An approach to normalizing micro-CT depth profiles of mineral density for monitoring enamel remineralization progress

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To indicate the possibility of a new approach to creating mineral density profiles, and to examine longitudinal changes in ‘the rate of remineralization (Rₐ)’ and ‘the mineral density (Dₐ,s) at 4 different depths’ (surface zone: SZ, lesion body: LB, middle zone: MZ, deep zone near to sound area: DZ) in enamel subsurface lesions, eight demineralized bovine enamel-dentin blocks were remineralized for 1 to 4 week and investigated using Micro-focus X-ray CT (micro-CT). After CT scanning, mineral density profiles were created. Mineral densities at each depth after demineralization were SZ<LB<MZ<DZ. Increase in Rₐ was the greatest in the first week of remineralization and it decreased over time. Increments of the mineral density were greater in the order of SZ<LB>MZ<DZ. This study indicated a new approach to create a mineral density profile and suggested the greater the value of the mineral density before the remineralization, the smaller the mineral density increments.

Keywords: Micro-CT, Normalization, Remineralization, Mineral density at different depths

INTRODUCTION

Micro-CT system is a novel and developing technology. The most notable advantage of micro-CT is its non-destructiveness. Caries formation is very complicated phenomenon that involves de- and remineralization¹. Using the advantage of micro-CT, it is possible to measure and visualize longitudinal mineral changes during de- and remineralization in the same lesion. Neves et al.² reported a satisfactory finding that there existed a linear correlation between CT intensity (or gray scale value) and the mineral density using threeapatite phantoms, the linearity covering a range of 0.25 to 3.14 g/cm³. Similarly, Schwass et al.³ found a good linearity using six phantoms covering a range from 0.07 to 2.95 g/cm³. These reports supported the quantitative analysis of the de- and remineralization based on CT intensity data.

There have been many studies concerned with de- and remineralization. Most of these studies were carried out using traditional Transversal Micro Radiography (TMR) method⁴,⁵. Although TMR has been used as a gold standard for quantifying mineral gain and loss, the method is usually destructive to enamel and dentin tissues, and they did not show any longitudinal mineral changes in exactly the same lesions. Thus, it can be said that the method is not suitable for observation of the longitudinal mineral changes in the same specimen. To overcome this disadvantage, some researchers used a ‘single thin section technique’, where incipient lesion is artificially produced in the enamel section with a thickness of approx. 100 μm and monitored by TMR analysis⁶, to study the longitudinal mineral change by applying TMR method. However, the section of specimen for this method was fragile. So, it would be quite difficult to perform a long term period of de- and remineralization experiments⁷.

Dowker et al. reported the development of subsurface enamel lesions using micro-CT⁸. Huang et al. showed mineral density profile of enamel white spot lesions using micro-CT⁹. However, they did not investigate longitudinal change in remineralization or longitudinal process of mineral change probably due to difficulty with accurate alignment of the 3D images. However, this difficulty was overcame using a software in the present study (TRI/3D-BON, Ratoc system engineering, Tokyo, Japan). Quantitative and longitudinal analysis of de- and remineralization using micro-CT was expected to provide new findings of a remineralization phenomenon, for example, which part of the lesion would be preferably remineralized under some experimental condition, or what experimental conditions would accelerate remineralization. If such things be clear, these findings would be valuable for better understanding of the dynamic mechanism of de- and remineralization. Thus, the first aim of this study was to indicate a possibility of normalizing approaches to creating depth profiles from the CT images during de- and remineralization. The second was to examine behaviors of the longitudinal changes in ‘the rate of remineralization (Rₐ)’, and ‘the mineral density (Dₐ,s) at 4 different depths’ of enamel subsurface lesions using micro-CT.
MATERIALS AND METHODS

Preparation of the specimens
Eight bovine enamel-dentin blocks with an approximate size of 5×4×2 mm³ were cut from bovine incisors using a water-cooled diamond-edged blade (Isomet® Low Speed Saw, Buehler, IL, USA). Buccal surfaces were polished flat with 2000-grit silicon carbide paper (Fujistar, Sankyo Rikagaku, Saitama, Japan). On the enamel surface, four small defects were prepared at the edges. These defects were used as one of an aid for alignment of 3D images of the specimens. The specimen blocks were then painted with water-resistant nail varnish except for a window (approximately 3×2 mm² in size) for de- and remineralization treatments.

