Demineralization and Remineralization Phenomena of Human Enamel
in Acid Erosion Model

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Abstract: In this study, in vitro experiments were conducted using an acid erosion model to investigate and compare changes in enamel demineralization over time in different age groups. A total of 34 human extracted teeth with no caries were used, 17 of which came from subjects in their 20s at the time of extraction and 17 from subjects in their 60s. To measure the depth and the volume of enamel demineralization, the teeth were immersed in demineralization solution (0.1 N HCl) for durations of 30 seconds, two minutes, one hour and five hours. In terms of the results, one important finding was that on the outermost layer of the enamel exposed to the acidic solution, an acid resistance layer was observed after only 30 seconds of exposure and after up to five hours of exposure. Detailed investigation of this hypermineralized layer revealed that the molar ratio for Ca/P was 1.16 ± 0.02, the width of the layer was 0.9 ± 0.2 μm, and dense depositions of large and small quadrilateral crystals were observed. Another important result is that the volume of enamel lost per second of exposure to the demineralization solution initially declined exponentially over time, with the largest rate of loss observed at 30 seconds of demineralization, after which the demineralization time increased and a tendency was seen for a state of equilibrium to be reached. Based on the results of this study using an acid erosion model, we conclude that while the demineralization solution penetrated into the interior of the enamel even after only a few seconds of exposure, minerals eluted from the crystals were confirmed to have been remineralized. In terms of age, enamel demineralization was not considerably influenced by age since no statistically significant differences in demineralization depth or volume were observed between the 20s and 60s age groups.

Key words: Human enamel, Acid erosion, Demineralization, High-mineralization layer, Enamel loss

Introduction

Caries and acid erosion are two of the primary conditions that cause human enamel to dissolve. Keyes1 identified three factors in caries lesions: host and teeth, microorganisms, and substrate. Later, Newbrun2 added a fourth factor, that of time. Currently, cariogenesis is thought to occur when these four factors are concurrent. Two dynamic mechanisms that cause onset and progression of enamel caries have been identified in the interface between enamel surface and saliva: demineralization and remineralization3. Acid erosion4, however, rather than involving microorganisms, is understood to be a progressive condition in which acids introduced from outside the human body, such as foods and beverages, and acids present in the stomach, such as gastric acids, act directly on tooth enamel to cause erosion. With respect to acid erosion, numerous studies have examined various aspects, from in vivo studies to in situ assessment of the erosion of human enamel caused by acidic foods and beverages. Imfeld5 has reported an increase in the incidence of acid erosion in step with increases in consumption of acidic beverages such as soft drinks and fruit juices. Owens6 et al. evaluated the pH levels of soft drinks and reported that the pH of carbohydrate drinks was between 2.4 and 3.1, and that of sports drinks was between 3.1 and 3.4. Enamel was then exposed to various beverages and the tooth surfaces were examined morphologically, using a scanning electron microscope. Bartlett7 et al. examined the teeth of 3,187 patients between the ages of 18 and 35 years and found signs of acid erosion in 29 %, thus showing a relationship between acid erosion and the amount of fruits and juices consumed. Many of the studies performed to date, however, did not quantitatively evaluate acid and the volume of enamel lost, or the time involved, and none of them evaluated the phenomenon of tooth enamel demineralization at the crystal level. In our study, we used human enamel of a known age and conducted in vitro studies using the acid erosion model in a detailed investigation that also compared different age groups to track changes in enamel demineralization over time. The aims of the study were: 1) to examine the relationship between enamel demineralization time and the volume...
of demineralization; 2) to observe changes in the microstructure of the area in which enamel demineralization occurred; and 3) to investigate the interaction between sound enamel and acid. As a result, we hoped to clarify the process of enamel demineralization and remineralization caused by acid erosion.

Materials and Methods

Teeth used for experiment

From human extracted teeth with no caries owned by the Department of Oral Anatomy of the Tsurumi University School of Dental Medicine, we used a total of 34 teeth; 17 from subjects in their 20s at the time of extraction and 17 from subjects in their 60s. The teeth, which had been preserved in a 10 % formalin solution, were taken out of the formalin solution prior to the experiment and were washed for 24 hours under running water. Use of the teeth in this study was approved by the Ethics Committee of the Tsurumi University School of Dental Medicine (approval No.: 1306).

Demineralization solution

The demineralization solution used was 0.1 N HCl (pH 1.8). Demineralization times were 30 seconds, two minutes, one hour and five hours. As shown in Fig.1, a peristaltic pump was used to maintain the volume of supplied demineralization solution at 800 ml in a 1000-ml beaker. The demineralization solution in the beaker at room temperature was stirred using a magnetic stirrer, and the enamel specimens were suspended in such a way that they were constantly in contact with fresh demineralization solution.

