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Effects of zinc fluoride on inhibiting dentin demineralization and collagen degradation *in vitro*: A comparison of various topical fluoride agents

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Root caries is developed because of demineralization followed by enzymatic collagen degradation. This *in vitro* study aimed to examine the inhibitory efficacy of ZnF_2 on dentin demineralization and collagen degradation. Bovine dentin specimens were treated either with ZnF_2 or HCl-acidified ZnF_2 (ZnF_2/HCl) and then demineralized. Anti-demineralization efficacy was assessed by TMR as mineral loss (ΔZ). The efficacy was compared with silver diammine fluoride (SDF), KF, and acidulated phosphate fluoride (APF). For evaluating anti-collagen degradation, EDTA-demineralized dentin specimens were treated by one of four fluoride agents [SDF, APF, ZnF_2/HCl, NaF] followed by collagenase challenge. The eroded depth of collagen layer in the lesion was assessed using optical microscope. ΔZ of SDF, KF, ZnF_2/HCl, and APF were significantly lower compared with ZnF_2 and Control (no treatment). Regarding anti-collagen degradation, SDF and ZnF_2/HCl demonstrated a significant difference in the eroded depth compared with Control. Although SDF possessed higher efficacy, ZnF_2/HCl might be beneficial as a staining-free agent.

Keywords: Dentin, Zinc fluoride, Demineralization, Collagen degradation

INTRODUCTION

With an increasing number of elderly people and teeth being retained within the elderly population, the prevention and treatment of root caries are becoming increasingly important in current dentistry[1]. A pathogenesis study of caries lesions demonstrated this multifactorial disease to be initiated from biofilms, in which bacterial acids demineralize tooth substrate followed by collagen degradation from host-derived collagenases[2]. To inhibit the progression and promote the healing of root caries, prevention and treatment strategies should focus on inhibiting collagen degradation and promoting remineralization.

Topical fluoride agents have been widely studied with regard to their anti-caries effects in dentin root surfaces. Among treatments of NaF, acidulated phosphate fluoride (APF), and silver diamine fluoride (SDF), SDF was the most effective[3,4]. It is unique in terms of anti-bacterial[5-7] and anti-enzymatic effects[8] with silver ions (Ag^+) in addition to the demineralization inhibition. These higher efficacies of SDF would be attributed to high concentrations of Ag (25.5%) and fluoride (44,880 ppm), leading to the acquisition of these ions on and into the dentin tissue[9]. However, its drawback of brown and black staining of treated teeth and gingiva is of high concern in terms of esthetics, thus its use must be limited.

Despite several studies regarding SDF efficacy, the understanding of how silver compounds exert the preventive effect on root caries has not yet been established[10], whereas the use of a high concentration fluoride paste with 5,000 ppm fluoride was reported to remineralize incipient root carious lesions[11].

Considering the composition of dentin volume, which is approximately 50% minerals, 30% collagen, and 20% water, collagen degradation appears to significantly contribute to caries progression. The role of collagenase, particularly host matrix metalloproteinases (MMPs), has been indicated in the caries process[2,12], whereas the MMP inhibitor prevented dentin caries in rats[13]. Another study revealed that mineral loss in dentin that was cyclically treated with acid and collagenase enzyme was greater than that of treated only with acid *in vitro*[14]. The collagen degradation by collagenase contributed to caries progression because the collagen network that acted as a barrier on the demineralized dentin was diminished. With this barrier, the acid diffusing inward and the dentin mineral diffusing outward were retarded, thereby inhibiting further demineralization of Ca and phosphate ions from the lesion body[15]. However, Klont revealed that there was no influence on lesion remineralization *in-vitro* when removing demineralized collagen by enzyme[16].

Present authors hypothesized that inhibiting collagen degradation leads to anti-demineralization in dentin. Thus, we further hypothesized that a new approach to prevent root caries by applying an active agent, which has fluoride efficacy (anti-demineralization and promotion of lesion remineralization), plus anti-

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collagen degradation would be more effective than an approach using only fluoride in preventing root caries.

