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In-air micro-proton-induced X-ray/gamma-ray emission analysis of the acid resistance of root dentin after applying fluoride-containing materials incorporating calcium

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This study employed an in-air micro-proton-induced X-ray/gamma-ray emission system to assess the effectiveness of fluoride-containing materials (FCMs) incorporating calcium in preventing root caries. Dentin surfaces of human third molars were coated with one of three FCMs: fluoride-releasing glass-ionomer cement (F7) and experimental materials in which half (P1) or all (P2) of the strontium in F7 was replaced with calcium. Dentin without FCM coating served as the control. Specimens were immersed in saline at 37°C for 1 month, sectioned, and then demineralized. Calcium loss after demineralization was lower in the Ca-substituted groups than in the Ca-unsubstituted groups ($p<0.05$). Calcium loss was negatively correlated with fluoride uptake ($p<0.01$). In the F7, P1, and P2 groups, the retraction of the dentin surface was significantly suppressed as compared with the control group. FCMs incorporating calcium improved the acid resistance of root dentin and could help prevent root caries.

Keywords: Root caries, PIXE/PIGE, Fluoride-containing materials, Acid resistance, Demineralization

INTRODUCTION

Root caries is a prevalent dental disease among elderly people, as root dentin has a higher critical pH for demineralization than does enamel[1,2]. Once a demineralized root surface develops a cavity, the carious lesion tends to expand and invade proximally to the subgingival areas along the cement-enamel junction (CEJ). The prevention of such lesions is therefore critical for promoting life-long oral health. Fluoride has been demonstrated to be effective in preventing root caries because it improves acid resistance and provides an antimicrobial effect[3-5]. Glass-ionomer cements (GICs) are fluoride-containing materials (FCMs) which are water-based, tooth-colored, chemically adhesive, and ion-releasing. Incorporated fluoride ions that are slowly released from the GICs provide significant anticariogenic properties[6-8]. In some studies, fluoride release from GICs has been limited and insufficient to prevent the development of caries[8-10]. Therefore, new functional restorative materials such as FCMs incorporating calcium have been developed to enhance the anticariogenic properties of FCMs[7,11,12]. However, further research is necessary to develop the optimal FCM that incorporates additional ingredients, such as calcium, to prevent caries.

Conventionally, caries progression has been measured for changes in mineral density of enamel and dentin lesions using transmission microradiography (TMR) or microcomputer tomography (μCT) as an effective standardized method[13-16]. However, the dynamic distributions of calcium and fluoride in root dentin within active carious lesions have not yet been clarified quantitatively at the molecular level, because it is not possible to achieve multi-elemental sequential analyses in caries-affected tissue using conventional methods such as TMR and μCT.

In a previous study[17], we successfully established a multi-elemental sequential measuring method using an in-air micro-proton-induced X-ray and gamma-ray emission (PIXE/PIGE) system to identify the distribution and concentration of calcium and fluoride in human dentin models. We found that PIXE/PIGE potentially offers a useful and advantageous technique for studying caries development in combination with conventional techniques such as TMR and μCT. The in-air micro-PIXE/PIGE system allows for qualitative and quantitative analyses of calcium and fluoride simultaneously, by measuring the intensities of characteristic X-ray and gamma rays generated by the bombardment of teeth with protons[18-20]. One of the reasons for the efficiency and precision of the PIXE/PIGE system is that its beam spot is very small (1 μm), which enables a high-resolution analysis of the distributions of each mineral element in

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dentin.

Other advantages of the PIXE/PIGE system include the fact that it is a multi-elemental measurement method conducted in normal atmospheric conditions, and it does not require any pretreatment of specimens. It was possible to superimpose mapping of the calcium and fluoride concentrations and distributions taken from identical specimens before and after demineralization by using the mathematical software and the copper reference point. This technique allows for authentic sequential comparisons of tooth components and was effective in assessing caries progression. Although electron probe microanalysis (EPMA) and energy dispersive X-ray spectrometry (EDS) are other techniques that can be used to characterize the distribution of major components such as calcium and fluoride in teeth[21-27], both EPMA and EDS require the specimens to be pretreated with destructive reagents. This means that PIXE/PIGE allows for serial measurements of calcium and fluoride at different time points during the demineralization and remineralization of carious lesions.

