and then the varnish-coated surface was subjected to an acid challenge. In group D, an initial lesion was created on the root surface, and varnish was applied. The tooth was then subjected to a second pH cycling to determine how the varnish-treated lesion would react to a persistent acid challenge. We applied pH cycling without agitation and produced a purely intact surface layer width of 10 – 15 μm and a demineralized subsurface layer with less than 20% mineral loss. Our second pH cycling extended the lesion, increasing the depth of both the surface layer and the demineralized layer. The surface layer remained well-mineralized, implying that the original minerals in the surface layer acted like a nucleus of mineral growth and an ion transport barrier.[15] In the subsurface layer, multiple electrodense bands

appeared in the SEM images, demonstrating the dynamic process of mineral loss and gain. This observation scope was relatively small, considering the resolution of conventional microradiography and light microscopy used in previous studies.[10,14] In this study, SEM was shown to be useful in detecting minute changes in initial caries development. Also, it may be applicable to elaborate a dose-response relationship of non-operative caries control protocols in future studies.

Quantitative analysis of the mineral composition of the root substrate is influenced by individual variation.[16] We split a single tooth into quarters, allocating the pieces equally into four groups, to minimize substrate differences. The window to which the varnish was applied was compared to the adjacent non-varnish window in order to decrease location variance. The baseline Ca and P contents were set at the inner dentin, where the Ca and P levels plateaued in scan analysis. Using these values, we determined the volume percentages of demineralization in reference to a standard value acquired from sound dentin, as other studies did in microradiographic analysis.[17] Our single cycling (5 days pH cycling plus 2 days remineralization) without agitation induced no significant loss of Ca or P in the surface layer, but a mild amount of demineralization in the subsurface layer. The second

cycle only increased the total width of the reactive zone, i.e. in both the surface layer and the subsurface demineralized layer. And Ca and P contents were at levels similar to those following the first cycling. We were interested in determining how fluoride would affect the surface-preserved demineralized lesion. On mineral-dissolved enamel and dentin, fluoride promotes the formation of fluoridated hydroxyapatite. However, a well-preserved surface layer may block ion transport to the deeper lesion, and the fluoride-driven remineralization process may not occur as readily as in lesions with a mineral-deficient surface layer.[15] In our study, no additional remineralizing patterns were demonstrated in the varnish subgroups of all three experimental groups. In other way, it was shown that varnish application dissolved more P from the surface layer in groups B and C and more Ca and P from the demineralized layer in group C ($p < 0.05$). Acidulated fluoride solutions dissolve enamel slightly and deposit substantial amounts of calcium fluoride on the tooth surface.[18] Similarly, the acidic Fluor Protector (pH 2.5, measured in our study) reacts with dentin which has half the mineral content of enamel.[19] The erosive changes from the acidic nature seemed to be recovered by further pH cycling. The levels of Ca and P were regained by the second cycling, and the Dv group did not show a significant difference from the Do group ($p < 0.05$). SEM demonstrated the unique profiles of cementum and dentin, although their elemental distribution did not differ (Figure 3). Since cementum naturally has the same mineral distribution as dentin, its demineralization pattern may not be different.[2,20] Our analysis supported the claim that cemental caries underwent a change in elemental composition similar to that of dentin caries.

We intentionally removed the varnish layer to create mechanical wearing that occurs after a certain period of time in an oral environment, and to simulate the appropriately brushed root surfaces of a low risk population. The peeled-off varnish layer may still be effective if the varnish had penetrated into porosities and remained as impregnated remnants. Fluor Protector has a thin consistency and good wetting effect on both dry and hydrated dentin substrates.[21] However, using CLSM, Arends *et al.*

