Short communication

Synchrotron radiation microbeam X-ray fluorescence analysis of zinc concentration in remineralized enamel *in situ*

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ABSTRACT

Objective: Remineralization is an indispensable phenomenon during the natural healing process of enamel decay. The incorporation of zinc (Zn) into enamel crystal could accelerate this remineralization. The present study was designed to investigate the concentration and distribution of Zn in remineralized enamel after gum chewing.

Methods: The experiment was performed at the Photon Factory. Synchrotron radiation was monochromatized and X-rays were focused into a small beam spot. The X-ray fluorescence (XRF) from the sample was detected with a silicon Si(lithium (Li)) detector. X-ray beam energy was tuned to detect Zn. The examined samples were small enamel fragments remineralized after chewing calcium phosphate-containing gum *in situ*. The incorporation of Zn atom into hydroxyapatite (OHAP), the main component of enamel, was measured using Zn K-edge extended X-ray absorption fine structure (EXAFS) with fluorescence mode at the SPring-8.

Results: A high concentration of Zn was detected in a superficial area 10 μm deep of the sectioned enamel after gum chewing. This concentration increased over that in the intact enamel. The atomic distance between Zn and O in the enamel was calculated using the EXAFS data. The analyzed atomic distances between Zn and O in two sections were 0.237 and 0.240 nm

Conclusion: The present experiments suggest that Zn is effectively incorporated into remineralized enamel through the physiological processes of mineral deposition in the oral cavity through gum-chewing and that Zn substitution probably occurred at the calcium position in enamel

hydroxyapatite.

Keywords: Enamel, Remineralization, Zinc, Synchrotron radiation, X-ray fluorescence

1. Introduction

Many trace elements were incorporated in the biologically mineralized tissues.[1,2] In particular, Zn is a dietary essential trace element known to be necessary for normal collagen synthesis and mineralization of bone.[3,4] It has long been said that zinc compounds, such as zinc chloride and zinc citrate are beneficial for teeth, and that zinc oxide inhibits dentine demineralization.[5] However, the mechanisms by which Zn inhibits dentine demineralization are not fully resolved. In men, dietary Zn intake and plasma Zn both have a positive relationship to bone mineral density.[6] Zn has also been demonstrated to have a potent stimulatory effect on osteoblastic bone formation[7,8] and an inhibitory effect on osteoclastic bone resorption.[9,10] Furthermore, Zn can stimulate protein synthesis in osteoblastic cells *in vitro* by active aminoacyl-tRNA synthetase.[11] Zn acts as a nucleator in the formation of the whitlockite phase *in vitro*.[12] The Zn incorporation in HA and whitlockite have also been investigated in *in vitro* conditions.[13,14] The Zn content decreased significantly from the outer to the inner enamel.[15] However, the exact distribution and concentration of Zn are still obscure in enamel remineralization.

The incorporation of Zn into enamel crystal could promote this remineralization. The present study was designed to investigate the Zn incorporation and concentration in a remineralized enamel surface. The experiment was performed by synchrotron radiation induced X-rays focused into a small beam spot. The XRF from the sample was detected with a Si(Li) detector. For the purpose of trace element detection, synchrotron radiation analyses bring sub-micron resolution and detection limits of the ppm range. We hypothesized that the distribution and concentration of Zn in mineral deposition could be elucidated using calcium-containing chewing gum to accelerate the remineralization. Thereafter, the local environment of Zn atom in remineralized enamel was analyzed by using the XAFS techniques.

2. Materials and methods

2.1. *Sample preparation*

Three third molars with a clinical diagnosis of pericoronitis without carious lesions were extracted with the patient's consent. After extraction, the enamel of the coronal region, about 1 x 1 x 1 mm was excised with a diamond disk under a stream of Ringer's solution. After coating 5 planes of these cubic fragments except the original enamel surface with nail varnish, artificial demineralization buffer which contained 0.1 M lactic acid, 3.0 mM $CaCl_2$, 1.8 mM KH_2PO_4 was used to create artificial enamel lesions for 3 days at 37^{o}C, pH 4.5. After demineralization, these fragments were fully rinsed with tap water and distilled water and dried at room temperature for 1 week. Afterward, these fragments were sterilized using low temperature