Zn compounds have been used as active ingredients in dentifrice for anti-calculus[17], anti-microbial/gingivitis[18], and anti-malodor[19] for a long time. Moreover, Zn compounds are anti-collagenase agents[20,21], although the detailed mechanisms are not well clarified. Moreover, it was clinically proven that Zn did not interfere with fluoride efficacy in cases where zinc citrate and sodium monofluorophosphate were included in the dentifrice[22]. Thus, this study aimed to examine the potential of a new agent to be used in preventing root caries using HCl-acidified ZnF_2 (ZnF_2/HCl), which does not cause the staining associated with SDF. Since ZnF_2 is less soluble in water, its preparation in diluted HCl solution makes it soluble and be able to provide Zn^{2+} and fluoride ions. ZnF_2/HCl solution was expected to produce several different precipitates on the apatite surface such as ZnF_2, zinc phosphates and CaF_2 when it is applied to apatite, because ZnF_2/HCl would be neutralized by dissolution of apatite which is alkaline in nature. Moreover, our preliminary study comparing anti-collagen degradation effect of various zinc compounds (acetate, lactate, chloride, sulfate and nitrate) indicated the similar anti-collagen degradation effect among these zinc compounds. The null hypothesis is that ZnF_2/HCl did not reveal a potential of anti-demineralization and anti-collagen degradation effects *in vitro*.

MATERIALS AND METHODS

Specimen preparation

To prepare dentin specimens, cervical two thirds of bovine root dentin was cut into blocks with surface areas of approximately 2.5×2.5 mm^2 and embedded in a resin (GC Ostron RII, GC, Tokyo, Japan). The surface was ground flat using a SiC waterproof abrasive paper of 800–1000 grit. After that, the ground surface was coated by a waterproof nail varnish leaving a window (approximately 2×2 mm^2) as an area for tested.

Anti-demineralization test

Sixty dentin specimens were prepared and ultrasonicated for 5 min to remove the smear layer. Then, they were divided into six groups (n=10) and treated for 3 min by one of the fluoride agents shown in Table 1. One drop of 5 μL fluoride agent was applied to the window and then the treated window was briefly rinsed with deionized water dispended from washing bottle one by one for several seconds to remove unreacted fluoride solution. After that, the specimens were demineralized for 5 days in a demineralization solution (2.2 mM $CaCl_2$, 2.2 mM KH_2PO_4, 50 mM Acetic acid, 0.02% NaN_3, and pH5.0 that was adjusted by NaOH) at 37°C. Each group of fluoride-treated specimens was separately immersed in the demineralization solution (10 specimens/100 mL). The solution was not refreshed during the demineralization period.

After finishing demineralization, the specimens were cut into approximately 220-μm-thick sections using a low-speed diamond saw (Isomet 5000, Buehler, Lake Bluff, IL, USA) and then immediately immersed in a 70% aqueous glycerin solution to prevent them from drying out[23]. Transverse microradiography (TMR) imaging was performed after removing the excess glycerin from the sections and placing them on an X-ray glass plate (High Precision Photo Plate, Konica Minolta Photo, Tokyo, Japan), along with a 15-step aluminum step wedge. A soft X-ray generator (SOFTEX CMR-2, Softex, Kanagawa, Japan) was used under the conditions of a tube voltage of 20 kV, tube current of 2.5 mA, and exposure time of 10 min[23]. The obtained TMR images were digitally photographed using an optical microscope (SMZ1000, Nikon, Japan) and CCD camera (DS-Fi1, Nikon), and the digitized images were analyzed using an image analysis software (Image J, version 1.42q, Wayne Rasband, NIH, USA) and customized image processing software to calculate the lesion depth (LD: μm) and mineral loss (ΔZ: vol%・μm) as shown in Fig.1. LD is defined as a depth from baseline of the sound dentin surface (referred to the dentin front covered by nail varnish) to the lesion front where the mineral content was 95% of the sound dentin mineral content, *i.e.*,

Table 1 Fluoride agents used in this study

Group	Agent	Composition
1. SDF	Silver diammine fluoride (Saforide®, Bee Brand Medico Dental, Osaka, Japan)	F=44,880 ppm, Ag^+=25.5%
2. KF	Potassium fluoride	F=44,880 ppm (neutral pH)
3. APF	NaF in 0.1 M H_3PO_4	F=9,048 ppm (pH 3.8)
4. ZnF_2/HCl*	4.18% ZnF_2・$4H_2O$ in HCl	F=9,048 ppm, Zn=1.56% (pH 2.7)
5. ZnF_2	4.18% ZnF_2・$4H_2O$ (partially dissolved)	F=9,048 ppm, Zn=1.56%
6. Control	No fluoride treatment	—

* ZnF_2/HCl solution was prepared by adding 1.6 mL of 1.0 N HCl into 10 mL of 4.18% ZnF_2・$4H_2O$ and was stable at least 1 month.