showed that retention of Fluor Protector was limited to the outer surface, which is similar to other varnish products.[21] They applied the varnish on the surface-softened lesions and produced a barrier with a thickness of 10 μm, which was consistent with our observations of the surface-preserved lesions. We attempted to detect any increased levels of fluoride in the surface zone, but were unable to do so, likely due to the low content level. Most studies using Fluor Protector employed a relatively severe acid challenge, resulting in some amount of surface disintegration, which may be more relevant to a high caries risk population.[3,5,6] In a high risk situation, the physical presence of the varnish enhances the redistribution of ions in the body of the caries lesion by creating a favorable gradient for inward fluoride diffusion and reduces the porosity of the lesion.[4] Under less severe conditions, the surface layer is well preserved, and an additional varnish barrier may not be advantageous. Silane, a bifunctional coupling agent with hydrophilic functional groups, is not stable in a hydrous environment.[22] A silane-based varnish may be as permeable as other polymerizable dentin adhesives and may provide an ion passage into the subsurface area. Our results were in accordance with those of a previous study that demonstrated that sound dentin with Fluor Protector applied was affected equally by pH cycling, relative to dentin without varnish.[23]

Recently, the use of not only fluoride varnish but also fluoride containing dentin adhesives and desensitizing agents have been advocated to protect root dentin from caries formation. The submicron level elemental analyses may be applicable to the quantification of anticarious activities afforded by physical protection of a film barrier and by the chemicotherapeutic effect of fluoride ions. Within the limits of this *in vitro* study, silane-based fluoride varnish did not affect demineralization patterns produced under a mild acid challenge. Varnish was restricted to the intact surface layer and did not penetrate into the subsurface area where mineral loss occurred.

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국문초록

불소 바니쉬 도포 후 초기 치근 우식 발현에 대한 정량원소분석

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연구 목적: 우식 저 위험군에서 저 농도 불소 바니쉬가 초기 치근 우식 예방 및 치료에 미치는 영향을 검증하고자 한다.

연구 재료 및 방법: 건전한 하악 소구치 10개를 준비하여 치관부를 제거하고 협설, 근원심 방향으로 치아 장축에 평행하게 절단 후 백악–법랑질 경계부 4 mm 하방에서 절단하여 40개의 시편을 제작하였다. 각각의 치근 표면에 형성된 4 mm × 1 mm 크기의 창에 Fluor Protector (1,000 ppm 불소 함유)를 도포하였다. 한 치아에서 나온 4 개의 시편을 다음과 같은 4개 실험군에 각각 배정하였다. A군: pH cycling 없이 불소 바니쉬 도포; B군: pH cycling시행 후 불소 바니쉬 도포; C군: 불소 바니쉬 도포 후 pH cycling시행; D군: pH cycling을 시행한 뒤 불소 바니쉬 도포 후 pH cycling 재 시행. 시편을 정중부에서 절단하고 단면 부위를 연마한 뒤 전자현미경으로 표면을 관찰하였다. X선 분광분석을 통하여 Ca과 P의 중량 비를 측정하고 공 초점 레이저 현미경으로 바니쉬가 도포된 치근 면을 관찰하였다.

결과: 치근 표면에는 평균 12.3 (2.6) μm (single cycling) 과 19.6 (3.8) μm (double cycling) 깊이의 우식이 형성되었다. 표층의 칼슘 함량은 정상 치근에 비해 유의할만한 차이가 없었으나($p > 0.05$), 표층 하 탈회 영역의 칼슘 함량은 유의할만하게 감소하였다($p < 0.05$). 불소 바니쉬의 적용은 건전한 치근이나 초기 우식이 있는 치근에 적용했을 경우 모두에서 유의할만한 차이를 가져오지 않았다($p > 0.05$). 바니쉬의 치근 상아질 투과 깊이는 표면 15 μm 이내에 한정되었다.

결론: 표층이 건전한 20 μm 이내의 초기 치근 우식 병소의 양상과 산 공격에 대한 변화를 관찰한 결과, 저 농도의 불소 바니쉬를 단기간 적용하는 것은 치근의 탈회 반응에 영향을 주지 못하였다.

주요단어: 바니쉬, 불소, 원소분석, 치근 우식증