INTRODUCTION

Dental caries is the result of tooth demineralization by acids that are produced through bacterial metabolism of sugars. Caries occurs through the breakdown of the dynamic balance between demineralization and remineralization of the highly mineralized enamel tissue. If the process is not interrupted in the early stage, substantial loss of minerals from enamel happens and leads to surface lesion. Therefore, improving the acid resistance of enamel surface is important to inhibit demineralization, particularly at high-risk tooth surfaces.

According to the minimal-intervention (MI) concept, early detection and prevention of enamel demineralization are important steps before taking the restorative approach. Fluoride and other substances such as casein phosphopeptide amorphous calcium phosphate (CPP-ACP) have been used to reduce enamel demineralization in experimental studies and clinical applications. The beneficial effects of topical fluoride application is based on a sizable body of evidence from randomized controlled trials. Fluoride can be incorporated incrementally into the tooth surface and form fluoridated apatite which decreases crystal solubility and make it more resistant to acid attack. There are several forms of fluoride used including NaF, acidulated fluorophosphates (APF) and stannous fluoride (SnF2) that may be used in various concentrations either for professional topical application as gel, paste and varnish, or for home use in the forms of toothpaste, mouthwash and gel.

Remineralization of demineralized enamel occurs in the presence of adequate concentration of mineral ions required to form apatite crystals. In recent years, several approaches have been investigated to increase the bioavailability of calcium and phosphate in the oral environment, i.e. in saliva and plaque and around the tooth surface. An ideal topical remineralization agent should diffuse and deliver calcium and phosphate into the enamel and increase the remineralization capability of saliva. From a chemical point of view, the theory behind prevention of demineralization and promotion of remineralization by these agents relies on local buffering of pH and supersaturation with respect to tooth minerals. Chow reported that dissolution of tetracalcium phosphate (TTCP) and dicalcium phosphate anhydrous (DCPA) would lead to a solution composition that is highly supersaturated with respect to hydroxyapatite (HA), resulting in HA precipitation. Recent in vitro studies have demonstrated that the application of a TTCP and DCPA based desensitizer material on the tooth surface may promote formation of HA crystal-like structure. This compound results in physical occlusion of dentinal tubules, and the obliteration of dentinal tubules might clinically decrease dentin hypersensitivity and reduce dentinal demineralization. Therefore, the TTCP and DCPA compound is an appealing anti-caries formula. However, the effects of topical TTCP and DCPA in prevention of enamel demineralization have not been investigated.

In the in vitro studies on preventive effects of topical agents on enamel demineralization, scanning electron microscope (SEM), confocal laser scanning
microscope (CLSM), transverse microradiography (TMR) and light microscopy have been routinely used to evaluate the cross-sectional morphological or mineral content changes in the tissue. In order to evaluate the effect of repeated applications of these agents, serial imaging is necessary. Nevertheless, in-depth evaluation of enamel using these techniques should need extensive specimen processing such as sectioning, coating, desiccating or polishing. Therefore, monitoring the effect of continuous applications of an agent on the same specimen using such invasive methods would be technically challenging.

In recent years, 3D and in-depth non-invasive imaging techniques have gained momentum in hard tissue research. Optical coherence tomography (OCT) is addressed as a technology which enables cross-sectional imaging of internal biological structures without the use of X-ray irradiation in a short time\textsuperscript{10,11}. It is a promising imaging modality, which does not require cutting and processing of the specimens and allows visualization of the microstructures of tissues and biomaterials almost in real time\textsuperscript{12}. As for clinical dental applications so far, OCT has been used to observe structures of caries, crack, fracture, restoration integrity and so on\textsuperscript{13}. The optical principle in evaluation of enamel demineralization by OCT is increased backscatter signal intensity, induced by multiple scattering and depolarization of the incident light through the demineralized enamel, typically projected from a near infrared laser source. This optical behavior of demineralized enamel is explained by the increased porosity and decreased density of the tissue\textsuperscript{14}.

The purpose of this study was to examine the potential anti-demineralization capacities of (a) TTCP and DCPA and 950 ppm fluoride paste, (b) CPP-ACP paste and (c) 0.21% sodium fluoride solution in comparison to (d) control solution (deionized water) in an artificial enamel demineralization model through in-depth monitoring of enamel using swept-source OCT followed by CLSM imaging. The null hypothesis was that none of the groups could prevent enamel demineralization when subjected to acidic challenge.

### MATERIALS AND METHODS

Three agents were used in this experiment to inhibit enamel demineralization: (a) AP Paste (Teethmate AP Paste, Kuraray Noritake Dental, Tokyo, Japan), a calcium phosphate based paste containing TTCP and DCPA and 950 ppm fluoride (as sodium fluoride), (b) MI Paste (MI Paste, GC Dental, Tokyo, Japan) containing CPP-ACP, and (c) 0.21% sodium fluoride solution (NaF, Wako Chemicals, Osaka, Japan) containing 950 ppm fluoride as a positive control. The composition of each material and application time of the treatment is listed in Table 1. The negative control group was (d) deionized water.