The enamel specimens were demineralized at 37°C using an acidified gel system for 10 days to create subsurface lesions: acidic gel (120 mL) with pH 4.6 containing 8% methylcellulose (Methocel® MC, Fluka, Buchs, Switzerland) and the gel being covered with 200 mL of 0.1 M lactic acid. After the lesion formation, the specimens were remineralized for 4 weeks at 37°C in a remineralization solution (20 mL per specimen) containing 1.5 mM CaCl₂, 0.9 mM KH₂PO₄, 130 mM KCl and 20 mM HEPES (pH 7.0)¹⁰. The solution was renewed every week (Fig. 1).

Micro-CT scanning, alignment of images and creation of the CT intensity profile
A cone-beam micro-CT scanner (TDM 1000, Yamato Kagaku, Tokyo, Japan) was used to scan the specimens. Each specimen was scanned 6 times: before demineralization (sound), after demineralization and after 1, 2, 3, and 4 weeks of remineralization. The micro-CT images were taken under the following conditions; tube voltage 90 kV, tube current 35 μA and voxel size 3.5 μm, with setting the axis of the tooth specimen orthogonal to the micro-CT specimen stage. Aluminum filter (0.5 mm) was installed in the beam path to cut off the low energy x-rays. Scanning was performed under 100% humidity to avoid dehydration of the specimens, where the specimens were placed in a small container with wet cotton.

Following the 3D image reconstruction, a median filter was applied to eliminate ‘random noise’. Then the 3D images were precisely aligned using the software¹¹. To evaluate the mineral changes at exactly the same site in the same lesion, commercial software was employed for the accurate 3D alignment of CT images in the present study (TRI/3D-BON, Ratoc system engineering, Tokyo, Japan). Each specimen had six 3D images: before demineralization (sound), after demineralization and after each week of remineralization (1 to 4 weeks). The software enabled real time precise alignment of 3D image by rotations and movement. The specimens were rotated at 0.1 degree rotation and were moved at 3.5 μm interval three dimensionally. The alignment was done referring to the 4 small defects, the shapes of the demineralized window and the 3D configuration such as enamel surface or dentinoenamel junction (DEJ).

Creation of normalized profiles from raw CT intensity profiles
After the alignment of the 3D images, the area (0.36 mm²) for evaluation was set on each lesion surface to obtain depth profiles. The profiles started from

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Fig. 1 Specimen preparation.
approximately 100 μm on the outer side of the lesion surface (air) and extended to the deeper sound region (until approximately 450 μm) through the lesion. Six raw CT intensity profiles per specimen were obtained from 8 specimens. Although CT scanning was done in the same conditions in the same specimens, considerable deviations in CT intensities were shown in each profile. So, to standardize these deviations, step-wise approaches were carried out (Fig. 2). First, as differences in the CT intensities between the sound (non-demineralized) enamel and air should be the same, these two values were standardized. Two reference points were defined on each CT intensity profile: CT intensity at the enamel surface (a boundary between air and the enamel surface) (p, f(p)) and CT intensity at the sound region (q, f(q)). Location of enamel surface (p, f(p)) was defined as the intersection point between two tangent lines drawn from the CT intensity profile: one line from the enamel surface zone, the other from air in front of the enamel surface. The sound region (q, f(q)) was set at 350 μm deep from the enamel surface (p, f(p)) in this study. Then the difference in CT intensity between sound enamel and air (f(q)−f(p)) was set to ‘15,000’ for convenience’s sake. ‘15,000’ was used because differences in the raw CT intensities, (f(q)−f(p)), were approximately ‘15,000’ in each profile in the present measurement.