Measurement of demineralization depth of enamel

Specimen preparation

A total of 18 teeth were used, nine from subjects in their 20s and nine from subjects in their 60s. Prior to the experiment, enamel surfaces of the crowns were cleaned using a rubber cup and pouring water over the surfaces. After teeth were dried, whole surfaces of the tooth roots were coated using various colors of nail varnish, and demineralization time of the enamel surface coated with nail varnish was controlled, as shown in Fig. 2. Demineralization times were set at 0 seconds, 30 seconds, two minutes, one hour and five hours. First, the part labeled “A” in Fig. 2 was coated with nail varnish, and was immersed in demineralization solution for 30 seconds. At that point, the part labeled “A” was designated as the surface having zero demineralization (control). Next, the tooth was taken out of the demineralization solution, and was washed with distilled water and dried. The part labeled “B” in Fig. 2 was coated with nail varnish and then immersed in demineralization solution for two minutes. At that point, the part labeled “B” was designated as the 30-second demineralization surface. This process of immersion in demineralization solution and coating with nail varnish was repeated for the parts labeled “C” and “D” in Fig. 2, and the surfaces were designated as the two-minute and one-hour demineralization surfaces, respectively. The part labeled “E” was not coated with nail varnish, and was designated as the five-hour demineralization surface. When nail varnish was applied to the demineralized tooth surfaces, different colors were used to indicate the various demineralization times. After demineralization was complete, acetone was used to remove the nail varnish, taking care not to scratch the enamel surfaces. Next, teeth were dehydrated using an ascending ethanol series, and were embedded using epoxy resin. Subsequently, a slow-speed hard tissue cutter was used to cut the resin-embedded tooth so that the center of the tooth crown had a thickness of 3 mm in the cross direction in relation to the tooth axis. This completed the preparation of the enamel specimens.

Measurement and calculation of demineralization depth

Enamel specimens were observed at an accelerating voltage of 15 kV under a scanning electron microscope (SEM) (JSM-5600LV; JEOL, Tokyo). Using the resulting SEM images, the distance from the enamel surface layer of the control to the demineralization fundus (deepest part) was measured using a digital caliper, and this value was used as the demineralization depth (μm). Demineralization depth was measured for each demineralization time for the nine teeth from subjects in their 20s and the nine teeth from subjects in their 60s, and the mean values and standard deviations were determined.
Specimen preparation

Measurement of cubic volume of enamel loss

Element (Ca, P) analysis of the enamel surface layer

Backscattered electron image observation of the enamel surface layer

Chemical analysis of fluoroapatite single crystals

Measurement of cubic volume of enamel loss
and for five-hour demineralization, 448.7 ± 172.6 µm. Statistically significant differences were found between all demineralization times. This indicates that demineralization depth increased with demineralization time. There were no significant differences in
demineralization depth between the two age groups, but at one hour and five hours of demineralization, there was a tendency for demineralization depth to be larger in the teeth taken from subjects in their 20s than those in their 60s. Moreover, calculations of the percentage of standard deviation in relation to the mean value (CV: coefficient of variation) showed no statistically significant differences between the teeth from subjects in their 20s and those in their 60s.

**Backscattered electron images of enamel surface layers**

Fig. 4 shows the perpendicular cross-sections of enamel surfaces observed as BEI. For subjects in their 20s and those in their 60s, at 30 seconds of demineralization, the outermost enamel layer showed a high backscattered electron density, indicating high mineralization. However, no layers with a low backscattered electron density indicating demineralization were seen directly

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**Table 1. Means of Demineralization Depth of Enamel**

<table>
<thead>
<tr>
<th>age group*</th>
<th>demineralized time</th>
<th>µm (S.D)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30sec</td>
<td>2min</td>
</tr>
<tr>
<td>20's</td>
<td>4.2 (2.3)</td>
<td>15.8 (4.8)</td>
</tr>
<tr>
<td>60's</td>
<td>3.5 (1.3)</td>
<td>14.1 (4.7)</td>
</tr>
</tbody>
</table>