Forty fresh bovine incisors were obtained from a local slaughter house (Yokohama, Japan) and stored frozen prior to the experimental procedure. Enamel blocks 6×3×3 mm\textsuperscript{3} (width×length×depth) were cut from the teeth using a low speed diamond saw (Isomet, Buehler, Lake bluff, IL, USA) under running water, and embedded in epoxy resin (Epoxycure resin, Buehler). The outer enamel surface was slightly polished with a 800-grit silicon carbide (SiC) paper (Sankyo, Saitama, Japan) until a flat area was obtained on the surface. This was aimed to expose enamel structure, eliminate any possible superficial defects and create a standard flat smooth surface. For each specimen, half of enamel surface area (3×3 mm\textsuperscript{2}) was coated with a one-step adhesive system (G-BOND PLUS, GC Dental) to serve as the reference unaffected surface with another half area covered by an adhesive vinyl tape to protect from G-BOND PLUS. The residual half area was treated using one of the above-mentioned three materials or deionized water as control (n=10 per group). All samples were subjected to a demineralization gel (Hydroxyethyl cellulose HEC 3%, CaCl\textsubscript{2} 1.5 mM, KH\textsubscript{2}PO\textsubscript{4} 0.9 mM, CH\textsubscript{3}COOH 50.0 mM, NaN\textsubscript{3} 3.08 mM) at pH 4.5 at 37°C for 1 h followed by a remineralization solution\textsuperscript{15} (CaCl\textsubscript{2} 1.0 mM, KH\textsubscript{2}PO\textsubscript{4} 3.0 mM, NaCl 100 mM, CH\textsubscript{3}COONa 100 mM, NaN\textsubscript{3} 3.08 mM) at pH 6.3 for 23 h. This experimental cycle (test material or control application, demineralization in gel and remineralization in solution) was repeated for 28 days. Samples were rinsed with deionized water.

### Table 1 Materials used in this study

<table>
<thead>
<tr>
<th>Group</th>
<th>Product</th>
<th>Active ingredients</th>
<th>Manufacturer</th>
<th>Application procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP Paste</td>
<td>Teethmate AP Paste</td>
<td>TTCP, DCPA, 950 ppm NaF</td>
<td>Kuraray Noritake Dental Tokyo, Japan</td>
<td>Applied by use of a rubber cup for 60 s and left for 180 s</td>
</tr>
<tr>
<td>MI Paste</td>
<td>MI Paste</td>
<td>10% CPP-ACP</td>
<td>GC Dental Tokyo, Japan</td>
<td>Applied by use of a microsponge for 60 s and left for 180 s</td>
</tr>
<tr>
<td>NaF</td>
<td>NaF solution</td>
<td>950 ppm NaF</td>
<td>Wako Chemicals Osaka, Japan</td>
<td>Soaked for 180 s</td>
</tr>
<tr>
<td>control</td>
<td>Milli-Q</td>
<td>—</td>
<td>Millipore, Billerica MA, USA</td>
<td>—</td>
</tr>
</tbody>
</table>
every time between application, demineralization and remineralization steps. The specimens were imaged using swept-source OCT (IVS-2000, Santec, Komaki, Japan) at baseline and at four stages (after 7, 14, 21, 28 days of de/remineralization cycles).

The OCT system utilizes a high-speed scanning laser, sweeping 1,260- to 1,360-nm (center: 1,310 nm) wavelength at a 20-kHz rate. The optical resolution is 20 µm transversally and 12 µm axially in air (7–8 µm in tissues with a refractive index around 1.5). This system has been described in detail elsewhere\textsuperscript{16,17}. Briefly, the laser beam scans the object in X and Z dimensions. Collected backscattered light is returned to the system, digitized in a time scale, and analyzed in the Fourier domain to form a depth-resolving scan (A-scan) at each point. A serial set of A-scans along a certain section creates a cross-sectional B-scan, from which a high-resolution, 2-dimensional image can be obtained by converting B-scan raw data into a gray-scale image. At each scanning time, the specimens were washed with deionized water and fixed on a micrometer metal stage with 5° tilt to decrease specular surface reflections. To standardize the hydration condition of the surface, a thin film of water-based gel containing 5% HEC was applied. For each specimen, cross-sectional images were monitored at three locations. To replicate the imaging location at each time, the specimen was marked by a small hole and placed in the same orientation as previous scans. For image analysis, a custom code in the image analysis software (ImageJ version 1.45S, National Institutes of Health, Bethesda, MD, USA) was used to import the raw data of the OCT. A noise reducing median filter (size 2) was applied to the data. In order to measure the lesion depth, an experimental plugin which was developed for ImageJ was used. Threshold function of the software allows to find appropriate intensity values that correspond to the visual boundary of the enamel lesions, suggesting the demineralization front or optical lesion depth. Lesion depth was calculated over a fixed region of interest (ROI, width 300 µm × optical depth 500 µm) as described in Fig. 1. Direct observation of the physical cross-sections was accomplished under CLSM (1LM21H/W, Lasertec, Yokohama, Japan). Enamel blocks were secondly embedded by enamel infiltrating resin (Icon, DMG, Yokohama, Japan). The specimens were cross-cut along the location that was previously imaged by OCT using the diamond saw, and fine polished to perform CLSM evaluation. Each sample was sequentially polished by SiC papers #600, #800, #1000, #1200, #1500, and #2000 in circular motion under copious cooling water, followed by diamond slurry with particle sizes of 6, 3, 1, 0.5, and 0.25 µm in a lapping machine (ML-160A, Maruto, Tokyo, Japan).