In the present study, the acid resistance of root dentin after the application of FCMs incorporating calcium was measured using an in-air micro-PIXE/PIGE measurement system at the Wakasa Wan Energy Research Center (Fukui, Japan), with the aim of developing an effective functional restorative material with anti-cariogenic properties. The null hypothesis of this study was that FCMs incorporating calcium would be ineffective in preventing root caries when evaluated by the in-air micro-PIXE/PIGE system.

MATERIALS AND METHODS

All experiments were carried out in accordance with protocols approved by the Research Ethics Committee of Osaka University Graduate School of Dentistry (H25-E28). Teeth were collected after written patient informed consent, under protocols approved by the Research Ethics Committee. Specimen preparations and measurements of the calcium and fluoride distributions and concentrations before and after demineralization were performed according to previously reported methods[17].

Specimen preparations

A total of 9 third molars free from caries that had been extracted for orthodontic and periodontal reasons at Osaka University Dental Hospital were used as specimens. These specimens were stored in 100% humidity for periods of less than 6 months after they were extracted.

The preparation of the specimens is shown in Fig. 1. Each tooth was sectioned perpendicularly to the tooth axis at 0.5 mm above and 7.0 mm below the CEJ using a low-speed saw (IsoMet, Buehler, Lake Bluff, IL, USA) with a diamond disc (15LC Diamond Wafering Blade, Buehler) under deionized running water. The root block was sectioned in half, parallel to the tooth axis (Fig. 1a). The buccal and lingual surfaces of the root block were cut until dentin was exposed, with the low-speed saw with the diamond disc under deionized water, and then further cut in two longitudinally at the center of the exposed dentin surface (Fig. 1b). Four root dentin specimens were taken from one root. The exposed dentin areas of three sections were coated with FCMs with approximately 500 μm thick. Three kinds of FCMs were prepared: F7 (Fuji VII, GC) and two experimental materials (P1 and P2, GC). The powder of F7 was composed of these compounds; silicon dioxide, aluminum oxide, fluorine, strontium oxide, and phosphorus pentoxide, not containing calcium ion. Half or all of the strontium in F7 was replaced by calcium in P1 and P2, respectively (Table 1). The powder to liquid ratio was 1.8 for all FCMs. When mixed at this powder to liquid ratio, both P1 and P2 were prepared so that the cement hardening time was the same as that of F7. One section without an FCM coating served as a control. Each specimen was coated with a sticky wax (New Sticky wax, GC), leaving the dentin area exposed in order to allow for fluoride uptake only from the exposed dentin (Fig. 1c). Finally, each specimen was immersed in isotonic sodium chloride solution (saline) for one month at 37°C, and the solution was changed every week.

After one month, the aforementioned acid-resistant varnish and the FCM were removed using hand instruments under a microscope. Each dentin block was sliced parallel to the longitudinal axis and perpendicularly to the exposed root dentin area to make a 500 μm section to be used for calcium and fluoride measurements (Fig. 1d). A copper foil of 4 μm thickness was placed on the cutting plane of each specimen as a reference point to compare the calcium concentrations and fluoride uptake before and after demineralization (Fig. 1e). The copper foil was set 800 μm in from the outer edge of the exposed root dentin.

Measurements of calcium and fluoride distributions and concentrations before demineralization

The calcium and fluoride distributions and concentrations in specimens were measured using the PIXE/PIGE system at the Wakasa Wan Energy Research Center according to a previous report[17]. A 2.5 MeV proton beam was used to bombard specimens in normal atmospheric conditions. The beam spot size was less than 10 μm, with a beam current of approximately 50 pA. The nuclear reaction $^{19}F(p, \alpha\gamma)^{16}O$ was used to measure the fluoride content. The gamma rays in this reaction were detected using a 3"bismuth germanium oxide detector, located just behind the sample stage. The calcium concentration was measured by the PIXE system. X-rays were simultaneously detected with a pure germanium detector without an X-ray absorber, which was placed at a 130° angle with respect to the beam axis, in a vacuum. The beam current was monitored by counting silicon K X-rays passing a silicon nitride window using a silicon positive-intrinsic-negative X-ray detector. Quantitative results were obtained by calibrating the PIGE yield using hydroxyl apatite (HA)