For all the depth profiles, following equations were employed.

1) \(|f(q)−f(p)|×a=15,000\)
where ‘a’: coefficient for each profile to standardize these deviations in the CT intensities. Then all the CT intensity profiles were converted to arbitrary scale-unit (the range from 0–100 unit, hereafter ‘A-unit’). This unit is a substitute for ‘mineral density’ in the present study.

2) \(15,000×β=100\) (A-unit)

3) \(\beta=x(αf_{raw}(x)−f(p))\)=\([15,000×f_{raw}(x)−f(p)×f(q)−f(p)]/150\times(f(q)−f(p))\)
where \(f(x)\): normalized equation for all the profiles, \(x\): depth along the x axis, \(f_{raw}(x)\): raw CT intensity profile, \(β\): coefficient to convert CT intensities into A-unit values. Finally, each point (p, f(p)) of all the profiles was set to origin coordinate, A-unit 0 and 100 represents f(p) and f(q), respectively (Fig. 2).

The normalized profiles were regarded as the mineral density profile (g/cm³) based on a previous finding that strong linear correlations existed between the CT intensity and the mineral density²,³.

Analysis of normalized profiles
From these normalized profiles, longitudinal changes in ‘the rate of remineralization (RΔ)’ and ‘the mineral density (DΔ)’ at 4 different depths’ in subsurface enamel lesions were calculated.

\[RΔ(%)=\frac{\text{integrated area (A-unit}×\mu m) \text{ after each week of remineralization}}{\text{integrated area (A-unit}×\mu m) \text{ before the remineralization}} \times100\]

\[DΔ(%)=\frac{\text{A-unit value at each depth after each week of remineralization period}}{\text{A-unit value before the demineralization (sound enamel) at each depth}} \times100\]

The \(RΔ\) is a representative parameter to evaluate the remineralization progress, and by examining the change in \(RΔ\) over time, one can monitor longitudinal change in remineralization process. The integrated area (A-unit×μm) was calculated corresponding to so-called integrated mineral loss (IML, or \(ΔZ\): vol%×μm) in TMR method. For \(DΔ\), 4 different depths were selected as representative depths for characterizing the lesion profile: at surface zone showing maximum mineral density (SZ), at lesion body showing minimum mineral density (LB), at one third (middle zone: MZ) and two thirds (deep zone: DZ) of the distance between LB and the lesion depth. The lesion depth was defined as a distance from the enamel surface to the depth at maximum A-unit in each profile. The locations of 4 different depths (SZ, LB, MZ and DZ) were fixed before the remineralization for each specimen. Thus change in the mineral densities (DΔs) was observed at the same locations. A-unit values for each DΔ were calculated by averaging the values measured in sequential 3 slices at the each depth position (SZ, LB, MZ, and DZ) and its anteroposterior slices.

Statistical Analysis
Regression analysis was used to examine the relations between mean \(RΔ\) or mean \(DΔs\) and the remineralization periods. \(DΔs\) were also tested by two-way analysis of variance (ANOVA) and t-test. Two factors were
evaluated, ‘remineralization periods’ and ‘the depths from the enamel surface’. To avoid an accumulation of errors due to multiple comparisons, the significance level was modified by Bonferroni correction. All statistical procedures were performed at a 95% level of confidence with the Statistical Package for Medical Science (SPSS ver.11 for Windows, SPSS Inc., Chicago, IL, USA).

RESULTS

**Micro CT image and the raw CT intensity profiles**

The representative micro-CT cross-section images: before and after demineralization, after 1 to 4 weeks of remineralization were presented in Fig. 3. These cross-section images were cut from exactly the same site of a same specimen. In each image, enamel surface showed slightly bright due to beam hardening effect. However, typical subsurface lesion with a well-defined surface zone which showed high CT intensity was confirmed (Fig. 3b). The CT intensity in the lesions increased with the increase in the remineralization period (Figs. 3b–3f).