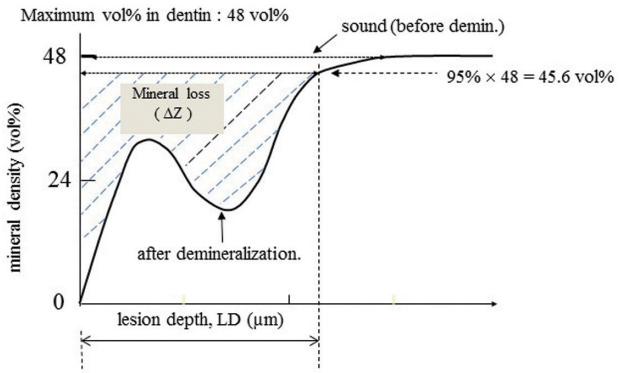

Fig. 1 The graph shows mineral density profile of demineralized dentin specimen.
LD: lesion depth and ΔZ: mineral loss.

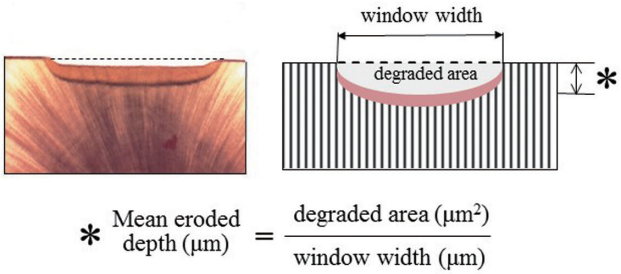

Fig. 2 Mean eroded depth measurement of the degraded collagen matrix by optical microscopic observation of ca.
200 μm-thick section.

45.6 vol%. On the other hand, ΔZ is defined as an area surrounded by the baseline and the mineral profile.

Anti-collagen degradation test

Ninety dentin specimens were prepared as described above. To expose the collagen network prior to fluoride agent application, the specimens were demineralized in 0.5 M EDTA with a pH of 7.4 at 4°C for 6 days. They were then allocated to four different fluoride groups (n=20) [SDF, APF, ZnF_2/HCl, and NaF (F=9,048 ppm at neutral pH)], leaving 10 specimens for the Control group (no fluoride treatment). The method of fluoride agent application was the same as in anti-demineralization test. The specimens in fluoride groups were further divided into two subgroups (n=10) after fluoride application. One subgroup was immersed for 30 s in distilled water (30-s immersion), and in order to examine the substantivity of fluoride compounds on treated surfaces, the other subgroup was immersed in artificial saliva (1.0 mM $CaCl_2$, 3.0 mM KH_2PO_4, 100 mM NaCl, 0.02% NaN_3, and pH 6.5) for 24 h (24-h immersion). There was no treatment in Control group, so no specimen was examined for 24-h immersion. After that, all specimens were immersed in a collagenase solution comprising of 12.5 unit/mL *Clostriticum histolyticum* enzyme (Type 1A, Cat# C9891, Sigma-Aldrich, MO, USA) in 50 mM HEPES buffer solution (0.36 mM $CaCl_2$, pH7.4) at 37°C for 6 h. The specimens were gently thin sectioned, including the lesion body. The depth of the degraded collagen layer in the lesion body was measured as demonstrated in Fig. 2. Under optical microscopy, the dentin thin section placed between the glass plate and coverslip in a wet condition was imaged. The microscopic image was then processed in ImageJ program to measure the degraded area (μm²) and the window width (μm). The mean eroded depth was calculated by dividing the degraded area by the window width.

Statistical analysis

For comparing dentin demineralization among the fluoride and Control groups, one-way ANOVA with Tukey's HSD test was performed with a significance level of p<0.05. For comparing the eroded depth of the collagen layer degraded by enzyme among the fluoride and Control groups, two-way ANOVA with Bonferroni *post-hoc* was performed with a significance level of p<0.05. Since this study did not carry out to examine the eroded depth for 24-h immersion in the Control group, the data of 30-s immersion were diverted to those of 24-h immersion.

