In vitro remineralization of enamel with a solution containing casein and fluoride

Keiki NAKAMURA, Hidenori HAMBA, Yoshihito MIYAYOSHI, Hisako ISHIZUKA and Takashi MURAMATSU

Department of Operative Dentistry, Caniology and Pulp Biology, Tokyo Dental College, Tokyo, Japan
Corresponding author, Keiki NAKAMURA; E-mail: nakamurakeiki@tdc.ac.jp

The aim of the present study was to investigate the effects of casein in a remineralization solution on enamel remineralization. Bovine blocks were demineralized for 21 days, then, allocated into four groups. The specimens were remineralized for 21 days in the following artificial saliva solutions: 1) 0 µg/mL casein, 0 ppm fluoride (F) (C10–F0); 2) 0 µg/mL casein, 1 ppm F (C0–F1); 3) 10 µg/mL casein, 0 ppm F (C10–F0); and 4) 10 µg/mL casein, 1 ppm F (C10–F1). Micro-CT analyses were performed once a week. Specimens were characterized by scanning electron microscopy (SEM) and X-ray photoelectron spectroscopy (XPS). The present results suggest that casein by itself inhibits remineralization, whereas the coexistence of casein and F promotes the remineralization of caries bodies by interrupting mineral deposition on the enamel surface.

Keywords: Casein, Fluoride, Demineralization, Remineralization, Enamel

INTRODUCTION

One potential difficulty associated with the remineralization of dental enamel lesions is the preferential remineralization of the outer enamel surface, which may slow or inhibit complete remineralization in deeper regions of lesions by restricting mineral ion diffusion. A less supersaturated calcifying solution has been shown to remineralize enamel lesions more completely than a highly supersaturated calcifying solution, which induced the preferential remineralization of the outer enamel surface. Furthermore, conventional in vitro remineralization studies lacked salivary proteins and, thus, did not fully reproduce the oral environment.

Saliva and the dental pellicle have been shown to alter the caries process. The protein-containing layer of the dental pellicle is formed within a few minutes on the surface of a tooth and functions as an anti-erosive barrier and buffer zone. Pellicle-precursor proteins, namely, statherins, proline-rich proteins (PRPs), and histatins, are important salivary phosphoproteins in mineral regulation processes. They suppress calcium phosphate crystal nucleation and mineral precipitation by being adsorbed onto the apatite of the enamel surface. When these proteins coexist with fluoride (F), they promote mineral recovery within caries lesion bodies, whereas artificial saliva lacking these proteins, but containing F leads to mineral deposition on the enamel surface. The coexistence of salivary proteins and minerals in artificial saliva has frequently been overlooked in in vitro remineralization research.

Casein is one of the most abundant proteins in milk, and its biological function is, for the most part, nutritional. In oral health, casein has been shown to inhibit erosion and demineralization. Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) has been developed and shown to bind to hydroxyapatite (HAp) and saliva-coated Hap, and has been effectively used for remineralization. Casein derivatives have also been employed as agents that promote remineralization and inhibit demineralization. Casein phosphopeptides are potential salivary phosphoprotein homologues that have been used in in vitro remineralization research on the tooth surface. However, the combined effects of casein and F on the remineralization of enamel lesion bodies have not yet been elucidated in detail.

Therefore, we herein investigated the effects of remineralization solutions containing casein with or without F on the remineralization of enamel lesion bodies in vitro using micro-computed X-ray tomography (micro-CT), scanning electron microscopy (SEM), and X-ray photoelectron spectroscopy (XPS).

MATERIALS AND METHODS

Specimen preparation

Twenty non-damaged bovine incisors were purchased from Yokohama Meat (Yokohama, Japan), cryopreserved, and then defrosted using running tap water immediately before use. The procedure for specimen preparation is shown in Fig. 1. After mechanically removing the surrounding periodontal tissues, coronal pulp tissues and the roots were also removed. The crowns of teeth were cut into 3×3×2 mm pieces and enamel-dentine blocks were prepared using a water-cooled low-speed diamond saw (Isomet, Buehler, Lake Bluff, IL, USA). Flat enamel surfaces were prepared with 600- to 2000- grit silicon carbide papers (Fuji Star, Sankyo Rikagaku, Saitama, Japan) under running water. Nail varnish (680, Revlon, New York, NY, USA) was applied to the surface edge (diameter of 1 mm, depth of 0.5 mm) at the side of each stained with Calcite Green (0.1%, Alfa Aesar, Karlsruhe, Germany). The specimens were allocated into four groups: 1) 0 µg/mL casein, 0 ppm F; 2) 0 µg/mL casein, 1 ppm F; 3) 10 µg/mL casein, 0 ppm F; and 4) 10 µg/mL casein, 1 ppm F. Micro-CT analyses were performed once a week. The specimens were characterized by scanning electron microscopy (SEM) and X-ray photoelectron spectroscopy (XPS).