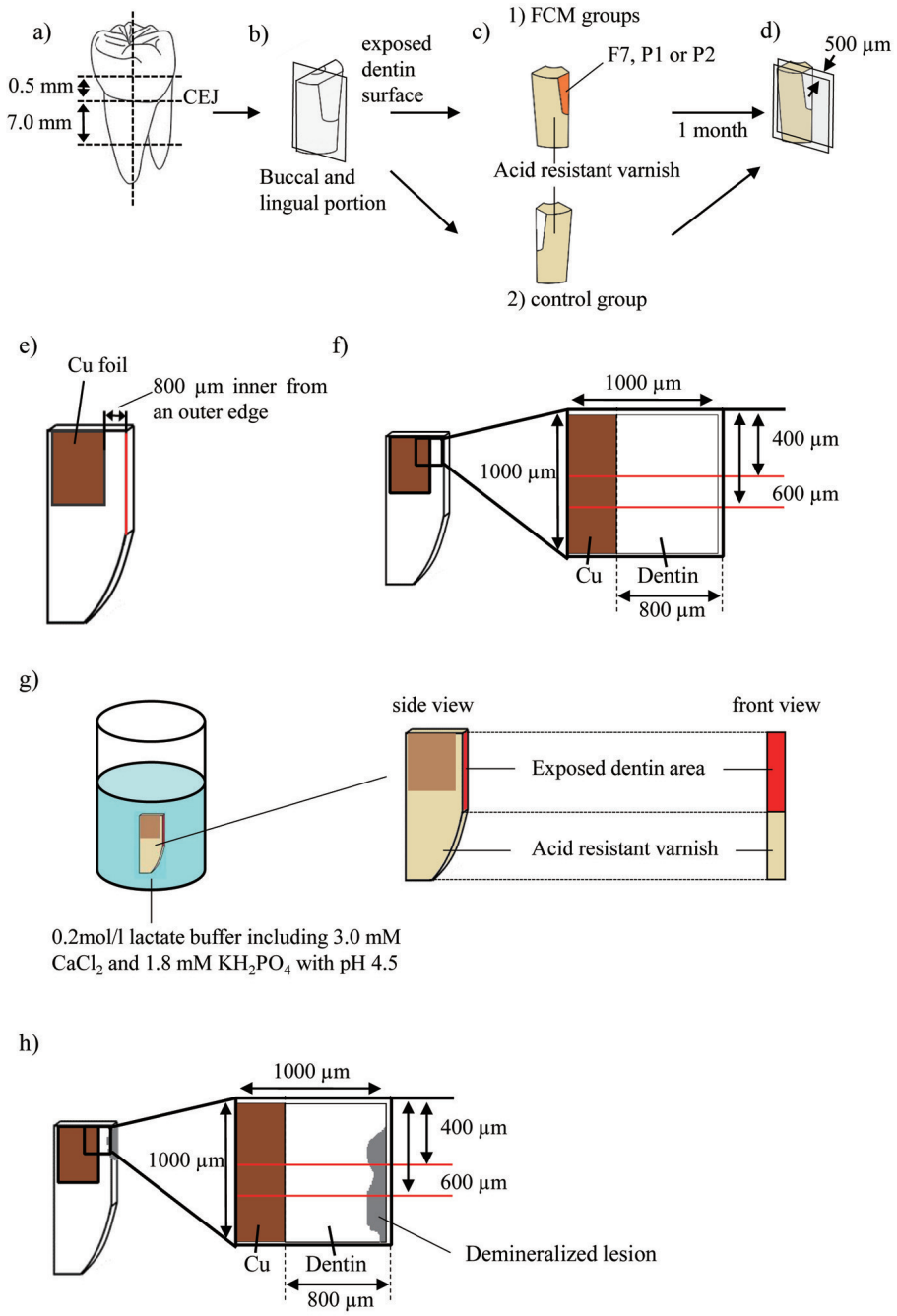

Fig. 1 Preparation of root dentin specimens and Ca and F measurements using PIXE/PIGE (Modified from Ref. 17).
(a) Each tooth was sectioned perpendicularly to the axis in the mesio-distal direction. (b) The buccal and lingual surface of the root block was cut until dentin was exposed, and the root block was sectioned in two longitudinally at the center of the exposed dentin surface. (c) The exposed root dentin area of the mesial block was covered with FCMs (c-1: F7, P1 and P2 groups), and that of the distal block served as a control without FCM (c-2: CO group). Every specimen was coated by an acid resistant varnish leaving the exposed dentin area. The specimens were immersed in saline for 1 month at 37 °C. (d) The aforementioned acid resistant varnish and the FCM were removed and each dentin block was sliced parallel to the longitudinal axis and perpendicularly to the exposed root dentin area to make a 500 µm section for the Ca and F measurement. (e) A 4 µm Cu foil was placed on the cutting plane of specimen at 800 µm inner from an outer edge of the exposed root dentin. (f)The first PIXE/PIGE measurement was performed before demineralization. (g) Demineralization of dentin: immersed in 10 mL of demineralization solution for 3 days. (h) The second PIXE/PIGE measurement was performed after the demineralization.

Table 1 Materials used in this study

Name	Composition	Sr/Ca (mol/mol)	Lot No.
Fuji VII (GC)	(Powder) Fluoroaluminosilicate glass	1/0	(Powder) 1507161 (Liquid) 1506021
P1 (GC)	(Liquid) Polyacrylic acid Distilled water	1/1	(Powder) 150805 (Liquid) 1506021
P2 (GC)	Polycarboxylic acid	0/1	(Powder) 150805 (Liquid) 1506021

with varying levels of fluoridation as reference materials ($Ca_{10}[PO_4]_6[OH]_{2-2x}F_{2x}$ with x=0, 0.25, 0.5, 0.75, 1; HOYA Technosurgical, Tokyo, Japan).

For the calcium and fluoride distribution and concentration measurements, a 1,000×1,000 μm^2 area of the measuring surface including the copper foil was scanned on a sample stage in normal atmospheric conditions (Fig. 1f). For each specimen, two lines at distances of 400 and 600 μm from the top edge of the scanning area were selected and subsequently analyzed.

Demineralizing treatment

