Effects of experimental pastes containing surface pre-reacted glass ionomer fillers on inhibition of enamel demineralization

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This study aimed to evaluate the inhibitory effect of experimental pastes containing surface pre-reacted glass ionomer (S-PRG) fillers on enamel demineralization. Bovine blocks were treated twice a day for 4 days by 7 groups; experimental pastes containing 0–30 wt% S-PRG filler (S00, S01, S05, S10, and S30), deionized water (DW) as negative control, and NaF paste (MP) as positive control. The surfaces were demineralized by acetic acid for 3 days. Mineral loss (ML) was calculated by micro-computed X-ray tomography. The treated surface was finally investigated with scanning electron microscope (SEM) and micro-focused particle induced X-ray emission (micro-PIXE). S05, S10 and S30 demonstrated significantly lower ML than S00, S01 and DW (p<0.05). S10 showed the greatest inhibitory effect, which was significantly greater than MP. The S-PRG filler containing experimental pastes demonstrated a potential to inhibit enamel demineralization. Sr ion incorporation was confirmed on the enamel surface with the experimental pastes.

Keywords: Demineralization, Enamel, Micro-CT, Micro-PIXE, S-PRG filler

INTRODUCTION

Regular brushing with fluoride containing experimental paste can lower the rate of dental caries incidence¹,². Topical applications of fluoride gel and varnish are also well established as therapeutic agents, providing protection against acid challenges as well as repair of demineralized enamel³–⁵. Fluoride has been shown to reduce enamel mineral loss following acid erosion challenges via in-vitro and in-situ studies²,⁶,⁷.

Recently, surface pre-reacted glass ionomer (S-PRG) filler has been developed, which is composed of fluoroboroalumino-silicate glass and polyacrylic acid solution and is produced through acid-base reaction⁸. The S-PRG filler has been incorporated into resinous materials, such as dentin bonding agents⁹,¹⁰, fissure sealants¹¹,¹², and resin composites¹³. The S-PRG containing resinous materials can release multiple ions, such as aluminium (Al), boron (B), fluoride (F), sodium (Na), silicon (Si), and strontium (Sr), in neutral and acidic conditions¹⁴. It was reported that among the ions released from the S-PRG fillers, Sr had the highest amount, followed by F and Si¹⁴.

Micro-computed X-ray tomography (micro-CT) can capture three-dimensional (3D) architectural information from samples non-destructively¹⁵. The technique has been shown as a promising method to assess demineralization or remineralization of enamel or dentin¹⁶–¹⁹. The use of micro-CT has been validated for quantitative assessment of enamel demineralization²⁰.

Particle induced X-ray emission (PIXE) analysis uses the characteristic X-rays generated by accelerated proton (or a charged particle) to detect elements. By focusing the incident beam down to micron size and scanning over the specimen surface, “micro-PIXE” can also provide images of the trace elemental distribution with high spatial resolution (μm scale) and high sensitivity²¹,²². The method has been used to analyze the distribution of trace heavy elements contained in various soft and hard tissues²³–²⁶.

The purpose of this study was to evaluate the inhibitory effects of experimental pastes containing S-PRG fillers on bovine enamel demineralization using micro-CT, scanning electron microscope (SEM) and micro-PIXE. The null hypothesis was that the experimental pastes could not prevent enamel demineralization when subjected to acid challenge.