Received Oct 20, 2020: Accepted Jan 11, 2021

specimen as a reference landmark for micro-CT scans. Specimens were separately immersed in 10 mL of a demineralizing solution (2.2 mmol/L CaCl₂, 2.2 mmol/L KH₂PO₄, 50 mmol/L acetic acid, and 0.02% NaN₃, pH 5.3) at 37°C with constant agitation for 21 days without monitoring or maintaining the degree of saturation for the duration of the demineralization period. Micro-CT was performed as described below. Specimens were randomly allocated into four groups (n=7 each) with equivalent mean mineral loss (ΔZ). ΔZ was defined as integrated mineral loss from the sound enamel surface to the bottom of the lesion.

As described by Fujikawa et al., artificial saliva solutions (1 mmol/L CaCl₂, 3 mmol/L KH₂PO₄, 100 mmol/L NaCl, 100 mmol/L Na acetate, and 0.02% NaN₃) containing the following: 0 µg/mL casein and 0 ppm F (C₀–F₀), 0 µg/mL casein and 1 ppm F (C₀–F₁), 10 µg/mL casein and 0 ppm F (C₁₀–F₀) or 10 µg/mL casein and 1 ppm F (C₁₀–F₁), were used to remineralize pre-demineralized enamel specimens. Hammarsten grade casein (FUJIFILM Wako Pure Chemical, Osaka, Japan) was employed in the present study. Remineralization solutions were replaced daily, and micro-CT was performed on all specimens once a week. According to a previously described procedure, 5-mL vessels with screw caps were used for demineralization and remineralization processes.

**Micro-CT scanning**

The mineral density (MD) of demineralized specimens was assessed using a micro-CT system (HMX225-Actis 4, Tesco, Yokohama, Japan) that generates polychromatic X-rays with cone-beam geometry. The beam-hardening effect was attenuated by installing a 0.2-mm thick brass (Cu–Zn) filter in the beam path. Specimens were mounted on a turntable controlled by a computer to synchronize rotation and the axial shift. This set-up had a nominal isotropic resolution of 8.1 µm and integration time of 120 s. During the scanning process, specimens were rotated 360° at rotation steps of 0.6°. The tube voltage and current were 100 kV and 120 µA, respectively. Distances from the X-ray source to the sample and to the detector were 39.2 and 300 mm, respectively. Specimens were mounted on the table such that X-ray beams were perpendicular to the treated enamel surface.

Three HAp disks (Ratoc, Tokyo, Japan) were scanned as mineral reference phantoms to calibrate MD: one disk each with 0.50 and 0.70 g HAp/cm³ of HAp crystals embedded in epoxy resin (Epoxicure Resin, Buehler) and one pure HAp disk (3.16 g HAp/cm³) (Cellyard, Hoya, Tokyo, Japan).

**Micro-CT image analysis**

Each scan provided 100 images in the 16-bit TIFF format. To visualize samples and perform quantitative volumetric measurements, 3D analysis software (TRI/3D-BON, Ratoc) reconstructed a 3D image stack from 2D images at a resolution of 512x512 pixels and isotropic volumetric pixel (voxel) size of 8.1 µm. A linear calibration curve based on grey values from the three mineral reference phantoms (linear regression, $R^2>0.9994$) was used to convert CT values into MD values (g HAp/cm³). To reduce noise, data were run through a median filter and the background was removed by the exclusion of pixels with a CT value less than the zero MD value according to the calibration curve. The software then manually translated and rotated the 3D volumes rendered in order to visually match the baseline image, which was used as the reference. The following features of each image were used for the matching process: the sound enamel surface, specimen edges, and reference landmarks (holes made in dentine by the dental bur). A volume of interest (VOI) of 486x486x486 µm³ in the center of the demineralization window was subjected to assessments. Mean MD values in the VOI were calculated at depth intervals of 8.1 µm. MD (vol%) was then plotted to provide the MD profile of each specimen. All maximum MD in the baseline (sound enamel) specimen were normalized to 100 vol% against depth.