* n=9 each age group, tooth n=18, one same sample was used demineralized time
below that layer. After five hours of demineralization, however, there was a layer in the outermost enamel layer with a high backscattered electron density indicating high mineralization, while directly under that, a layer with a low backscattered electron density indicating demineralization was observed. Moreover, the lower layer, which was the field thought to be sound enamel, had a middle backscattered electron density that was between the outermost enamel layer and the layer directly below it, so we clearly identified three layers. In specimens that had undergone demineralization for 30 seconds and five hours, the width of the high-mineralization layer of the outermost enamel layer was measured at 11 points. The results indicated that in the teeth from subjects in their 20s, the width was 0.9 ± 0.2 μm at 30 seconds of demineralization and was 0.8 ± 0.2 μm at five hours, while in the teeth from subjects in their 60s, the width was 0.8 ± 0.2 μm at 30 seconds of demineralization and was 0.9 ± 0.2 μm at five hours. In addition, in specimens that underwent five hours of demineralization, when the width of the layer with low backscattered electron density indicating demineralization was measured at 11 points, it was approximately 4.8 ± 0.7 μm for teeth from subjects in their 20s, and approximately 7.7 ± 1.7 μm for teeth from subjects in their 60s.

**Element analysis (Ca, P)**

When the Ca/P ratio (CPS/CPS) was determined for specimens from subjects in their 20s that had been subjected to demineralization solution for five hours, the results were as follows: for the outermost enamel layer: 1.16 ± 0.02; for the layer directly under the outermost enamel layer: 1.37 ± 0.06; and for the field thought to be sound enamel: 1.60 ± 0.02 (Fig. 7). The Ca/P ratio was lowest in the outermost enamel layer. Moreover, the Ca/P ratio obtained as a result of chemical analysis of the natural fluoroapatite single crystals was 1.63, and a tendency was seen for specimens in the field thought to be sound enamel to approach the values obtained from chemical analysis of the natural fluoroapatite single crystals.

**Measurement of the cubic volume of enamel loss**

When the volume of enamel lost each second (×10⁻³mm³/s) was calculated from the cubic volume of enamel lost (mm³), the results were as follows. For teeth from subjects in their 20s, the
results were 69.8 ± 28.7 after 30 seconds of demineralization, 34.3 ± 11.6 after two minutes, 3.6 ± 0.7 after one hour and 2.8 ± 0.7 after five hours. For teeth from subjects in their 60s, the results were 116.8 ± 73.0 after 30 seconds of demineralization, 46.2 ± 18.5 after two minutes, 4.1 ± 1.5 after one hour and 2.5 ± 0.6 after five hours (Table 2). When the progression of enamel loss was observed three-dimensionally on micro-CT images, a tendency was observed for the acid to act on the surface of the tooth, resulting in dissolving of the enamel over the entire crown (Fig. 5). For teeth from both subjects in their 20s and 60s, the volume of enamel lost each second was largest after 30 seconds of demineralization, and as the demineralization time increased, a tendency was seen for a state of equilibrium to be reached (Fig. 6). Significant differences were seen in the volume of enamel lost for each of the demineralization times. Looking at 30-second demineralization, the volume of enamel lost was larger for teeth from subjects in their 60s than those in their 20s, but no statistically significant differences were observed. Furthermore, no statistically significant differences were seen in the volume lost between the two age groups. Therefore, based on the dissolution rate with acid shown in Figure 6, the cubic volume of enamel lost each second could be expressed by the approximation logarithmic function: \[ V = - \log[H^+] \cdot \logT, \] in which \( \text{pH} = - \log[H^+] \) then, \( V = \text{[pH]} \cdot \logT \) when, \( V \): volume of enamel loss (mm³/s); \( T \): duration of acid exposure; and \( [H^+] \): concentration of Hydrogen ions (mol/l).

Transmission electron microscope observation

Fig. 7 shows transmission electron microscope images of the enamel demineralization surfaces of teeth from subjects in their 20s on which demineralization solution had acted for five hours, as well as the corresponding BEI. Looking at the crystal morphology, dense deposition of large and small quadrilateral crystals and crystals in other shapes was observed in the outermost enamel layer. There were no hexagonal shapes indicating hydroxyapatite crystals in any of the images (Fig. 7-B-a, H-a). Inter-crystal spaces were seen directly under the outermost enamel layer (Fig. 7-B-b), and some images were seen in which hexagonal crystals were adhered to each other in the field thought to be sound enamel (Fig. 7-B-c). Also, high-magnification images of the layer directly under the outermost enamel layer showed crystals accompanied by central perforations (Fig. 7-H-b). Measurements of the width of the crystals deposited on the outermost enamel layer (Fig. 7-A-a, B-a) taken at 11 locations showed the width to be 0.8 ± 0.2μm.