MATERIALS AND METHODS

Specimen preparation

Twenty extracted, non-damaged permanent bovine incisors were stored frozen until the experiment. The teeth were thoroughly cleaned and washed under running water to remove all adherent soft tissues. The specimens were prepared by cutting into 3×3×2 mm enamel-dentin blocks using a low-speed diamond saw (Isomet, Buehler, Lake Bluff, IL, USA) under...
water as a coolant. The enamel surfaces were ground flat with 600- to 2000-grit silicon carbide (SiC) papers (Fuji Star, Sankyo Rikagaku, Saitama, Japan) under running water. The surfaces were then covered by a nail varnish (680, Revlon, New York, NY, USA) leaving a window (2×2 mm) to expose the polished enamel surface of each specimen. In order to set a reference landmark for the micro-CT scans, a hole (1 mm in diameter, 0.5 mm in depth) was made at the side of the specimen by a diamond bur (440SS ISO # 010, Shofu, Kyoto, Japan). They were then divided into seven treatment groups to give a total of 70 specimens listed in Table 1; deionized water (Direct-Q, Millipore, Billerica, MA, USA) (DW) as a negative control, 950 ppm F (as NaF) commercially available paste (Merssage Plus, Shofu) (MP) as a positive control and experimental pastes containing 0, 1, 5, 10, and 30 wt% of S-PRG fillers (Shofu) (S00, S01, S05, S10, and S30, respectively). Experimental paste suspensions were prepared at dilution of 1:2 (paste: deionized water), thoroughly stirred and mechanically agitated for 1 min by means of a vortex mixer (MF-71, TGK, Tokyo, Japan) according to the procedures described in the previous study27. The specimens were immersed in each suspension at 37°C for 5 min twice a day, rinsed off using deionized water for 10 s and returned to the storage in deionized water in the incubator at 37°C. The treatment cycle continued for 4 days, and the suspensions were prepared daily. After the treatment cycle was completed, the specimens were scanned by micro-CT to establish a baseline. Each specimen was then separately immersed in 10 mL of a demineralizing solution (2.2 mM CaCl₂, 2.2 mM KH₂PO₄ and 50 mM acetic acid adjusted at pH 4.5 with 10 M KOH) at 37°C for 3 days28. The demineralizing solution was refreshed daily. After the demineralization, the specimens were removed from the solution and scanned by micro-CT again (Fig. 1).

### Micro-CT scanning

A micro-CT system (InspeXio SMX-100CT, Shimadzu, Kyoto, Japan) was used to evaluate mineral density (MD) of the demineralized specimens. The micro-CT system can generate polychromatic X-rays with cone-beam geometry. A 0.2-mm thick brass (Cu-Zn) filter was installed in the beam path to reduce beam-hardening effect19,29. The specimen was mounted on a computer-controlled turntable, which synchronized the rotation and the axial shift. The nominal isotropic resolution of the setup was 8.1 μm with an integration time of 120 s. The scanning was performed with the specimen being rotated 360° at rotation step of 0.6°. The tube voltage was 100 kV at a current of 120 μA. The distance from X-ray source to the sample was 39.2 mm, and that from X-ray source to the detector was 300 mm. The specimen was mounted so that the X-ray beam was perpendicular to the treated enamel surface.

For MD calibration, a series of mineral reference phantoms were also scanned which included three hydroxyapatite (HAp) disks in total (Ratoc, Tokyo, Japan); two with different concentrations (0.50 and 0.70 gHAp/cm³) of HAp crystals embedded in epoxy resin (Epoxicure Resin, Buehler), and one pure HAp disk (3.16 gHAp/cm³) (Cellyard, Hoya, Tokyo, Japan).

### Micro-CT image analysis

The data in each scan were acquired as 100 images in 16-bit TIFF format, and used to reconstruct a 3D image stack from the 2D images, with a resolution of 512×512 pixels and an isotropic volumetric pixel (voxel) size of 8.1 μm by a 3D analysis software (TRI/3D-BON, Ratoc), which was used for visualization and quantitative volumetric measurements. CT values were converted into MD values (gHAp/cm³) using a linear calibration curve based on the grey values obtained from the mineral reference phantoms (linear regression, \( R^2 >0.9994 \)). A noise reducing median filter was applied to the data,

### Table 1 Materials used in this study

| Group | Product         | Ingredients                                      | Lot     | Manufacturer   |
|-------|-----------------|--------------------------------------------------|---------|----------------|
| S00   | Experimental    | S-PRG filler 0 wt%                               | 081101  | Shofu, Kyoto, Japan |
| S01   | Experimental    | S-PRG filler 1 wt%                               | 081101  | Shofu, Kyoto, Japan |
| S05   | Experimental    | S-PRG filler 5 wt%                               | 081101  | Shofu, Kyoto, Japan |
| S10   | Experimental    | S-PRG filler 10 wt%                              | 081101  | Shofu, Kyoto, Japan |
| S30   | Experimental    | S-PRG filler 30 wt%                              | 081101  | Shofu, Kyoto, Japan |
| MP    | Merssage Plus  | Silicic acid, Glycerin, Carboxymethyl cellulose, Purified water, etc. | PN0575  | Millipore, Billerica, MA, US |
| DW    | Direct-Q        | Deionized water                                  | —       | Millipore, Billerica, MA, US |