After the calcium and fluoride distribution and concentration measurements, the surface of each specimen, except for the exposed root dentin area, was coated again with the acid-resistant varnish to allow for demineralization only from the exposed root dentin. Each specimen was immersed in 10 mL of demineralizing solution for 3 days (Fig. 1g). The demineralizing solution was a 0.2 mol/L lactate buffer including 3.0 mM $CaCl_2$ and 1.8 mM KH_2PO_4 at pH 4.5[28]. After 3 days, the exposed dentin area was washed with deionized water for 30 s, and then the acid-resistant varnish was removed from the specimens. The specimens were stored in 100% humidity until the second PIXE/PIGE measurement.

Measurements of calcium and fluoride distributions and concentrations after demineralization

After demineralization, the calcium and fluoride distributions and concentrations in the same specimens were measured again at the same locations as before demineralization, using the PIXE/PIGE system (Fig. 1h).

Comparisons of calcium and fluoride distributions before and after demineralization

The distributions and concentrations of calcium and fluoride before and after demineralization were calculated from calibration curves obtained from reference materials using the Tooth line scan analyzer software (Wakasa Wan Energy Research Center). The outermost surface of the dentin was defined as the position containing 5% of the average calcium of intact dentin; the innermost surface of the demineralized lesion was defined as the position containing 95% of the average calcium of intact dentin.

When analyzing the demineralization of root dentin, the calcium distribution results obtained from line analysis of the same specimen before (Fig. 2A) and after (Fig. 2B) demineralization were superimposed based on the reference point of the copper foil (Fig. 2C). On the superimposed image, the calcium loss (wt%・μm) and the fluoride uptake (ppmF・μm) was calculated according to previously reported methods[17].

The distributions of morphologies

The distributions of morphologies were classified into 'no demineralization' (Fig. 3a); most of the surface layer remained even after the demineralization, 'subsurface demineralization' (Fig. 3b); the surface layer remained, however the lower layer was lost, and 'lost surface' (Fig. 3c); the surface layer was completely lost by demineralization, by the superimposed image. In each group, the ratio of the number of samples showing each morphology to the total number of samples was calculated.