A typical 2D image of the control group after demineralization is shown in Fig. 2. The enamel
subsurface region was observed as a mottled demineralized image.

MD profiles were used to calculate mean integrated mineral loss ($\Delta Z$) (vol% $\mu$m) after each demineralization period. The axial position of the sound enamel surface at baseline was used as the reference point of the depth axis (0 $\mu$m). As shown in Fig. 3, $\Delta Z$ in each specimen was calculated from MD profiles by subtracting the area under the curve after demineralization from that before demineralization. These calculations were performed by importing MD data for each depth of demineralization into a spreadsheet software package (Excel for Windows, Microsoft, Redmond, WA, USA). Similar $\Delta Z$ analyses were performed for all groups using the original surfaces as a reference. Mineral recovery vol% was computed as follows: ($\Delta Z$ baseline–$\Delta Z$ after 7, 14, 21 days)/$\Delta Z$ baseline×100.

$\Delta Z$ baseline: $\Delta Z$ at baseline of intact enamel; $\Delta Z$ after 7, 14, 21 days: $\Delta Z$ after 7, 14, 21 days of remineralization

**SEM observations**
Specimens were prepared as described for micro-CT. Following a protocol to process specimens for SEM involving desiccation and gold sputter coating, SEM (SU6600, Hitachi, Tokyo, Japan) was conducted at a magnification of ×3,500 on additional specimens in each group (after 21 days of remineralization), and morphology perpendicular to the enamel surface in the treatment window was observed.

**XPS observations**
Extra specimens from the C0-F1 and C10-F1 groups (after 21 days of remineralization) were examined by XPS (Kratos Axis Ultra spectrometer, Kratos Analytical, Manchester, UK) using Al-Kα as an X-ray source at 8 kV and 30 mA to measure the intensity of F ions. The depth of F ions was investigated from the enamel to dentine direction. The depth profiles of chemical compositions were measured by electron beam with a probe area of 2.5×4.0 $\mu$m was accelerated by a 5-kV acceleration voltage with continuous Ar ion etching of 2 keV at the low 10⁻⁹ torr (etching rate for F ions: 4.3 nm min⁻¹).

**Statistical analysis**
Mineral recovery vol% was shown as the mean±standard deviation. Data were analyzed using a repeated measures
ANOVA and the Bonferroni post-hoc test. Statistical analyses were performed using a statistical software package (EZR, Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). The significance level of all tests was set at $\alpha=0.05$.

RESULTS

Mean MD profiles
The MD profiles of enamel in each experimental group are shown in Fig. 3. Following remineralization, mineral recovery was the lowest in the C10-F0 group, and were followed by slight increases in subsequent weeks.

Remineralization level (m)
Mean mineral recovery vol% over time is shown in Fig. 4. Mineral recovery vol% increased in all groups, except for the C10-F1 group (blue profile in Fig. 4), in a time-dependent manner and showed a similar pattern of remineralization. Mineral recovery vol% was in the order of C10-F1, C0-F0, C0-F1, and C10-F0 after 21 days. Mineral recovery vol% was significantly higher in the C10-F1 group than in the C10-F0 or C0-F1 group ($p<0.05$).

SEM observations
Figure 5 shows representative SEM micrographs perpendicular to the enamel surface after 21 days of remineralization. After remineralization, prismatic enamel structures were clearly observed in the C0-F0 group. Furthermore, the C10-F0 and C10-F1 groups had visible aggregates on their enamel surfaces. The structure of the conventional enamel prism differed in the C0-F1 group.

XPS observations
Survey spectra and sputter-cleaned surfaces showed F peaks at approximately 685 eV (Fig. 6). Peak intensities after sputter cleaning decreased for F. At 460 s, the C10-F1 group showed a peak (arrow) at approximately 685 eV, whereas the C0-F1 group did not.

Fig. 4 Mineral recovery vol% in remineralization groups after 7, 14, and 21 days. The same lowercase letters indicate significant differences with a repeated measures ANOVA and the Bonferroni correction ($p<0.05$).

Fig. 5 SEM micrographs of enamel cut surfaces treated with C0-F0, C0-F1, C10-F0, and C10-F1. The structure of the conventional enamel prism differed in the C0-F1 group (arrow). Deposits were observed on the enamel prism in the C10-F0 and C10-F1 groups (arrowheads).