S-PRG: Surface reaction-type of pre-reacted glassionomer, NaF: Sodium fluoride
and the background was removed by excluding those pixels with a CT value lower than the zero MD value according to the calibration curve. The rendered 3D volumes were manually translated and rotated in the software to visually match the baseline image, which served as the reference. The features on each image used for the matching process were the sound enamel surface, specimen edges and reference landmarks (holes made by a bur on dentin). The assessment was performed in a volume of interest (VOI) of 486×486×486 μm³ at the center of the demineralization window. Mean MD values were calculated in the VOI at each 8.1 μm depth. The MD profile in each specimen was obtained by plotting the mineral density (vol%). All maximum MD in baseline (sound) specimen was normalized to 100 vol% against the depth.

Mean integrated mineral loss (ML) (vol% μm) after each demineralization period was calculated from the MD profiles; with the reference point of the depth axis (0 μm) set at the axial position of the sound enamel surface at baseline. The ML on each specimen was calculated from the MD profiles by subtracting the area under the curve after demineralization from the area before demineralization as shown in Fig. 2. These calculations were performed by importing the MD data for each depth of demineralization into spread sheet software package (Microsoft Excel for Windows version 2013, Microsoft, Redmond, WA, USA).

**SEM observation**
Specimens were prepared in the same manner as for micro-CT observation. Morphology of enamel surface at the treatment window was observed by SEM (JSM-5310LV, JEOL, Tokyo, Japan) under ×3,500 magnification on extra specimens in each group before and after demineralization, following a specimen processing protocol for SEM, which included desiccation and gold sputter coating.

**Micro-PIXE observation**
Additional specimens from the S10 and DW groups (before and after demineralization) were subjected to
the micro-PIXE analysis. The specimens were cut into 0.5×3×2 mm enamel-dentin slices at the center of the treatment window using a low-speed diamond saw (Isomet) under water as a coolant. The cross-sectional surface was then ground flat with 2000-grit aluminum oxide carbide papers (Lapping paper, 3M ESPE, St. Paul, MN, USA) under running water for observation.

Micro-PIXE analyses were conducted at the National Institute of Radiological Sciences (Chiba, Japan). An accelerated and micro-focused proton beam (3.0 MeV, 2 μm beam diameter) with raster scanning was applied over the target area of the specimen (maximum area of 2×2 mm) on two surfaces; on the demineralized enamel surface (imaging from top) and on the polished cross-section. The generated characteristic X-rays were collected using Si (Li) and CdTe detectors to obtain the elemental distribution images and the characteristic X-ray spectra. The obtained data were processed with analysis software (OMDAQ2007, Oxford Microbeams, Bicester, UK), and the elemental distribution images were obtained.

Statistical analysis
The ML results were expressed as mean and standard deviation. The collected values were statistically analyzed using one-way ANOVA with Tukey’s test. The significance level of all tests was set at α=0.05. All statistical tests were performed with a computerized statistical program (SPSS for Windows Ver. 11, SPSS, Chicago, IL, USA).

RESULTS

Micro-CT analysis
A typical 2D image of the control group after demineralization was shown in Fig. 3. The DW group revealed approximately 100 μm thickness of demineralized enamel.

Mean mineral density profiles
The MD profiles of enamel in each experimental group were summarized in Fig. 2. After demineralization, the DW group revealed the most aggressive mineral loss pattern among all the groups. In the S00 and S01 groups, the profiles showed patterns similar to that of the DW group. The S05 and S30 groups showed mineral profiles similar to each other, representing less mineral loss compared to the DW group. On the other hand, the S10 group showed the least mineral loss among all the treatment groups. In the MP group, the profile showed an intermediate pattern between the S30 and DW groups.

Mean mineral loss
The mean ML value of each group was summarized in Fig. 4. The highest ML value was obtained in the S00, S01, and DW groups, which were not significantly different each other (p>0.05). The S05, S10 and S30 groups showed significantly less ML than the S00, S01 and DW groups (p<0.05). In terms of nominal values, S10 was the most effective in inhibition of enamel demineralization, followed by S05 and S30, however, there was no statistically significant difference among them (p>0.05). The MP group showed significantly larger mineral loss compared to the S10 group (p<0.05); there was no significant difference between MP and any other groups (p>0.05).