Repeated measures analysis of variance (ANOVA) was used to compare the progress of lesion depth at different demineralization times among different materials and their interaction. This was followed by post hoc comparisons with Bonferroni correction. Further one-way ANOVA was used to compare the lesion depth between materials at different demineralization times. The statistical procedures were performed at a significance level of α=0.05 with the statistical package for social science (SPSS for windows, Version 16.0, SPSS, Chicago, IL, USA).

**RESULTS**

Mean optical lesion depth of different experimental groups are shown in Fig. 2. Repeated measures ANOVA revealed that demineralization time ($F=82.5, p<0.001$) and material ($F=43.9, p<0.001$) significantly affected the optical lesion depth. Their interaction effect was also significant ($F=24.8, p<0.001$). There was a significant difference in lesion depth at different demineralization times within all groups ($p<0.05$). Overall comparisons revealed that the lesion progress in AP Paste and NaF groups was significantly different from that in MI Paste and control groups ($p<0.05$), but there was no significant difference among AP Paste and NaF ($p>0.05$).

When materials were compared at each demineralization time by further pairwise comparisons, there was no significant difference between materials at 7 days ($p>0.05$). At 14 and 21 days control was significantly different from AP Paste, MI Paste and NaF ($p<0.05$) but there was no significant difference among AP Paste, MI Paste and NaF ($p>0.05$). At 28 days MI Paste and control was significantly different from AP Paste and NaF ($p<0.05$). However, no difference was found between AP Paste and NaF at any of the periods ($p>0.05$).

Representative OCT images of each experimental group are presented in Fig. 3. For all demineralized groups, superficial enamel showed a visible boundary between bright and dark areas on the grayscale OCT image, which is associated with depth of lesion. The boundary is most prominent for control group after 21
Fig. 2 Optical lesion depth calculated by OCT of different groups through demineralization days (n=10). The lesion progress in AP Paste and NaF was significantly different from that in MI Paste and control (p<0.05) but there was no significant difference among AP Paste and NaF (p>0.05). When materials were compared at each demineralization time, there was no significant difference between materials at 7 days (p>0.05) (black horizontal bar). At 14 and 21 days control was significantly different from AP Paste, MI Paste and NaF (p<0.05) (dotted black horizontal bar and white horizontal bar). At 28 days MI Paste and control was significantly different from AP Paste and NaF (p<0.05) but no difference was found between AP Paste and NaF (p>0.05) (dotted white horizontal bar).

Fig. 3 (a1–d1) B-scan images of reference surface and measuring surface after 7 days demineralization; (a) AP Paste, (b) MI Paste, (c) NaF, (d) control (a2–d2) after 14 days demineralization (a3–d3) after 21 days demineralization (a4–d4) after 28 days demineralization. The boundaries under the demineralized enamel was observed after 28 days demineralization (white arrows).

Fig. 4 Representative cross-sections confirmed by the CLSM for each material after 28 days of demineralization; (A) AP Paste, (B) MI Paste, (C) NaF, (D) control. The scale bar indicates 84.4 µm. The precipitation of minerals over the surface (white blank arrow) was observed. A thicker surface layer (white solid arrow) was observed.
Figure 4 shows the representative cross-sectional CLSM images for each material after 28 days of demineralization. In all experimental groups, similar lesion trends were found using CLSM compared with OCT. MI Paste and control groups showed the deeper lesion depth than AP Paste and NaF groups. AP Paste group showed a demineralization-resistant surface layer measuring about 15 μm in thickness, followed by a moderately demineralized subsurface zone beneath the surface layer (Fig. 4-A). CLSM images of NaF group showed precipitation of minerals over the surface and a thicker surface layer (Fig. 4-C). In line with OCT findings, CLSM images indicated that a demineralization-resistant surface layer was not observed in control group, and a deeper lesion was formed than all other groups. MI Paste group occasionally showed formation of a low-density demineralization-resistant surface layer (Fig. 4-B).