plasma sterilization

2.2. *Oral appliance preparation*

Three demineralized enamel fragments were set on the occlusal plane of each appliance which was made of acrylic resin. These appliance were set in the 11 volunteers' (no smokers, at least 28 natural teeth with no current caries activity and periodontal disease, no antibiotic medication, with 1 mL/min of salivary flow rate) oral cavity for remineralization experiments through gum chewing *in situ*.[16] Approval for this study was obtained from the Nagasaki University Graduate School of Biomedical Sciences Human Research Committee, and all subjects provided their writtem informed concent.

2.3. *Remineralization experiment in situ*

Two different types of calcium phosphate-containing xylitol chewing gums on the market were examined with at least one week as a rest period between treatments. One was Xylitol® (Lotte Co.; L gum), which contained xylitol, funoran and calcium hydrogen phosphate. The other was Poscam® (Glico Co.; G gum), which contained xylitol and calcium phosphorylated oligosaccharide. For the pellet gum study, the subjects set the appliance in the mouth chewed for 10 minutes and the appliances were retained for another 10 minutes in a subject's mouth; 5 times daily for seven days (sufficient for quantitative comparison using energy dispersive X-ray microanalysis (EDX)).[16] This *in situ* study was carried out by the double blind method.

2.4. *Specimen preparation*

After the completion of each treatment, the enamel pieces were removed

from the appliances, fixed and dehydrated in ethanol. The ethanol was replaced with *t*-butanol and the specimens were freeze-dried and preserved in the container until analysis. For SEM and EDX of the enamel sections, each enamel fragment was divided into two pieces using a razor blade. One piece of enamel section with relatively smooth surface was used for the Zn analysis without carbon coating.

2.5. *Synchrotron experiment*

The experiment was performed on a beam line (BL)-4A (the station for XRF analyses) at the Photon Factory, High Energy Acceleration Research organization (KEK) in Tsukuba, Japan. Synchrotron radiation from a bending magnet was monochromatized using a synthetic multiplayer monochromator and X-rays were focused into a small beam spot of about 4 μm diameter using a Pt-coated ellipsoidal mirror. The sample was moved by an x-y-z stepping motor (1 mm) while being observed by the optical microscope. The XRF from the sample was detected with a Si(Li) detector (Princeton Gamma-Tech. Inc., Princeton, NJ, USA). X-ray beam energy was tuned to be 14.2 keV to detect Zn. Zn spectrum was analyzed at 6 points, the 10 μm step of interval from the superficial layer (0 μm deep) to the inner layer (50 μm deep) and in 5 files (100 μm interval) in each piece. A total of 59 pieces {18 pieces for L gum, 23 pieces for G gum, 4 pieces for control without gum-chewing (C), 7 pieces for only demineralization (D) and 7 pieces for intact enamel (I)} were measured. The standard reference material (SRM) was used during the calibration process. The area density of each spot was precisely determined by comparing it to that of a reference thin film. SRM consists of

a 64 μg/cm^2 density of zinc which has deposited on the Mylar membrane (2.5 μm thick) (Micromatter Co., Deer Harbor, WA, USA).

Furthermore, the possibility of substitution of Zn atom into OHAP as a main component of enamel crystal was preliminarily analyzed in 2 specimens (one remineralized and one intact pieces) using Zn K-edge EXAFS with fluorescence mode on BL37XU (the station for XRF analyses) at the largest synchrotron radiation facility in the world (SPring-8, Hyougo, Japan). X-ray microbeam (1 μm diameter) energy was tuned to be 10 keV to detect Zn. Three points were measured in each piece at 4-5 μm deep from surface. A 5% Zn-substituted OHAP powder was used for the reference.[17] The EXAFS signals were analyzed using a special software program (REX2000, Rigaku Co., Japan).

2.6. Statistical analysis

Each Zn concentration was expressed as the mean $\pm$ SE. Paired Student's *t*-test was applied to assay any significant difference among the data measured. Significance was set at a probability level of $p<0.05$.