SEM observation
The representative SEM micrographs of the enamel surfaces before and after demineralization are shown in Fig. 5. The smear layer covering the enamel surface was visible after the treatment cycle and before demineralization in all the groups. After the
Fig. 5 SEM micrographs of enamel surface treated with the paste S00 (a), S10 (c), MP (e), groups and DW (g) groups before demineralization. S00 (b), S10 (d), MP (f), and DW (h) groups after demineralization. The residues of the experimental pastes were remained on the enamel surfaces in S00, S10, and MP groups. The smear layers resulting from SiC paper polishing were observed on the enamel surface in DW group (g). The honey-comb like structures were observed in S00, MP and DW groups. The surface texture of S00 and DW groups were more distinct compared with that of MP group. The smear layer was removed, but a smoother surface was observed in S10 group. The scratches are created by SiC paper polishing.

demineralization, the prismatic enamel structures were exposed in the S00 (Fig. 5b), MP (Fig. 5f), and DW (Fig. 5h) groups. However, the enamel prism structures were not observed after demineralization in the S10 group (Fig. 5d), while the smear layer was removed and distinct scratches (due to SiC polishing) were observed.

Micro-PIXE observation
The elemental distribution images of Ca and Sr on the enamel surfaces after the treatment in the DW and S10 groups obtained by the micro-PIXE analysis are shown in Fig. 6. In both specimen, Ca was homogeneously distributed on enamel surface and no difference could be
Fig. 6   Elemental distribution of Ca and Sr on the surface of DW and S10 treated groups obtained by micro-PIXE. The two specimens were placed next to each other and scanned to facilitate direct comparison. Ca distribution on the enamel surface was similar between DW and S10 groups. On the other hand, elemental concentration of Sr on surface of S10 group was slightly higher than that of DW group. The line profiles correspond to cumulative distribution of each element through the square area of interest.

Fig. 7   Elemental distribution images of Ca and Sr of the cross-section obtained after S10 treatments (A) and after demineralization (B). E: enamel, D: dentin. Sr adsorption on the enamel surface was clearly obtained after S10 treatment (white arrows in A), but not after demineralization (B).
observed between the DW and S10 groups. In contrast, Sr could be detected on enamel surface in the S10 group, but not in the DW group. Figure 7 depicts the representative distribution images of Ca and Sr on the cross section of enamel surface in the specimen of the S10 group before and after demineralization (Figs. 7A and B, respectively). Ca distribution through the cross-section of enamel was similar distribution before and after the demineralization. Sr accumulation was detected on the enamel surface after the S10 treatment; however, the accumulated Sr on the enamel surface disappeared after demineralization.

DISCUSSION

Micro-CT is a powerful research tool that yields 3D information about hard tissues, allowing dynamic detection of mineral change after demineralization and remineralization of enamel and dentin. Micro-CT can be used as a nondestructive alternative to the 'gold standard' method of transverse microradiography, with comparable parameters for the study of mineral content and lesion depth. In addition, its accuracy can be increased by reducing beam-hardening effects and using appropriate mineral density calibration curves.

Experimental pastes are daily oral care products to prevent caries, periodontal disease, or acid erosion. Experimental pastes are recognized as the best source of fluoride, which are the most effective treatment to protect both deciduous and permanent teeth from caries. Fluoride concentration in the whole saliva is related to the efficiency of caries prevention and experimental pastes are normally diluted in the process of brushing; therefore, the experimental pastes used in this study were diluted three times with distilled water. The suspensions were used to simulate oral situation.

The effect of different concentrations of the S-PRG fillers in the experimental pastes on inhibition of enamel demineralization was evaluated in this study. Concentrations of the S-PRG fillers in the experimental paste ranged from 0 to 30 wt% in the experiment. A commercially available fluoride paste containing 950 ppm F (MP) was used as a positive control.