RESULTS

Anti-demineralization test

Figure 3 compares the measured values of LD (a) and ΔZ (b) among the fluoride and Control groups. A statistically significant difference in LD was noted between SDF and the Control group, but not in the other fluoride groups. In contrast, significant differences in ΔZ were observed between the Control and the four fluoride groups, except ZnF_2 group. Overall, SDF revealed the highest efficacy among the groups; however, no significance was observed between SDF *vs.* KF and ZnF_2/HCl. Figure 4 indicates representative TMR images of Control and fluoride groups. The Control group indicated cavitated lesions, while the fluoride groups generated subsurface lesions with relatively intact surface layers.

Anti-collagen degradation test

Figure 5 shows the eroded depth of each group after 30-s and 24-h immersions. Two-way ANOVA did not indicate significant differences between 30-s and 24-h immersion in any studied groups. The differences in lowercase letters indicate the statistically significant differences among groups, regardless of immersion time. No statistical significance between the Control and APF or NaF groups. The SDF group of 30-s and 24-h immersion clearly demonstrated significant reduction in the eroded depth compared with the Control group (p<0.05), with the mean reduction rates of 96 and 87%, respectively. Moreover, the ZnF_2/HCl group exhibited significantly less erosion depth compared with the Control group (p<0.05), with the mean reduction rate of

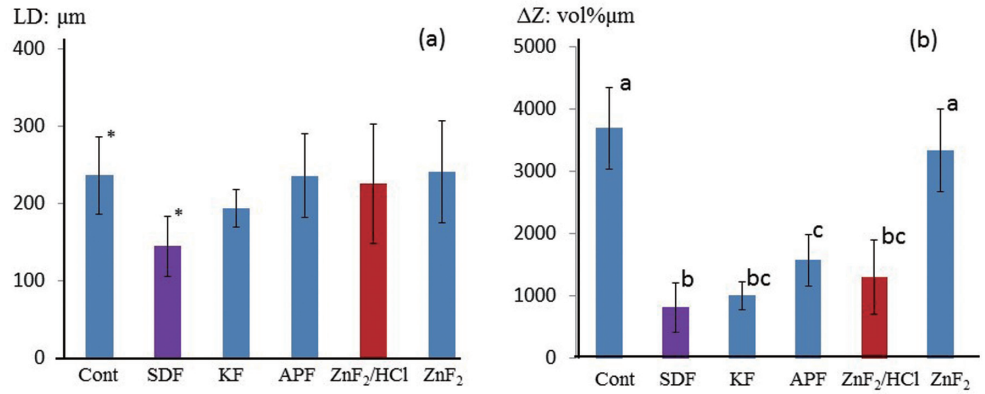

Fig. 3 Comparison of the lesion depth (LD: a) and mineral loss (ΔZ: b) among the 6 groups. (a) Lesion depth, LD. Asterisk (*) indicates the significant difference (p<0.05). (b) Mineral loss, ΔZ. Different lowercase letters indicate the significant differences (p<0.05).

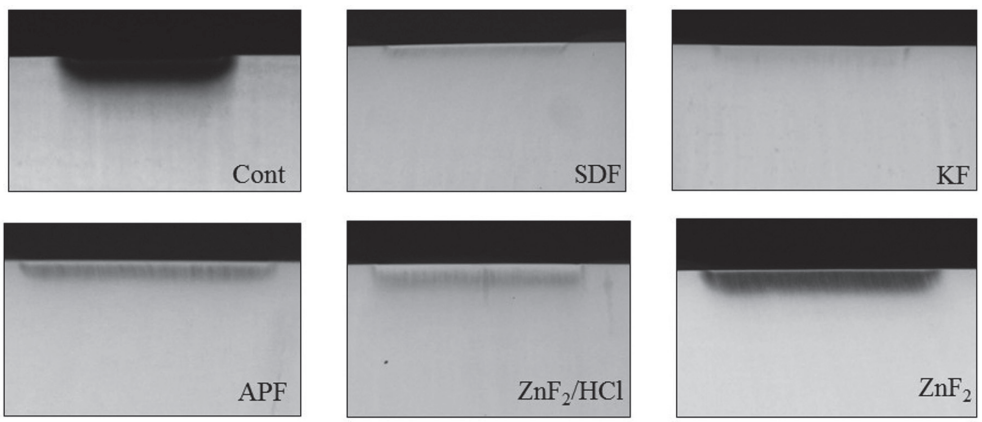

Fig. 4 Representative TMR images of Fluoride and Control groups. Note that Control group exhibited cavitated lesion, whereas Fluoride groups exhibited subsurface lesions with relatively intact surface layer.