Measurement of calcium and fluoride release from FCMs

The FCMs (n=4 for each group) were prepared according to the manufacturer's recommended instructions: materials were packed into silicon molds (9 mm diameter and 2 mm deep), the top surface was then covered by cellophane strips (Shofu, Kyoto, Japan), and allowed to set for one hour in 100% humidity at 37°C. Each specimen was then immersed in 50 mL of deionized water at 37°C. After 12 h, the specimens were removed and placed into a new vial containing 50 mL of deionized water. Each solution was collected and diluted 5-fold. This procedure was repeated at 1, 5, 7, 14, and 28 days, and the cumulative calcium and fluoride release measurements were then obtained. An inductively coupled plasma (ICP) emission spectrophotometer (ICPS-8000, Shimazu, Kyoto, Japan) was used to measure the calcium release. A fluoride ion-selective electrode (Model 9609BN, Orion Research, MA, USA) was used to measure the fluoride release.

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics 22 (International Business Machines, Armonk, New York, USA). The calcium loss and fluoride uptake were compared between groups using the Steel-

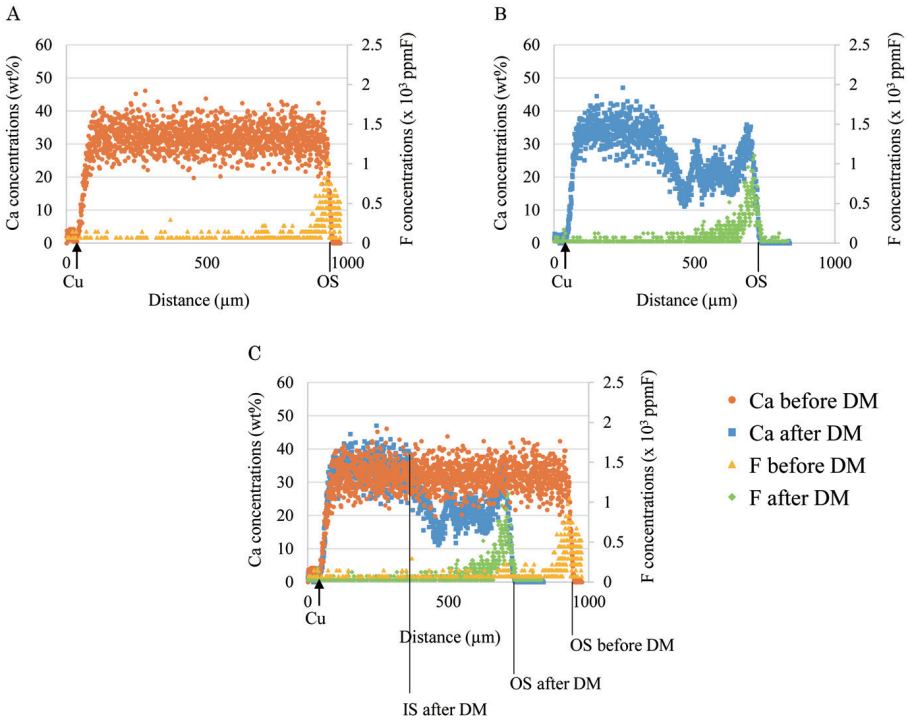

Fig. 2 Distributions and concentrations of Ca and F before and after demineralization (DM). A: Distributions and concentrations of Ca and F before DM obtained from line analysis. B: Distributions and concentrations of Ca and F after DM of same tooth indicated by (A) obtained from line analysis. C: Distributions and concentrations of Ca and F before and after DM, superimposed on the basis of the reference point of the Cu foil (arrow). OS: outer surface. IS: inner surface.

