

Remineralizing efficacy of fluoride in the presence of oral microcosm biofilms

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ABSTRACT

Objectives: The oral biofilm structure or composition can affect the penetration of remineralizing agents. Therefore, this study evaluated the remineralizing efficacy of fluoride using a pH-cycling model with oral microcosm biofilms.

Methods: Artificial carious lesions were formed in 80 bovine incisors. The pH-cycling conditions with or without oral microcosm biofilms were applied to 40 specimens each. The pH-cycling scheme was repeated for 12 days. Fluorescence loss (ΔF ,%) of early carious lesions was measured for all specimens using a quantitative light-induced fluorescence-digital camera, before and after fluoride application. Biofilms on specimens were further analyzed for red fluorescence intensity (red/green ratios) and colony-forming unit counts. The effects of pH-cycling conditions and treatments on changes in ΔF ($\Delta\Delta F$) and the effects of interactions between factors were analyzed using two-way analysis of variance.

Results: The fluoride-treated group with oral biofilms had an approximately 0.89-fold lower $\Delta\Delta F$ than the fluoride-treated group without oral biofilms ($p < 0.0001$). When oral biofilms were absent, the fluoride-treated group showed a 1.31-fold greater $\Delta\Delta F$ compared to that in the distilled water-treated group ($p < 0.0001$). When oral biofilms were present, the fluoride-treated group showed a 1.14-fold greater $\Delta\Delta F$ compared to that in the distilled water-treated group; however, this difference was not statistically significant.

Conclusions: There was a significant difference in fluoride remineralizing efficacy based on the presence of biofilms on early carious lesions. Therefore, fluoride remineralization assessment in the absence of oral biofilms could lead to an overestimation of efficacy.

Clinical Significance: Fluoride application might not have a robust remineralization effect on early carious lesions in the presence of a mature biofilm on the tooth surface.

1. Introduction

It is highly likely for early caries to be present underneath oral biofilms that have not been removed for a long time [1]. These mature biofilms will have more complex three-dimensional relationships among the cells, matrix, space, and substrate than younger biofilms and will have a heterogeneous architecture [2]. This structure of the biofilm might impede the fast penetration of remineralizing materials, and the presence of a biofilm on the early carious lesion could thus reduce the remineralizing efficacy [3]. A recent study proved that the presence or absence of biofilms can affect the efficacy of anticariogenic agents [4]. However, this finding was based on evaluations using a biofilm formed with only one strain. The structure of the oral biofilm is built up through

the interactions of numerous bacteria. In this process, bacteria induce oral biofilm-associated diseases through ecological changes [5]. Therefore, oral biofilms made of certain types of bacteria cannot adequately reflect the physiological situation in the oral cavity [6].

An *in vitro* biofilm model should reflect the natural diversity and characteristics of oral biofilms [7]. Recently, a novel pH-cycling model was proposed using oral microcosm biofilms [8]. This pH-cycling model was based on the inoculation of human saliva, which simulates the oral microbial environment under controlled experimental conditions and reproduces the heterogeneity and variability of oral biofilms [9]. Nevertheless, the results of this previous study did not find a significant difference in the remineralizing efficacy of 2% NaF solution according to the presence or absence of a biofilm. Previous studies reported that the

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rate and extent of agent penetration can depend on factors including the biofilm structure and composition [10,11]. Therefore, The aim of this study was to evaluate the remineralizing efficacy of fluoride using the pH-cycling model in oral microcosm biofilms, based on the hypothesis that the remineralizing efficacy of fluoride in early carious lesions would differ in the presence of the simulated biofilms.