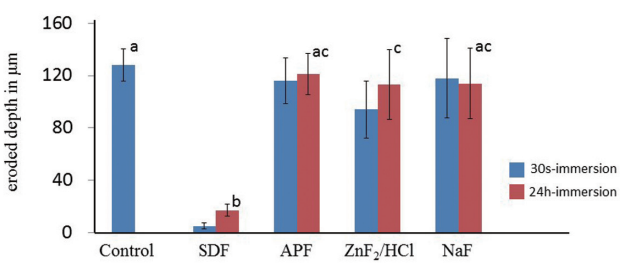

Fig. 5 Comparison of the eroded depth of collagen layer among the 5 groups. Different lowercase letters indicate the significant differences among fluoride application and Control groups (p<0.05). No statistically significant differences between 30 s- and 24 h- immersion were indicated.

27% for 30-s immersion. Regarding color staining, SDF treated specimens exhibited dark staining on treated surface, whereas no coloring was observed at the treated surface in ZnF_2/HCl group.

DISCUSSION

SDF comprises of high concentrations of fluoride and Ag, thus one can anticipate greater efficacy in preventing root dentin caries[3]. However, SDF results in a strong browning of dentin surface and gingiva, thereby causing esthetic concerns. We investigated the potential of using ZnF_2 to inhibit dentin demineralization and collagen degradation without the browning of tooth surfaces.

The rationale behind using ZnF_2 was that Zn compounds, such as $ZnCl_2$ and zinc acetate, were studied as a combination to lower fluoride concentration in NaF and were surprisingly found to promote lesion

remineralization[24,25] by acting as a mineral regulator to control the porosity at the lesion surface. It was of interest to include fluoride and zinc together because they are both powerful elements for remineralization. However, the obstacle of using ZnF_2 was that $ZnF_2 \cdot 4H_2O$ is less soluble in water (1.52%). Therefore, a minimal amount of HCl was added to dissolve it. The acidic ZnF_2/HCl solution would be neutralized when applied to the dentin surface. Thus, ZnF_2/HCl was expected to produce precipitations of Zn^{2+}-containing compounds, such as phosphate and fluoride, and CaF_2-like materials[26]. These compounds are inherently less soluble at a range of neutral pH, providing a reservoir for zinc and fluoride.

Regarding anti-demineralization efficacy, a similar tendency was observed between LD and ΔZ in this study. However, the statistically significant difference in LD was noted only between the Control and SDF groups. In contrast, several significant differences were detected in ΔZ among the fluoride groups, thereby implying less sensitivity of LD than ΔZ in the demineralization process. Therefore, the discussion would be made based on the ΔZ data. The result revealed that the SDF group possessed the highest efficacy on anti-demineralization. This might be attributed to a higher concentration of fluoride (44,880 ppm) in SDF compared with the lower concentration (9,048 ppm) in the APF and ZnF_2/HCl groups. However, the differences in efficacy were not obvious, despite the 5 times difference in fluoride concentration. It should be noted that the high Ag concentration (24%) in the SDF solution was not responsible for the anti-demineralization effect as a previous study reported almost complete inhibition of enamel demineralization using 2.36 M AgF (25.5% Ag, 44,840 ppm F) or KF (44,840 ppm F), but no significant difference between 2.36 M $AgNO_3$ and the untreated (control) groups was observed[27].

An overall finding indicated that the anti-demineralization efficacy was ranked in the order of SDF≒KF>ZnF_2/HCl>APF>ZnF_2. It is interesting that ZnF_2/HCl exhibited the highest efficacy among lower fluoride concentration groups. Although the investigation regarding the quantitative elemental analyses of Ca, F, or Zn on the treated dentin surface was not performed in this study, one could speculate that the application of ZnF_2/HCl might produce a larger amount of CaF_2-like material because of a much lower pH (2.7) than that of APF (3.8). The generated CaF_2, in turn, became a fluoride reservoir and impeded further demineralization[28]. Moreover, the precipitation of the less soluble ZnF_2 from the ZnF_2/HCl solution yielded the same possible long-term efficacy as CaF_2-like material. In contrast, less efficacy of the ZnF_2 group in anti-demineralization would be because ZnF_2 was less soluble in water and the non-sustainability of precipitates, resulting in less soluble fluoride for anti-demineralizing action.