**DISCUSSION**

OCT is a noninvasive imaging system that can provide cross-sectional images of the dental structure nondestructively. Demineralization and remineralization processes on enamel are difficult to detect at early stages by visual inspection alone. In previous studies, it was shown that OCT had the potential in assessment of the early enamel lesions as well as the remineralization process\(^{19}\). The effectiveness of OCT in *in vitro* and *in vivo* observation of the internal structure and surface layer characteristics of a white spot lesion have been demonstrated\(^{19,20}\). The current results demonstrated the excellent potential of OCT imaging for observing the minimal changes in enamel subsurface lesion after demineralization. OCT enables cross-sectional imaging of internal biological structures in real time at baseline and monitoring of the continuous lesion progress.

In this study, TTCP and DCPA paste was applied to investigate if it could act as a bioactive material to protect enamel from demineralization. The AP paste along with NaF showed significantly lower lesion progress compared to other groups. Enamel surface reflectivity remained strong throughout the 28 days of demineralization in the AP Paste and NaF groups, which indicated that enamel surface had resisted demineralization; however, in MI Paste and control groups the surface gradually showed decreased specular reflectivity which indicates lower density of enamel surface. In the meantime, increased scattering at the subsurface zone, which gradually progressed deeper (especially in the control group) indicated gradual loss of minerals and increased porosity of enamel lesion. The demineralization-resistant zone at superficial enamel lesion was also confirmed on CLSM image. It has been reported that low concentrations (up to 1 ppm) of fluoride in a solution can reduce enamel demineralization\(^{21}\). When fluoride ions come into contact with free calcium and phosphate ions in a solution supersaturated with respect to tooth minerals (apatite), fluoridated apatite and fluoroapatite would rapidly form in the surface layer\(^{22}\). Hence, the demineralization-resistant zone may contain such apatite in the current experiment. Furthermore, AP Paste group contains TTCP and DCPA compound which has been shown to form minerals with Ca/P ratios close to that of HA\(^{23,24}\). In the scanning electron microscopy study using calcium phosphate desensitizer containing TTCP and DCPA, HA-like precipitates were observed on the dentinal surface and also in the dentinal tubules\(^{25}\). Another *in vitro* experiment suggested that the application of this desensitizer to dentin sample in acidic challenge inhibited to decrease ultrasonic velocities of dentin, suggesting that the application of this desensitizer prevented dentin demineralization\(^{26}\). Using the *in vitro* transmission electron microscopy (TEM), Chiba *et al.* evaluated that the remineralization effect of the tested desensitizing paste (AP Paste) on demineralized enamel\(^{24}\). They suggested that TTCP and DCPA could react with water to release calcium and phosphate ions, and both ions clearly promoted crystal growth in demineralized enamel\(^{24}\). In this study, TTCP and DCPA may contribute to inhibit the enamel demineralization by promoting apatite crystal growth. However, there is still little information available on the added clinical benefits of TTCP and DCPA in the AP Paste material. Further study is needed to identify such benefits and characterize the minerals formed by the calcium-releasing material.

Moreover, NaF group showed the precipitation of mineral-like structures onto the enamel surface. This precipitation may be caused by high fluoride concentration in the NaF solution; fluoride ions can drive the remineralization of enamel lesion and precipitation of new minerals if adequate calcium and phosphate ions are available\(^{28}\).

MI Paste group showed inhibition of surface lesion partially on CLSM image. Once present in the enamel subsurface lesion, the CPP-ACP would release the weakly bound calcium and phosphate ions that have a high binding affinity for apatite\(^{25}\). In this regard, MI Paste resulted in significant inhibition of lesion progress measured by OCT images compared to control.

Present study was an *in vitro* trial to compare the effect of daily application of anti-demineralization materials in an artificial enamel demineralization model. Within the limitations of the research, which included a narrow study design, the proposed null hypotheses were rejected as anti-demineralization materials could prevent enamel demineralization, and these anti-demineralization effects depended on the material used. Clinical management for the protection of sound enamel against demineralization is essential to realize the MI concept. For the patients at high-risk of dental caries such as dry mouth patients, such a product could help to reinforce enamel against demineralization. Further *in vitro* and *in vivo* studies on the assessment of bioavailable calcium and fluoride compounds could help to establish the simple preventive approach.
CONCLUSION

Application of calcium-releasing anti-demineralization pastes or sodium fluoride solution to the enamel surface could contribute to the protection of enamel surface against an acid challenge, and that potential was depended on the material used. OCT appears to be an effective tool for monitoring enamel lesion depth and surface layer over time.

CONFLICT OF INTEREST

The authors declare no conflicts of interest with respect to the authorship and/or publication of this article.

REFERENCES