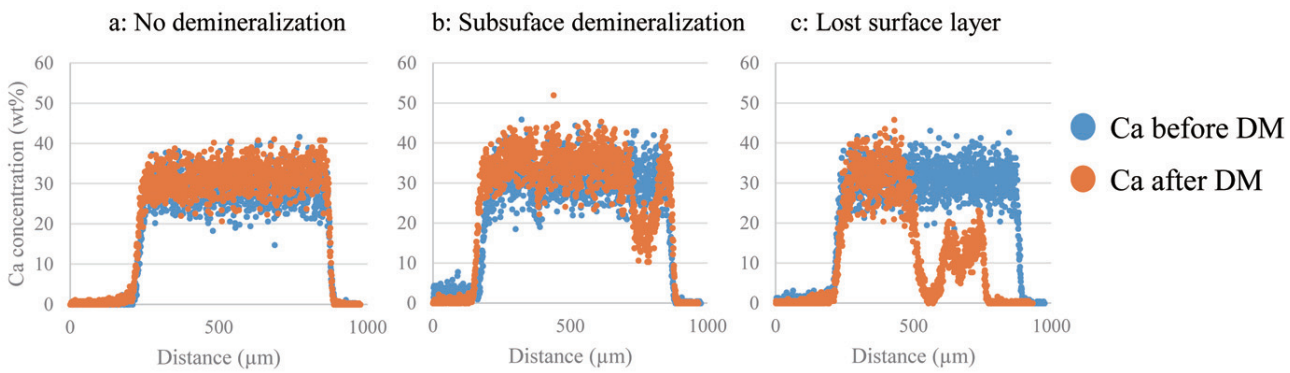

Fig. 3 Morphologies of demineralized dentin surface.
A: No demineralization, B: Subsurface demineralization, c: Lost surface layer. DM: demineralization.

Dwass test at a 95% level of confidence. The correlations between calcium loss and fluoride uptake were evaluated using Spearman's rank correlation coefficient at a 99% level of confidence. The morphologies of the demineralized surfaces of all groups were compared by using the Kruskal-Wallis test at a 95% level of confidence. Calcium and fluoride release from FCMs were evaluated by two-way analysis of variance (ANOVA) and Tukey's test at a 95% level of confidence.

RESULTS

The median calcium losses in the F7, P1, P2, and control groups were 2.8×10^2 (range: 8.4×10 to 5.2×10^2), 1.2×10^2 (range: 0.0 to 4.0×10^2), 7.0×10 (range: 0.0 to 2.3×10^2), and 4.1×10^2 wt%•μm (range: 2.2×10^2 to 8.1×10^2), respectively (Fig. 4A). The P1 and P2 groups showed significantly smaller losses of calcium than the F7 and control groups (Steel-Dwass test, $p<0.05$).

The median fluoride uptakes before demineralization

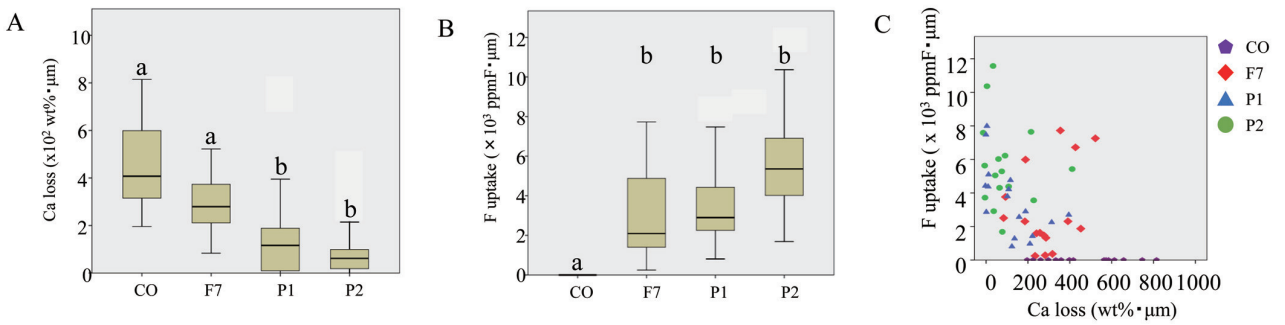

Fig. 4 The Ca loss and the F uptake before and after demineralization (DM).
A: Ca loss after DM, B: F uptake before DM, n=9. No statistically significant differences among the groups indicated with same letters (Steel Dwass test, p<0.05). C: Correlations between the Ca loss and the F uptake. Significant negative correlation between the F uptake before demineralization and the Ca loss was detected (Spearman's rank correlation coefficient, p<0.01, R=0.63).