2. Materials and methods

2.1. Preparation of specimens with artificial carious lesions

This study was approved by the Institutional Review Board of our institution (Approval number: 1,044,396–201,805-HR-118–02). The sample size was calculated based on a two-way analysis of variance (ANOVA) using G*power 3.1 (Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany). According to the results of a study by Hwang et al. [8], the required sample size was 80, with an effect size (f) of 0.368, an alpha level of 0.05, and 90% power. Enamel blocks having a 6 mm diameter and 5 mm height were sectioned from lesion-free anterior bovine teeth (Fig. 1). All blocks were ground sequentially using 400–1200-grit sandpaper and a polishing machine (M-PREP 5TM, Allied High Tech Products, Inc., Compton, CA, USA) and covered with an acid-resistant nail varnish on all surfaces except for one-half of the facial surface (Trendy Nails, The Face Shop, Seoul, Republic of Korea). After the varnish dried, the blocks were immersed in 45 mL of demineralizing solution at pH 4.8 for 40 days at 37 °C. The solution contained 50% hydroxyapatite and 1% Carbopol (Carbopol® ETD 940 polymer, Rowellrico, Goyang, Republic of Korea) in a 0.1 M lactic acid base, which was changed at 5-day intervals. After 40 days, a quantitative light-induced fluorescence-digital camera (QLF-D Biluminator™2+, Inspektor Research Systems BV, Amsterdam, Netherlands) was used to measure fluorescence loss (ΔF ,%) in each block. In total, 80 blocks with ΔF in the range of -37.5 to -26.3 (mean, -31.08 ± 2.09) were selected. After early carious lesions were formed, the blocks were embedded in round acrylic molds, with a space of 1 mm above each block; specimens were then placed in 24-well cell culture plates (SPL Life Sciences, Gyeonggi-do, Republic of Korea).

2.2. Preparation of oral microcosm biofilms

Stimulated saliva was collected from a healthy adult donor with no history of antibiotic use within the previous 3 months and no active caries or periodontal disease. This was used to inoculate 40 of the plated bovine specimens, with 1.5 mL per well in the 24-well plates with enamel specimens [12], whereas the remaining 40 specimens were kept as controls. The plates were then incubated at 37 °C for 4 h in an atmosphere of 10% CO₂, following which the remaining saliva was carefully removed from the bottom of each well. Subsequently, to form the biofilms, each specimen was supplied with 1.5 mL of growth medium

containing 0.3% sucrose and basal medium mucin at pH 7.0 containing 2.5 g/L porcine mucin (Type III, Sigma Aldrich, St. Louis, MO, USA), 10.0 g/L proteose peptone (KisanBio, Seoul, Republic of Korea), 5.0 g/L trypticase peptone (KisanBio), 5.0 g/L yeast extract (KisanBio), 1 mmol/L urea (Georgia Chemical, Norcross, GA, USA), 1 mmol/L arginine (Georgia Chemical), 2.5 g/L KCl (KisanBio), and 1 mg/L menadione (Sigma Aldrich). This medium was changed at 24 h intervals over the 6-day period during which the oral microcosm biofilms matured.

2.3. Fluoride treatment under pH-cycling conditions

All specimens were placed in a demineralizing solution for 2 h daily and subsequently immersed in the remineralizing solution for 2 h (Fig. 2). They were then treated with either distilled water (DW) or 2% sodium fluoride (NaF, 9050 ppm F) solution for 4 min daily, according to experimental groupings. Four different groups were established, with 20 specimens per group. Group 1 was made up of non-biofilm-inoculated control samples that did not receive fluoride treatment; DW was used as a substitute. Group 2 included non-biofilm-inoculated samples treated with 2% NaF solution. Group 3 included biofilm-inoculated samples treated with DW. Group 4 included oral biofilm-inoculated samples treated with 2% NaF solution. All groups were then immersed in remineralizing solution for the remaining 24 h. This pH-cycling scheme was repeated for 12 days at 37 °C. Equal volumes (1.5 mL/well) of remineralizing, demineralizing, and treatment solutions were used and replaced daily.

The compositions of the remineralizing and demineralizing solutions varied depending on the presence or absence of biofilms, to maintain biofilm viability [8]. In specimens subjected to pH-cycling without biofilms (i.e., groups 1 and 2), the remineralizing solution was composed of 0.22% gastric mucin, 6.5 mM NaCl, 1.45 mM CaCl₂, 5.42 mM KH₂PO₄, and 14.94 mM KCl in 1 L DW, with a final pH of 7.0. The composition of the demineralizing solution was identical to that of the solution used to create artificial lesions, but its pH was adjusted to 4.5. In specimens subjected to pH-cycling with biofilms (i.e., groups 3 and 4), the remineralizing solution was composed of 0.2% sucrose, 300 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.3% yeast extract, 130 mM KCl, 0.9 mM K₂HPO₄, 1.5 mM CaCl₂, 4 mM L-glutamic acid, 1 mM L-arginine HCl, 1.3 mM L-cysteine HCl, and 0.1 mM tryptophan in 1 L DW at pH 7.0 [4,13]. The demineralizing solution (final pH 4.5) was composed of the same ingredients as the remineralizing solution, excluding sucrose and HEPES, and combined with 50 mM acetic acid.