We must be cautious when we compare TMR data of the specimens, which are treated by solutions containing heavier metal ions, such as Ag and Zn than Ca, in this study. These metal ions, when incorporated in dentin tissues, will demonstrate higher radiopacity, which manifests as a higher mineral density than the actual mineral density based on Ca, thereby overestimating the mineral density. Lambert-Beer's law predicts greater X-ray absorption in Ag and Zn than in Ca when exposed to 20 kV X-ray, therefore a supplemental experiment was conducted to estimate the influence of Ag in SDF and Zn in the ZnF_2/HCl solution on the real dentin mineral density. The analysis of TMR images taken just after the treatment did not indicate higher radiopacity at the lesion surface than that in the control area, which was not exposed to the solutions (data not shown), probably because of these ions accumulating in lesser amounts. Moreover, the TMR images revealed no substantial mineral loss during the treatment, which may be because of short time of treatment, *i.e.*, 3 min and application of fluoride rich solution which inhibits demineralization. EDTA-demineralized dentin specimens were used for evaluating anti-collagen degradation efficacy because minerals were not detectable by TMR analysis in the demineralized lesions compared with the dentin specimens demineralized in an acetic acid buffer, which produced a variation of mineral content that remained in the lesion (data not shown). This scenario of acetic acid buffer demineralization could interfere with the function of enzymes in the collagen degradation process.

Similar to anti-demineralization efficacy, SDF demonstrated an almost perfect inhibition of collagen degradation, even after a 24-h immersion. This could be attributed to the high Ag concentration (24%) as neither APF nor NaF demonstrated the inhibition of collagen degradation (Fig. 5)[29]. Previous study comparing the anti-degradation efficacy among the 38% SDF, 10% NaF, 42% $AgNO_3$, and water (Control) showed that both SDF and $AgNO_3$ similarly exhibited better efficacy compared to Control; whereas NaF did not[30].

There was a concern that no direct comparison was performed between 30-s immersion and 24-h immersion in the Control group. It should be noted that the immersion of EDTA-demineralized dentin in artificial saliva for up to several days or weeks did not induce remineralization[31-33]. On the other hand, there is a study revealed that demineralized dentin immersed in artificial saliva for up to 21 days showed constant (slight) activity of endogenous collagenases, comparing to 1-day immersion[34]. Therefore, it could be assumed that activity of endogenous collagenases would be minimum and the properties of collagen in 24-h immersion of Control group would not be different from 30-s immersion.

Although fluoride was reported to potentially inhibit the activity of MMPs from human saliva, the inhibition was time dependent[35]. The application time of fluoride agents in this study may not be sufficient. However, the ZnF_2/HCl group exhibited a significant reduction in the eroded depth compared with Control group. Although Zn was reported to be an essential element for collagenase activity[36], the excess amount of Zn was hypothesized to inhibit the collagenase enzyme by

binding to collagen at the cleavage sites[20,21]. The present statistical analysis of the eroded depth among the fluoride groups was not able to detect a difference in immersion factor (30-s and 24-h immersion). This is most probably due to greater deviation and insufficient specimen numbers. However, simple comparison in terms of the mean values between 30-s and 24-h immersion in SDF, APF and ZnF_2/HCl groups suggests a tendency of the lower efficacy in the 24-h immersion group than the 30-s immersion group. This might imply the labile adsorption of Zn^{2+} to collagen molecules.

In general, the hydroxyproline assay technique[37] was used to evaluate the degradation of collagen in biological tissues as a gold standard. The microscopic technique employed in this study was a new trial in collagen degradation assessment, therefore this new technique had to be validated to the hydroxyproline assay technique. Our previousstudy demonstrated the correlation between the hydroxyproline technique and microscopic technique in quantifying the degraded (eroded) collagen layer with Pearson's correlation coefficient of 0.94[38]. Therefore, in this study, we employed a microscopic observation technique for evaluating the anti-collagen degradation efficacy (Fig. 2), which was more beneficial for visual illustration in demineralized dentin lesion.

CONCLUSION

This study revealed that SDF seemed to possess higher efficacy than that of ZnF_2/HCl regarding anti-demineralization. Regarding anti-collagen degradation efficacy, SDF exhibited very strong efficacy followed by ZnF_2/HCl. These results suggested that ZnF_2/HCl might have potential benefit as a staining-free agent to tooth substrate for root caries prevention.

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