The representative CT intensity profiles from a specimen were shown in Fig. 4. The profiles showed beam hardening effect at the surface area of the specimen: higher CT intensities than that of at the inner enamel. And there were also inclinations at the beginning of the profiles (around the border between air and enamel). Although CT scanning was done in the same conditions in the same specimens, considerable deviations in CT intensities were shown in each profile.

**Normalized profile**

Representative normalized profiles were shown in Fig. 5. Due to X-ray scattering, the profiles were inclined at the beginning of the profiles (ĉ in the figure). A-unit values larger than 100 were shown near the enamel surface. This characteristic, which showed larger value than the actual value, differed from those obtained by TMR method. However, the profile after demineralization indicates a typical subsurface lesion with a peak at the surface zone. In each profile, the A-unit values at any depth in the lesion were increased with increasing remineralization period (p<0.05).

**Rate of remineralization**

Mean longitudinal changes (N=8) of the R₃ (%) were plotted as a function of remineralization period (Fig. 6). A logarithm equation \[ R₃ = α ln(t) + C \] was fitted, and a highly significant relationship was found between R₃ and remineralization period (R²=0.977, p<0.05).
**DISCUSSION**

To investigate dynamic mineral behavior in the caries lesion, Micro-CT is considered to be one of the suitable methods. However, at the same time, it is known that micro-CT technology bears inherent artifacts which require careful handling such as ‘beam hardening effect’, ‘random noise’ and ‘partial volume effect’. Beam hardening effect is the phenomena caused by polychromaticity of the X-ray. Owing to the artifact, the surface of the object tends to be seen brighter than it naturally should be. So, in this study, the surface of the enamel was seen brighter than the actual condition. Random noise is minimized by median filter. This operation contributed to make the CT images smoother.

**Longitudinal mineral changes at 4 different depths**

Mean longitudinal changes of the D<sub>A,s</sub> (A-unit value) at the 4 different depths were plotted (Fig. 7). Similar to R<sub>A</sub>, logarithm equations [D<sub>A</sub>s=αLn(t)+C] were fitted. Highly significant relationships were found between D<sub>A</sub>s at the 4 different depths and remineralization period (R<sup>2</sup>&gt;0.957, p&lt;0.05).

In each remineralization period, the greater increment in D<sub>A</sub>s was observed at SZ and LB followed by MZ and DZ (p&lt;0.05).

Table 1 shows statistical significances in terms of A-unit value between each of remineralization periods for the 4 different depths (upper table), and among the 4 different depths during 4-week remineralization (lower table).
Table 1  Statistical significances of mineral densities (A-unit values) among the 4 different depths, and remineralization periods. (*: p≤0.05, N: p>0.05)

<table>
<thead>
<tr>
<th>Periods</th>
<th>SZ</th>
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<th>MZ</th>
<th>DZ</th>
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<td>3</td>
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When the Cone-beam micro-CT is used, uncertainty of the surface position of the specimen is inevitable mainly because of partial volume effect and effects of penumbra. When the materials which have the different absorption coefficient exist in a certain minimum voxel, the intensity of the voxel represents in its average. This phenomenon is called partial volume effect. The plate-like X-ray source causes penumbra. Penumbra is also caused by such as unevenness of the sensitivity of the sensor, misalignment of the central axis, inclination of the specimens and problems during reconstruction. These artifacts were troublesome to evaluate actual mineral density in the lesion. To convert CT intensity into mineral density, apatite phantoms are generally used \(^2,^9,^{12}\). However, it is not easy to obtain reliable and long-term usable phantoms covering wide range of mineral density because of the difficulty to prepare them. Some phantoms are available but expensive. So far many efforts have been explored to solve the problems \(^2,^9,^{12}\), but the technologies have not yet completely established to solve these problems.