2.4. Analysis of cariogenicity of oral microcosm biofilm

Before and after pH-cycling, oral microcosm biofilms were observed in the dark room using blue light at 405 nm with the QLF-D camera at a shutter speed of 1/45 s, aperture value of 10.0, and ISO sensitivity of

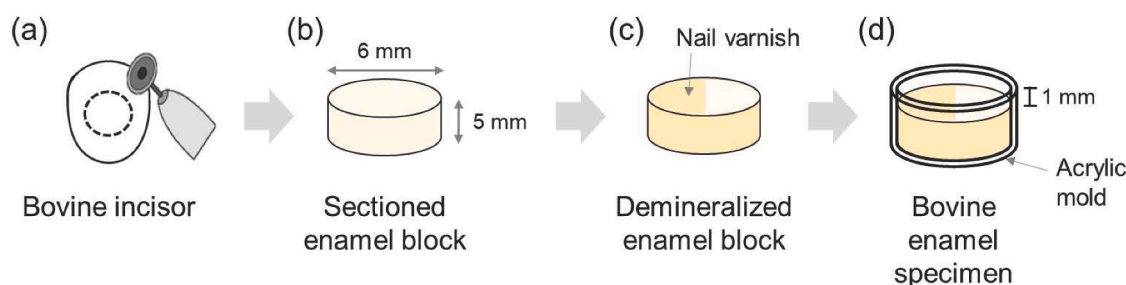


Fig. 1. Preparation of bovine enamel specimen. (a) A sound bovine incisor without defects such as cracks and white spots was selected. (b) The labial surface of the bovine incisor was sectioned and polished. (c) All surfaces excluding half of the labial surface were painted with acid-resistant nail varnish*; the enamel block was then demineralized. (d) The demineralized block as embedded in an acrylic mold with a space of 1 mm above the block for the formation of a dental microcosm biofilm as the final step of specimen preparation.

*In this study, transparent nail varnish was used; however, the varnish is illustrated in yellow to facilitate easier comprehension.

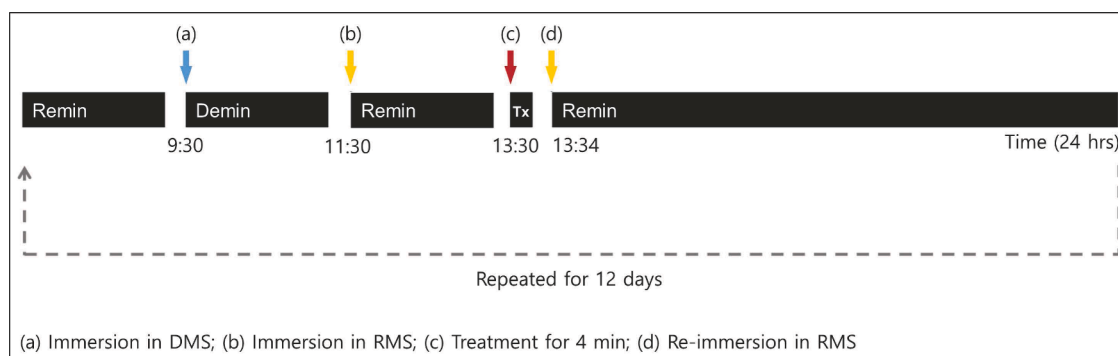


Fig. 2. Treatment schedule for 12 days.

Remin, remineralization; Demin, demineralization; Tx, treatments; DMS, demineralizing solution; RMS, remineralizing solution.