Table 2 The ratio of the number of samples showing each morphology to the total number of samples after the demineralization.

Group	Number of samples (ratio to the whole)		
	a	b	c
CO	0 (0.0%)	0 (0.0%)	16 (100.0%)
F7	0 (0.0%)	8 (50.0%)	8 (50.0%)
P1	5 (31.3%)	10 (62.5%)	1 (6.2%)
P2	7 (43.8%)	9 (56.2%)	0 (0.0%)

After the demineralization, 50, 94 and 100% of the dentin surface in F7, P1 and P2 groups was maintained, respectively; while 0% in CO group was preserved. Significant differences were observed in the distributions among different groups (Kruskal-Wallis test, p<0.05).

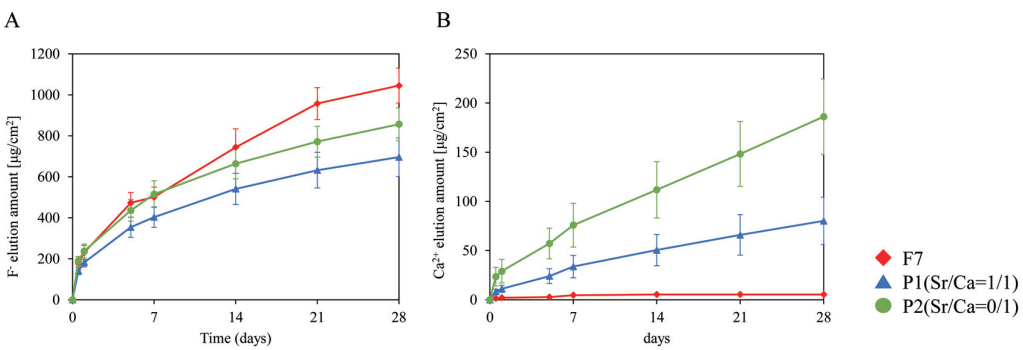

Fig. 5 Ion elution amount from FCM in 28 days.
The F (A) and Ca (B) elution concentrations from FCMs.

in the F7, P1, P2, and control groups were 2.1×10^3 (range: 2.5×10^2 to 7.7×10^3), 2.9×10^3 (range: 8.1×10^2 to 7.4×10^3), 5.5×10^3 (range: 1.7×10^3 to 1.0×10^4), and 0.0 ppmF•μm (range: 0.0 to 0.0), respectively (Fig. 4B). The F7, P1 and P2 groups showed significantly higher fluoride concentrations than the control group (Steel-Dwass test, p<0.05).

As shown in Fig. 4C, fluoride uptake and calcium loss showed a significant negative correlation (Spearman's rank correlation coefficient r=−0.63, p<0.01).

The morphologies of demineralized surfaces significantly differed between the experimental groups (Kruskal-Wallis test, p<0.05) (Table. 2). In the F7, P1, and P2 groups, the number of samples whose surface was maintained was 50%, 94%, and 100%, respectively, of the total number of samples. In contrast, in the control group, the number of samples whose surface was maintained was 0% of the total.

As shown in Fig. 5, the cumulative fluoride ion release in the F7, P1, and P2 groups at 28 days was 1.0×10^3, 7.0×10^2, and 8.6×10^2 μg/cm^2, respectively. There was no significant difference in fluoride ion release between the groups (two-way ANOVA and Tukey's test, $p>0.05$). The cumulative calcium ion release from the F7, P1, and P2 groups by 28 days was 5.3, 8.0×10, and 1.9×10^2 μg/cm^2, respectively. Significant differences were observed between all groups (two-way ANOVA and Tukey's test, $p<0.05$).