To convert CT intensity into gray scale value, it is also essential to remove beam hardening effect. For this purpose, it is necessary to make a polychromatic source closer to monochromatic source using metal filters. However, at the same time, use of the metal filters also reduces the quantity of the X-ray and as a result, it reduces S/N ratio. This study aimed at observation of the mineral change, so the conversion of CT intensity into Bone mineral density (BMD) wasn’t employed. To obtain high sensitivity and S/N ratio, the usage of metal filters was kept to a minimum.

Considering such background factors, the authors tried to apply a new method. This approach was the method that used the depth profiles during the de- and remineralization obtained from CT intensities directly, i.e. without converting the CT intensities into mineral density or gray value. The method did not yield the absolute value of the mineral density, but it was considered reasonable for relative and longitudinal comparison of changes in mineral density in the same specimen. Even though we did not use any phantoms, appropriate standardizations procedures were needed. To achieve the quantitative and longitudinal comparison of the CT intensities corresponding to the mineral densities, normalization approaches to correct the depth profiles created from CT images were crucial. In addition, to investigating de- and remineralization longitudinally in the same area of the same lesions, accurate 3D alignment of the CT images was also essential \(^11\).

In the present study, from one specimen, six 3D images from different time points were accurately aligned and thus the longitudinal micro-CT measurement was achieved. Alignment of the images was successfully done using the software. The six cross-sectional images shown in Fig. 3 were from exactly the same site in the same specimen. It might be mentioned that those images were similar to conventional TMR images, or if not they were not very clear compared to those obtained by TMR method.

Even if the 3D images were aligned precisely, there were some difficulties in aligning and normalizing the depth profiles. Because when the profile was in the order of 3.5 μm, more minute attention was needed to align the profiles in the graphs than to align 3D images in the monitor. Furthermore, one of the perplex phenomena was considerable deviations of CT intensities even in a single specimen in this longitudinal observation (Fig. 4). This was thought to be due to the effect of X-ray power drift, X-ray detector sensitivity or slight error in the
positioning of the specimens when CT scanning was done. On the other hand, in some literature and ongoing experiments, this has been one of the most difficult issues to solve, i.e. to determine the edge of an object (enamel surface in this study). Thus, the alignment of the profiles toward depth direction was normalized by referring to the reflection point around the enamel surface in the profiles (Fig. 2). The authors accept that the present alignment approach could include uncertainty in the determination of the enamel surface due to subjective judgment, and thus a mathematical (objective) approach would be required. However, the uncertainty inherent in determining the points by eye was not considered to be a crucial bias in the present analysis, because the positions of the enamel surface in the profiles were fixed by the same procedure throughout all of the profiles. In addition, $R_A$ and $D_A$s were not calculated as absolute values but relative values. Therefore we thought the analyses of $R_A$ and $D_A$s were not much affected by the subjective judgment of enamel surface determination, it not being easy to ascertain the enamel surface.

Even after meticulous alignment of the profiles, they were not similar to the profiles of TMR method. Usually, the beam hardening effect is partly compensated for by using a variety of metal filters. Although 0.5 mm Al plate was used in the present study, considerable beam hardening effect still remained at the enamel surface. It resulted in inclination (X-ray attenuation) of the A-unit value from the enamel surface toward the inner sound region. Considerable reductions in CT intensity were observed from the surface toward deeper site in the sound region, although theoretically, mineral density should be the same both at the surface and deeper site in the enamel. Though it was not easy to eliminate beam hardening effect completely, as a result, we thought the normalization processes used to analyze mineral density in a ratio compared to sound enamel (control) were acceptable for our purpose, since the present analysis indicated reasonable order (remineralization rate was $SZ = LB > MZ > DZ$, $p<0.05$) about the longitudinal changes during 4 weeks of remineralization (Fig. 5) as supported by the following study by Exterkate et al.