1600. During imaging, a constant distance of 10 cm was maintained between the specimen and the lens. Red and green intensities in the identified area were analyzed using Image-PRO software (Version 10, Media Cybernetics, Inc., MD, USA). The mean red intensity/green intensity (R/G) ratios in each biofilm were calculated to compare the red fluorescence intensity in the biofilms between groups 3 and 4 (Fig. 3).

After obtaining fluorescent images of biofilms, biofilm-covered enamel specimens were rinsed with 1.5 mL of cysteine peptone water (CPW) to remove free-floating bacteria loosely attached to the enamel block surface. Next, 40 specimens were moved to a conical tube containing 1 mL of CPW. The tubes were sonicated for 60 s and then vortexed for 60 s. The suspensions were serially diluted (10^{-1} to 10^{-7}), pipetted onto brain heart infusion agar plates at pH 4.8, and incubated for 72 h at 37 °C and 10% CO₂. Colonies of aciduric bacteria were then counted.

2.5. Analysis of changes in fluorescence loss on the early carious lesions

All enamel specimens, after removing the oral microcosm biofilms, were dried for 15 s using an air-dryer. Imaging of carious lesions on the specimens was performed as described using a QLF-D camera, and the ΔF for each lesion was analyzed using the white spot patch function of the QA2 program (Version 1.24, Inspektor Research System BV, Amsterdam, Netherlands) (Fig. 4). Changes in ΔF ($\Delta\Delta F$) before and after fluoride treatment were calculated. Larger $\Delta\Delta F$ values were considered to indicate a greater remineralization effect on early carious lesions.

2.6. Statistical analyses

All dependent variables were subjected to assessment of normality using the Shapiro-Wilk test. In accordance with a 2×2 factorial design, the main effects of oral biofilms and fluoride treatment on $\Delta\Delta F$ values were assessed using two-way ANOVA. A Bonferroni post hoc test was conducted to examine the effects of biofilms on $\Delta\Delta F$ in each treatment group. The Mann-Whitney U test was performed to compare red fluorescence intensities and aciduric bacterial counts in biofilms between treatments. All data were analyzed using PASW Statistics ver. 24.0 (SPSS Inc., Chicago, IL, USA), with differences considered significant at $p < 0.05$.

3. Results

There was a significant interaction between the biofilm and fluoride treatment based on the $\Delta\Delta F$ ($F = 7.377$, $p = 0.008$, Fig. 5). In the fluoride-treated group without an oral microcosm biofilm (group 2), $\Delta\Delta F$ was higher by 5.01 ± 3.91 compared to that in the DW-treated group without an oral biofilm (group 1; $p < 0.0001$, Table 1, Fig. 5). In the presence of an oral biofilm, the $\Delta\Delta F$ did not differ significantly between the DW-treated group (group 3) and the fluoride-treated group (group 4; $p > 0.025$, Table 1, Fig. 5). Following fluoride treatment, the $\Delta\Delta F$ for early carious lesions in group 2 without oral biofilms was higher by 2.76 ± 3.40 compared to that in group 4 with oral biofilms ($p < 0.0001$, Table 1, Fig. 5). When the R/G ratios and colony-forming unit (CFU) counts of the oral microcosm biofilms were compared between groups with