Discussion

The purpose of this experiment was to elucidate the acid-dissolution phenomenon of human enamel under the conditions that do not elicit a remineralization. Cariogenic bacteria produce various acids such as lactic and formic acid those have mild acidity. For acid-erosion test when mild acids are used as decalcification solution, remineralization tends to occur in the enamel. Based on the same concept Maron⁸ used HCl in his research work. As for pH of food and drink, citrus and sports beverages, for example, have pH around 3, and acidity of gastric acid is around pH1. From these pH data, we originally set the pH of decalcification solution as 1.5 in between pH3 and 1, however considering the time and amount of enamel loss during the acid challenge, acid concentration was set to 0.1 N and then the final
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pH resulted in 1.8. The most interesting finding from this study was that although the enamel was exposed to the strong acid (HCl, pH 1.8) for very short time (30 seconds), we found the hypermineralization layer, that is, the remineralization phenomenon, occurred on the outermost surface of the enamel. These unexpected phenomenon was confirmed by SEM and TEM observation. Relationship between enamel demineralization time and volume of demineralization; With respect to the relationship between time and amount of demineralization, both the demineralization depth and volumetric loss tended to increase over time. There were no significant differences in demineralization depth between the two age groups, but at one hour and five hours of demineralization, there was a tendency for the demineralization depth to be larger in the teeth taken from subjects in their 20s than those in their 60s. For teeth from both subjects in their 20s and 60s, the volume of enamel lost each second was largest after 30 seconds of demineralization, and as the demineralization time increased, a tendency was seen for a state of equilibrium to be reached. These findings indicated that while the demineralization solution penetrated into the interior of the enamel, and minerals eluted from the crystals were confirmed to have been remineralized in the outermost layer of enamel. The remineralized crystals do not dissolve easily in acid, and function as acid-resistant crystals in the surface layer of the enamel. Because of this, the amount of demineralization is thought to reach a state of equilibrium after a considerable amount of time has passed. For the outermost enamel that reached a state of equilibrium, acid calcium phosphate crystals were morphologically confirmed by TEM, and Ca/P ratio was evaluated by EPMA. Changes in microstructure in areas where enamel demineralization occurred; To date, there have been no reports describing detailed studies, such as BEI, on the degree of mineralization after human teeth are exposed to acid for periods of less than 30 seconds. The outcomes of numerous in vitro experiments pertaining to chronic caries have given rise to the theory that penetration of demineralization solution into the enamel causes reflux of H+ inside the enamel, and as Ca2+, (PO3)3-, OH- and the other ions perfuse the enamel, the demineralization of apatite crystals progresses. In the present study, a layer with high electron density could be confirmed on the outermost enamel layer even after only 30 seconds of demineralization (Fig. 4), and this was confirmed for both BEI (Fig. 4) and TEM (Fig. 7) observation. Therefore, there appears to be no correlation between the action time of the acid and the width in the depth direction. This could be explained by pH in the logarithmic function as described in the result. We assume that width of the high-mineralization layer is influenced by the type of acid. The Ca/P ratio of the layer formed on the outermost surface enamel was calculated to be 1.16. Generally, hydroxyapatite is the only stable calcium phosphate crystal in the neutral environment, and its theoretical Ca/P ratio is 1.67 as calculated with molar ratio of apatite. Based on earlier studies, the Ca/P ratio of human enamel is largely taken as 1.60. The reason that the Ca/P ratio of human hydroxyapatite crystals is not the 1.67 is thought to be that there are various lattice defects in human enamel apatite crystals, as well as impurities such as CO32- and HPO42- while stable calcium phosphate crystals in the acid environment are crystals such as di-calcium phosphate dehydrate (DCPD, Ca/P ratio: 1.0), octa calcium phosphate (OCP, Ca/P ratio: 1.3) and tri-calcium phosphate (TCP, Ca/P ratio: 1.5). Based on the results of the present study, a relationship between the Ca/P ratio and the deposited crystal morphology is speculated as follows. The Ca/P ratio of the outermost layer of the enamel is 1.16, which is similar to that of DCPD, 1.0. We believe that the reason is the presence of crystals other than DCPD, TCP, OCP and/or HAP between the crystals of the outermost enamel layer. In this outermost enamel layer, cross-sections of quadrilateral and polygonal crystals were observed by transmission electron microscopy (Fig. 7-B-a, H-a). For the Ca/P ratio of the layer directly under the outermost layer of the enamel, which is 1.37, the value is similar to the theoretical molar ratio of OCP, 1.30. This could be because apatite crystals with central perforations were observed, and other low molecular weight acid calcium phosphate crystals may be present in this field (Fig. 7-B-b, and H-b), while in layers not yet reached by ongoing demineralization, crystals that exhibited hexagonal shapes were confirmed (Fig. 7-B-c). With respect to the fact that 1.60 was indicated in deep areas of the enamel (Fig. 7 A-c), this was thought to be because, as noted above, hydroxyapatite contains various impurities.

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Conflict of Interest
The authors have declared that no COI exists.

References