According to the MD profiles of the micro-CT assessment, inhibition of the enamel demineralization was variable in the different groups (Fig. 2). As expected the MD profile of the negative control (DW) group indicated the most aggressive demineralization of enamel. The experimental pastes containing 5 wt% or more S-PRG demonstrated significantly lower ML values than the negative control group. In addition, S10 was the only group, which was more effective than the positive control, commercially available fluoride paste in inhibiting demineralization. Viscosity of the S30 group was higher than the other experimental pastes in our pilot study. Previous study reported enamel loss was dependent on viscosity of acid. High viscosity decreased the ion exchange and clearance of dissolution products. High viscosity of the S30 group suggested a prevention of ion exchange on tooth surface at a slow rate.

The representative SEM micrographs indicated different features of enamel surfaces among the groups (Fig. 5). The protective effects of S10 against demineralization were evident from the SEM images, where the typical honeycomb etching pattern of enamel was absent unlike other groups in which enamel surface was clearly demineralized (Figs. 5c and d). These findings are in line with the micro-CT results.

The S-PRG filler particles are produced by acid-base reaction of fluoroboroaluminosilicate glass and polyacrylic acid, which is known to have a potential to release multi-ions including Al, B, Na, Si, Sr, and F. Fujimoto et al. measured concentration of F ions released from the S-PRG filler in distilled water using inductively coupled plasma atomic emission spectroscopy (ICP-AES) and fluoride ion electrode methods. They reported that up to 90 ppm of fluoride ions were detected from the slurry of the S-PRG fillers with distilled water, which was smaller F concentration than expected from the glass ionomer filler. This fact suggests that the effect of inhibition of demineralization of the S-PRG filler containing experimental pastes was not only due to F ions, but also related to other ions released from the S-PRG fillers. A recent study on a S-PRG containing coating material also suggested that multiple ions release from these fillers increased the resistance of enamel against demineralization. It was shown that ions released from the S-PRG filler had antibacterial and pH neutralizing effects on acidic solutions.

In a previous study, over 250 ppm of ionized Sr was detected from a suspension of the S-PRG fillers diluted twice with water. Dedhiya et al. reported that Ca-Sr apatite complex could form at the apatite crystal surface in presence of Sr ions, which may play an important role in retardation of acid dissolution. Sr is a homologous element of Ca which is thought to promote mineralization in conjunction with F.

Therefore, micro-PIXE analysis was done in the current study to detect Sr on the enamel surface. Micro-PIXE detects multi-elemental distribution in biological samples with high spatial resolution (1 μm range) and high sensitivity (down to μg/g range). According to the surface analysis of enamel using micro-PIXE, intensity of Ca was similar between the DW and S10 groups, while intensity of Sr was higher in the S10 group than in the DW group. In the cross-sectional enamel specimens of the S10 group, intrinsic Ca was visualized in the whole enamel area, which was almost similar before and after demineralization. On the other hands, Sr was densely absorbed on the enamel surface before demineralization, however, this phenomenon disappeared after demineralization. The accumulation of Sr on the enamel surface after immersion in the S10 suspension suggested that the inhibitory effects of the experimental paste might be explained partially by the Sr incorporation. In order to reveal the mechanism of action of Sr and its interaction with enamel (e.g. chemical state, composition and structural changes) should be investigated in future studies.

The current study demonstrated that the S-PRG
fillers containing experimental paste could protect enamel surface from the acid attack. Further study should be carried out to evaluate the efficacy of multi-ion release from the S-PRG fillers on demineralization and remineralization of enamel and dentin.

CONCLUSIONS

Micro-CT and SEM examinations revealed that the S-PRG filler containing experimental pastes effectively inhibited demineralization of enamel surface. Micro-PIXE analysis demonstrated high concentration of Sr on the enamel surface treated with the 10 wt% S-PRG fillers containing experimental paste, suggesting that Sr may play an important role in inhibition of enamel demineralization.

ACKNOWLEDGMENTS

This work was supported by JSPS KAKENHI Grant Numbers 25870203 and by the grant from the Japanese Ministry of Education, Global Center of Excellence (GCOE) program, “International Research Center for molecular Science in Tooth and Bone Diseases”. Micro-PIXE analyses were supported by the MEXT Project for Creation of Research Platforms and Sharing of Advanced Research Infrastructure (2014-001) of the National Institute of Radiological Sciences (NIRS, Japan).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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