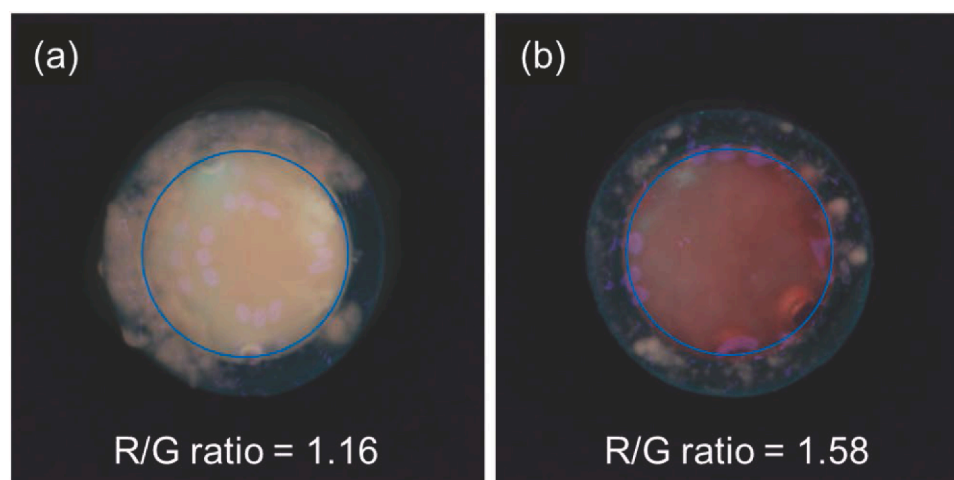


Fig. 3. Representative quantitative light-induced fluorescence-digital images showing the oral microcosm biofilm. The interior of the blue circle is the region of interest for the analysis of intensity of red and green. R/G ratio is the ratio of red intensity and green intensity in red fluorescence images of the oral biofilms. (a) Before pH-cycling; (b) after pH-cycling.

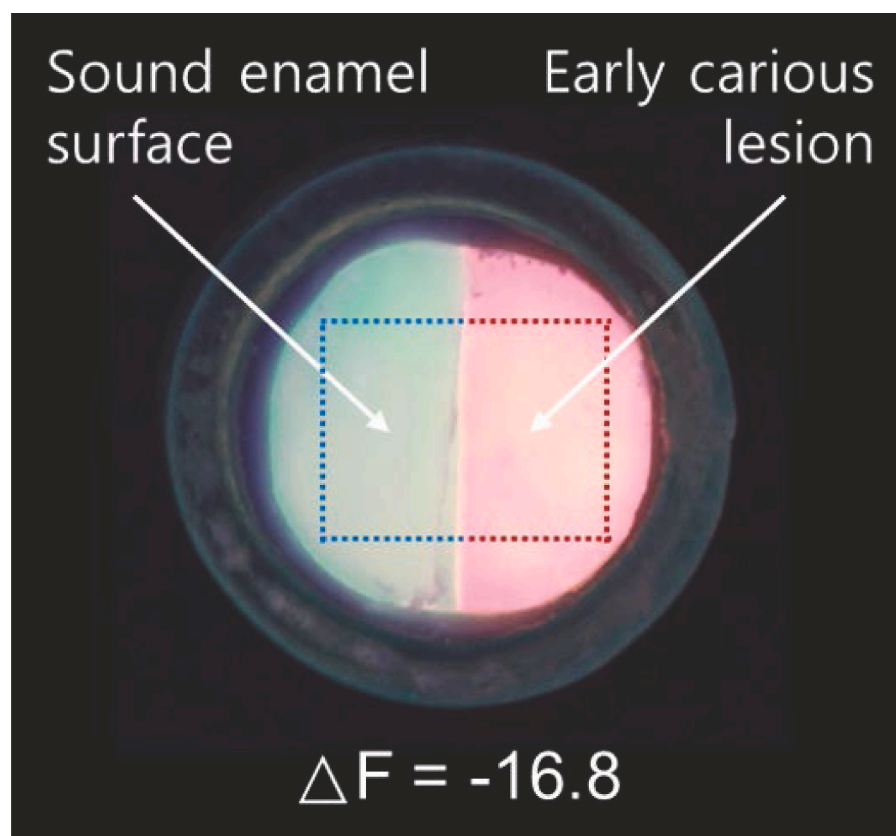


Fig. 4. Representative quantitative light-induced fluorescence-digital images showing early carious lesion. The interior of the rectangle is the region of interest for the analysis of the fluorescence loss in early carious lesion. A blue dotted line was placed on the sound enamel surface, and a red dotted line was placed on the surface of the early carious lesion using the white spot patch function of the QA2 program. ΔF (%) refers to the relative fluorescence loss of the surface of the early carious lesion compared to that of the sound enamel surface.