DISCUSSION

This study showed a negative correlation between the fluoride uptake from the FCM and calcium loss. Similar results showing the preventive effects of fluoride on the onset and progression of caries in enamel and dentin have been demonstrated in previous studies[3,29-31]. FCMs release a substantial amount of fluoride in acidic conditions rather than in neutral conditions; this is because fluoride is an anion that is easily eluted under acidic conditions in which a high concentration of hydrogen cations exist. The released fluoride may be absorbed into enamel and dentin through the surface because of its small ionic radius[32]. In our previous study, there was no significant difference in the fluoride uptake before and after demineralization, but the fluoride penetration after demineralization was greater than that before demineralization in the FCM group[17]. These results suggested that a considerable amount of fluoride was able to penetrate into deeper parts of the dentin through its porous structure after demineralization. The fluoride that penetrates into the tooth substrates could aid remineralization and thus have a protective effect against demineralization[33,34].

The FCMs incorporating calcium were effective in preventing cavitation after demineralization. In particular, the surface morphology after demineralization significantly differed between the experimental groups. The P1 and P2 groups demonstrated effective acid resistance: 94% of specimens in the P1 group and all specimens in the P2 group maintained their surfaces after demineralization. In contrast, 50% and 100% of the specimens in the F7 and control groups lost their surface layers, indicating a failure in preventing cavitation. In FCM groups, the uptake of fluoride in root dentin effectively improved its acid resistance. However, the difference in results between F7 group and P1, P2 groups is considered to be due to the action of Ca. Although the amounts of calcium released from the three FCMs differed at 28 days, the P1 and P2 groups demonstrated almost identical frequencies of cavitation. This suggests that replacing half of the calcium in the F7 material with strontium is enough to prevent cavitation effectively.

When a freshly mixed GIC is placed on wet dentin, an interaction between the two materials occurs in the form of an ion exchange[35,36]. Aluminum, fluoride, calcium, and strontium leach out of the cement as the glass is dissolved by the polyacid, while calcium and phosphate ions also move out of the underlying dentin as a result of the self-etching effect of the setting cement on mineralized dentin[35,37]. This ion exchange process creates an intermediate layer composed of ions derived from both substrates[36-38]. The release of fluoride, calcium, and strontium ions gives GICs the potential to aid the remineralization of carious tissues[39,40]: ion exchange could replenish the demineralized tissue ions, thus tipping the balance in favor of apatite formation.

Calcium is an ion that does not leach easily from GICs once the cement has set[41] due to its rapid binding, in an insoluble form, to the polyacrylic acid matrix[42]. Elution of calcium, when it does occur, has been attributed to acid erosion of the cement matrix[6,41]. When the calcium was replaced by strontium in the GIC, strontium was released as the acid in the acidic buffer eroded the cement. The acid-catalyzed release of calcium from the GIC was consistent with the protection of the adjacent dentin observed during the acid challenge.

In the present study, CaF_2-like deposits may form on the root surface after the application of FCMs incorporating calcium[43]. Many studies have identified CaF_2-like deposits as the most important labile source of fluoride on the tooth surface[42-46]. CaF_2-like deposits can form due to the reaction between the tooth-bound calcium and the topically applied fluoride, especially when the topical fluoride agents have a low pH and a high concentration, and are applied over a long time period[45,47-49]. Once the CaF_2-like deposits form, they have the desirable properties of a fluoride source[45], and continuously release fluoride during cariogenic challenges[44,50].

Mazzaoui *et al.* reported that the incorporation of 1.56% wt/wt casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) into the GIC significantly enhanced the release of calcium, phosphate, and fluoride ions under neutral and acidic conditions[7]. The release of CPP and fluoride from the CPP-ACP-containing GIC was associated with enhanced protection of the adjacent dentin during an acid challenge *in vitro*. Another novel material known as Glass Carbomer (GCP Dental, Mijlweg, The Netherlands) has been reported to contain nano-glass particles, hydroxyapatite/fluorapatite (HAp/FAp) nanoparticles, and liquid silica[12]. The glass has a much finer particle size compared to conventional GICs, a property that is thought to aid the material's dissolution and ultimate conversion to FAp and HAp.

Further studies are needed to identify the best methods for incorporating calcium and fluoride into GIC to maximize its anti-cariogenic properties. For future studies, the PIXE/PIGE system should be utilized as an effective tool to characterize the release of ions such as fluoride and calcium in order to develop effective restorative materials for preventing caries.

CONCLUSION

PIXE/PIGE measurements demonstrated that the application of fluoride and calcium in root dentin effectively improved its acid resistance.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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