Exterkate et al., found a relationship between changes in the integrated mineral loss (IML: vol% × μm) and in the mineral uptake (vol%) using ‘single thin section technique’ in 3 weeks remineralization protocol in vitro. They showed that the decrease in IML (increase in mineral density) in the lesion was the greatest during the first week of remineralization. And their lesion profiles revealed that mineral deposition was retarded in the deeper region of the lesion\(^7\). Similar trend was found in the longitudinal change of the mean $R_A$ in the present study: increase in $R_A$ was the greatest in the first week of remineralization and it decreased over time ($p<0.05$). Also similar trend was observed between $D_A$s in the present study and the longitudinal mineral uptake (vol%) at the different depths in the report of Exterkate et al.\(^7\). The ‘single thin section technique’ adopts TMR method for longitudinal monitoring of the same thin section. Thus these similarities were considered to be supportive to our approaches.

This study employed a logarithm equation to fit to the mean $R_A$ ($p<0.05$). However, the mean $R_A$ was observed as an almost linear progression over time, reaching up to approximately 86% after 4 weeks of remineralization ($p<0.05$). One reason for using logarithm equation was that the rate of remineralization at 4 weeks remineralization deviated from the linearity, and the other was that the rate of remineralization is supposed to level off at the late stage of the remineralization.

From a theoretical view point, the rate of crystal growth would be positively proportional to the active surface area of the crystal seeds. On the other hand, it would be negatively proportional to the pore volume that governs the rate of diffusion of the mineral ions and results in crystal growth\(^10\). These characteristics were considered to satisfy part of the requirement for logarthim fitting.

The logarithm equation was also employed for $D_A$s. The coefficient of the logarithm equation was thought to be associated with magnitude of the kinetics for both $R_A$ and $D_A$s. Theoretically, differential calculus of the equations of $R_A$: $dR_A/dt \cdot (1/t)$ and $D_A$: $dy_i /dt \cdot (1/t)$, i: 1-4 means kinetics of $R_A$ and $D_A$s for any time during 4-week remineralization. From these differentiated equations, it was obvious that the kinetics declined over time during the remineralization period ($p<0.05$).

Also, by comparing the coefficients (a) and the constant values (C) of the four equations (y1, y2, y3 and y4), following findings were indicated (Fig. 7). a) Trends of equation at $SZ$ and $LB$ were similar. It was probably due to the small distance between $SZ$ and $LB$. b) The coefficients of y1 and y2 were similar to those of y3 and y4. These implied that the growth rates of the mineral density at $SZ$ and $LB$ were greater than those at $MZ$ followed by $DZ$. This suggested that the large part of remineralization (crystal growth) in the lesion took place at $SZ$ and $LB$ in the present remineralization conditions.

In other words, mineral density ($D_A$) at $SZ$ and $LB$ would be major contributors to $R_A$. c) The constant values of y3 and y4 were greater than those of y1 and y2, while the coefficients of y3 and y4 were smaller than those of y1 and y2. It was thought that the coefficient and constant values were in an inverse relationship, meaning that the greater the constant value, the slower the kinetics of crystal growth as seen at $MZ$ and $DZ$. In other words, the lower the mineral density before the remineralization, the greater the rate of the mineral density increment.

These findings could be associated with other studies. The intra oral (in situ) study by Strang et al., demonstrated that the greater the mineral density at $SZ$ and $LB$ before the remineralization, the smaller the increment of mineral density\(^10\).

Although we have proposed the normalization approaches to creating the depth profiles corresponding to mineral density in this study, biases such as beam hardening effect or determination of the surface remain unsolved for the more accurate quantification of the mineral density. Future studies are needed to overcome the inherent biases associated with quantitative use of micro-CT.
CONCLUSIONS

This study indicated a possibility of normalizing approaches to create depth profiles corresponding to the mineral density from the CT images for the longitudinal study on enamel de- and remineralization. The results obtained from the depth profiles showed that 1) the increment of the mineral density at 4 different depths was greater in the order of surface zone > lesion body > middle zone > deep zone. 2) The greater the value of the mineral density before the remineralization, the smaller the increments of mineral density at 4 different depths.

ACKNOWLEDGMENTS

This study was supported by the Global COE program. The authors are indebted to Prof. Toshio Teranaka and Dr. Yoshiharu Mukai for their technical support.

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