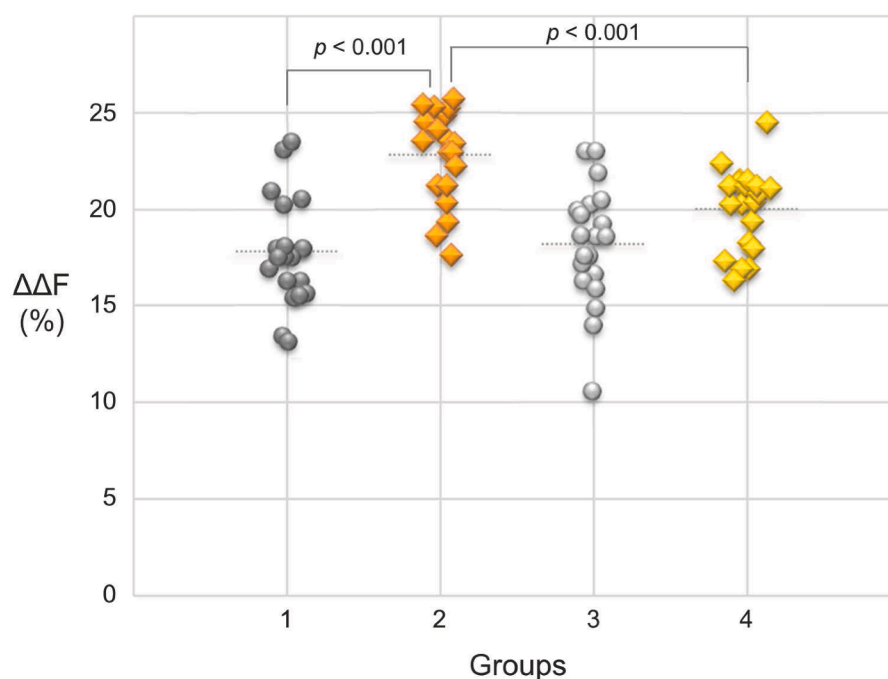


Fig. 5. Jitter plots illustrating the distribution of changes in fluorescence loss ($\Delta\Delta F$) among the groups. Dotted lines represent the mean values of the data (group 1, non-biofilm + distilled water (DW) treatment; group 2, non-biofilm+ 2% NaF solution treatment; group 3, oral microcosm biofilmDW treatment; group 4, oral microcosm biofilm2% NaF solution treatment).

biofilms (groups 3 and 4), there were no significant differences between the two groups ($p > 0.05$; Table 2).

4. Discussion

The results demonstrate that oral biofilms can affect the remineralizing efficacy of fluoride. Remineralizing efficacy was significantly

Table 1

Changes in fluorescence loss ($\Delta\Delta F$) values of early carious lesions after fluoride application.

		Treatments		*Adjusted p-values
		DW	2% NaF	
Oral microcosm biofilms	Absent	17.80 $\pm$ 2.81	22.80 $\pm$ 2.39	< 0.0001*
	Present	18.22 $\pm$ 3.04	20.05 $\pm$ 2.13	
† Adjusted p-values		0.649	< 0.0001†	0.034

Data are presented as meansstandard deviations.

* Adjusted $p < 0.025$: all values in rows were obtained using an independent t -test with an adjusted p -value.

† Adjusted $p < 0.025$: all values in columns were obtained using an independent t -test with an adjusted p -value

DW, distilled water; NaF, sodium fluoride.

Table 2

Red/green (R/G) ratios and viable counts of oral microcosm biofilms after fluoride application.

Treatments	N	R/G ratios	Viable counts (log CFU/biofilm)
DW	20	1.68 $\pm$ 0.29	6.95 $\pm$ 0.86
NaF	20	1.59 $\pm$ 0.37	6.84 $\pm$ 0.93
*p-value		> 0.05	> 0.05

Data are presented as meansstandard deviations.

* $p < 0.05$ based on Mann-Whitney test

R/G ratios were assessed in red fluorescence images of biofilms; CFU, colony-forming unit; DW, distilled water; NaF, sodium fluoride.