3. Results

The concentration of Zn was highest at the superficial layer in all samples (Table 1). Then, the statistical comparison of Zn distribution was carried out using the average concentration of both 0 and 10 μm deep areas from surface. The remineralized enamel outer layer after L gum chewing revealed the highest Zn concentration, which showed also significantly higher than that in intact enamel ($p<0.05$). There was no significant difference between G

gum chewing and the intact enamel groups. The Zn concentration in the remineralized enamel after either L ($p<0.01$) or G ($p<0.05$) gum chewing was significantly higher than that in the control group without gum chewing. The average Zn concentration in the remineralized enamel after chewing the two types of gum was not significantly higher than that in the intact enamel.

The atomic distance between Ca and the nearest O in OHAP is theoretically 0.258 nm.[18,19] The difference of an ionic radius between Ca and Zn is about 0.02 nm. Then, 0.238 nm (0.258 minus 0.02) was set up as the atomic distance between Zn and O in OHAP for the software analyses. The EXAFS data analyses showed that the average atomic distance between Zn and O was calculated to be 0.237 $\pm$ 0.004 nm in one piece of remineralized enamel and 0.240 $\pm$ 0.000 nm in one piece of intact enamel.

4. Discussion

This study is the first report that Zn is present in the superficial layer of remineralized enamel after gum chewing, and is consistent an earlier study in the intact enamel.[15] The highest concentration of Zn was detected in the superficial layer of the enamel section. We have already confirmed that EDX showed a tendency for high Ca/P molar ratio in the superficial layer of remineralized enamel.[16] The present study also shows that gum chewing promotes the Zn accumulation into the surface of remineralized enamel. These findings strongly suggest that Zn is closely related to the mineralization process.

The present data indicate that calcium phosphate-containing gum chewing for 1 week could restore the Zn concentration in the artificial demineralized enamel. This means that the incorporation of Zn originated from saliva into demineralized enamel is probably necessary for the remineralization phenomena and that the gum chewing could promote the remineralization process in enamel. Zn is thought to be an important and indispensable trace element for mineral deposition in enamel remineralization, as it is in osteoblastic bone formation.[7,8] Although further studies must be conducted to determine the interaction between Zn and Ca ions, this study supports the possibility that Zn is an indispensable trace element for tooth mineralization, especially the acceleration of mineralization.

This study is also the first XAFS examination of remineralized enamel. The analyzed atomic distances between Zn and O in two sections were 0.237 and 0.240 nm, which were very close to the theoretically shortest distance of 0.238 nm. However, the atomic distance between Zn and O in the standard zinc oxide crystal is 0.195-0.198 nm, whose distance is completely different from the present distance. The negative correlations show the possibility of substitutions among the elements Ca-Zn for the different types of teeth and dental mineral.[13,20] Although the present experiments for XANES and EXAFS was done using very limited specimens, the present data suggest that Zn should be incorporated into remineralized enamel through the gum chewing process for mineral deposition and that Zn substitution probably occurred at the calcium position in the hydroxyapatite crystal of remineralized enamel as in the superficial layer of an intact enamel .

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Table 1 – Zn concentration (ppm) at the superficial layer in each group

Group / μm deep	L	G	C	D	I
0	777.70±32.44	696.96±15.17	611.60±28.15	506.97±20.55	643.32±20.57
10	673.15±24.71	577.04±17.22	481.54±24.19	434.98±16.68	550.69±24.10
A	725.42±21.95	637 ±14.44	543.57±29.99	470.98±16.17	597.00±19.92
20	577.83±25.85	484.64±18.44	396.64±30.65	377.89±21.77	484.78±24.98
30	499.68±29.50	419.48±18.69	344.13±38.60	330.06±25.62	436.83±23.18
40	434.19±30.84	370.67±18.96	317.78±40.15	290.22±26.60	399.83±19.86
50	380.07±29.00	330.36±17.56	295.35±40.84	258.91±25.85	365.43±15.83

A, the average concentration of both 0 and 10 μm deep areas; C, control without chewing; D, only demineralized enamel; I, intact enamel.