Fig. 6 Line charts showing the sputtering time of the enamel surface. Ions were observed at deeper regions with increases in the sputter time. Dashed lines indicate the binding energy of peak positions on the enamel surfaces of F. At 460 s, the C0-F1 group had fewer F peaks, whereas the C10-F1 group had more (arrow), suggesting that F entered deeper regions in the C10-F1 group than in the C0-F1 group.
DISCUSSION

The present study investigated the effects of casein in remineralization solutions with or without F on enamel remineralization in vitro. The results obtained showed that remineralization solutions containing only casein inhibited mineral deposition on enamel surfaces, while those containing casein and F promoted remineralization at the lesion body.

Casein exists in micelles that stabilize calcium (Ca) and phosphate (P) ions in milk. Salivary proteins, including statherins, histatins, and PRP, form protein-Ca complexes that suppress apatite growth. Therefore, the availability of free mineral ions in solutions for lesion remineralization may be maintained at higher levels by preventing the binding of mineral ions deposited on surface enamel to apatite growth sites. Micro-CT data showed that mineral recovery vol% was significantly lower in the C10-F0 group than in the C0-F0 group. Furthermore, deposits were ultrastructurally observed on the enamel surface in the C10-F0 and C10-F1 groups. These results suggest that casein inhibited mineral deposition or the further growth of demineralized enamel crystallites at the superficial layer of enamel. C0-F1 groups were lower mineral recovery level than C0-F0. 1 ppm F alone had no significant effect on remineralization. Since F is as small as 1 ppm, it is considered that there was no significant effect on remineralization by itself.

XPS revealed the presence of F in deep enamel regions in the C10-F1 group. No F was observed on the surface at the sputtering time of 0 seconds in the C10-F1 group, indicating that F was not precipitate on the surface. The F peak near surface of the C0-F1 group is observed around 684 eV, and the internal F is observed around 685 eV, suggesting that F with different chemical compositions is present on the surface. Remineralization solutions containing F and casein achieved higher MD in deep regions of the lesion body, suggesting the deeper penetration of ions in the presence of F. Previous studies demonstrated that casein suppressed surface mineral precipitation, but promoted the remineralization of subsurface lesions. Fujikawa reported that when casein is not contained, mineral deposition occurs on the surface layer and minerals do not reach subsurface lesions. Casein was thought to deposit on the surface of the enamel and allow minerals to penetrate through the gaps. Although the underlying mechanisms remain unclear, casein deposits may not have blocked pores in demineralized enamel and allowed apatite-forming mineral ions, such as Ca, P, and F, to enter deep regions of the lesion body.

A previous study used a casein concentration of 0.5% w/v, which was 500-fold higher than that used in the present study, and demonstrated reductions in surface softening and erosion in an in vitro erosion model. Furthermore, the addition of 300 ppm F to a 0.5% w/v casein solution markedly reduced tissue loss and prevented softening. Collectively, these findings and the present results imply the synergistic effects of casein with F on the remineralization of lesion bodies. The dose and concentration of F have been shown to affect the cariostatic properties of F in milk. Mineral precipitation on the human enamel surface as well as on dentine markedly increased following the incorporation of 1 ppm F. In the present study, we added 1 ppm F, based on the estimated residual concentration of F after its application in the oral cavity. The present results demonstrated that the application of a low concentration of casein (10 µg/mL) and 1 ppm F was effective for lesion remineralization.

Milk fluoridation to prevent dental caries has been extensively examined since the early 1950s. The optimal daily intake of F from milk effectively prevents dental caries. The present results also support the effectiveness of milk fluoridation for the prevention of dental caries. Future investigations are needed to provide further evidence for milk fluoridation.

CONCLUSIONS

A remineralization solution containing casein only significantly suppressed remineralization by covering the enamel surface; however, the coexistence of casein and F promoted the remineralization of caries bodies by interrupting mineral deposition on the enamel surface.

ACKNOWLEDGMENTS

This work was supported, in part, by a Tokyo Dental College President’s Encouragement Research Grant. The authors would like to thank Ms. Eiko Watanabe (Oral Health Science Center, Tokyo Dental College) for her assistance with the XPS analysis.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

6) Bennick A, Kells D, Madapallimattam G. Interaction of calcium ions and salivary acidic proline-rich proteins with hydroxyapatite. A possible aspect of inhibition of