greater in the group without an oral biofilm (group 2) than in the group with an oral biofilm (group 4). This shows that oral biofilms negatively affected the recovery of early carious lesions via higher diffusion resistance to fluoride ions. This finding is different from the result of a previous study that reported no significant difference in the remineralizing efficacy of fluoride according to the presence of oral biofilms [8]. Greater biomass density of mature biofilms can result in low solute penetration rates, such as that of fluoride ions [14]. When a lesion is covered by an oral biofilm, the diffusion coefficient of fluoride ions is approximately one-third lower than that in the absence of a biofilm [10]. Biomass in a mature biofilm is considerably greater compared with that in a young biofilm, resulting in a lower diffusion coefficient for fluoride ions through the biofilm [15]. The penetration of fluoride ions, the halogen with the highest electronegativity, can be delayed via adsorption into biofilms [10]. These observations suggest that oral biofilms act as a diffusion barrier to fluoride, which might have led to the significantly lower recovery rate in group 4 compared to that in group 2. Nevertheless, unlike the result of a previous study that reported lesion progression in early carious lesions with biofilms [4], the net mineral change of the lesions was in the direction of remineralization, presumably because a high concentration of fluoride ions was stored in the biofilm during treatment, which was slowly released to the biofilm–enamel interface with a decrease in the environmental pH. In this pH-cycling model using oral microcosm biofilms, changes in inorganic ion kinetics were able to induce the recovery of carious lesions.

There were no significant differences in biofilm pathogenicity between groups 3 (treated with DW) and 4 (treated with fluoride). Based on these results, we explored why lesions could not be significantly remineralized by fluoride in the presence of a biofilm. The rate of fluoride ion penetration into a biofilm slows with increasing biofilm thickness [10], which could prevent fluoride ions from reaching the deeper layers of the biofilm and lead to a greater distribution of fluoride in the outer region of the biofilm [16]. Limited fluoride penetration would restrict the ability of fluoride ions to reduce the rate of oral microbial metabolism. Moreover, long-accumulated biofilms have been reported to show increased spatial heterogeneity [7], which could increase microbial resistance. This could explain why there were no

significant differences in R/G ratios or biofilm CFU counts in the presence of an oral biofilm, as well as why fluoride treatment did not induce the significant recovery of lesions in the presence of a biofilm.

This study had several limitations. First, the oral microcosm biofilms in this study were formed with a thickness of approximately 1 mm. A previous study reported that at least 30 min is required for fluoride, at a concentration of 1000 ppm, to penetrate a 1 mm-thick biofilm [10]; however, we had subjected specimens to fluoride treatment for only 4 min, which might have been insufficient for fluoride penetration. To overcome this limitation, a further study will be designed to modulate the time and frequency of fluoride treatment. Second, because bovine enamel is more porous than human enamel and has a more rapid rate of demineralization [17], the results of this study should be interpreted with caution when applied to humans. Nevertheless, as severe mineral loss results in faster recovery rates during the early stages of fluoride treatment [18,19], the results of this study permit cross-group comparisons. Third, the sixth day of biofilm culture was considered the point for a baseline comparison between groups in this study. A biofilm maturing over a 6-day period is similar in thickness to biofilms found in caries-susceptible sites [20]; mature biofilms can be expected to exhibit considerable resistance to fluoride [21]. In the future, younger biofilms should also be investigated for their influence on the remineralizing efficacy of fluoride. Finally, when analyzing the viability of the biofilm, it was not possible to present the state of bacterial survival according to fluorine treatment. In future studies, if the characteristics of the biofilm are additionally observed, the influence of the biofilm on the efficacy of the remineralizing materials can be explained in more detail.

Nonetheless, oral microcosm biofilms provide the experimental conditions closest to an intraoral environment. Oral biofilms are an important factor in the development of dental caries, and fluoride ions must penetrate biofilms to repair carious lesions. Therefore, when assessing the efficacies of caries-prevention agents, the presence of oral biofilms should be considered. Our findings indicate that the presence of oral biofilms can affect the efficacies of these agents. Further studies addressing the limitations of this study could improve the clinical applicability of new remineralizing agents.

5. Conclusion

The results of this study demonstrated that the presence of an oral biofilm can negatively affect the remineralizing efficacy of fluoride. Therefore, fluoride application should proceed after accurate prophylaxis for those with mature oral biofilm deposition. In addition, the efficacies of remineralizing agents such as fluoride could be overestimated when the influence of an oral biofilm is ignored during *in vitro* experiments.

Declarations of Competing Interest

There are no conflicts of interest to declare.

Ethics approval

This study was approved by the Institutional Review Board of Gachon University (Approval number: 1,044,396-201,805-HR-118-02).

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Conflicts of interest

There are no conflicts of interest to declare.

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