Acidic Calcium Phosphate Crystals Observed on the Outermost Layer of Human Enamel

Yuka Asada\textsuperscript{1,2}, Toshie Chiba\textsuperscript{2}, Shinji Shimoda\textsuperscript{2}, Yasuko Momoi\textsuperscript{3} and Takatsugu Yamamoto\textsuperscript{3,4}

\textsuperscript{1} Department of Operative Dentistry, Tsurumi University School of Dental Medicine, Kanagawa, Japan
\textsuperscript{2} Department of Oral Anatomy, Tsurumi University School of Dental Medicine, Kanagawa, Japan

Abstract: Many studies have been conducted to evaluate the timing of brushing and methods to suppress or halt the progression of acid erosion and initial caries formation, and to restore tooth enamel. However, the analyses in these studies were done from a macroscopic point of view, such as evaluating the change in the hardness of the enamel surface and the change in X-ray permeability, the morphological change due to crystal deposits on the enamel surface has not been elucidated. The purpose of this study was to investigate how crystals on the enamel surface differ when they are in contact with oral cavity liquids such as saliva versus no contact with these liquids. In addition, we assessed the condition of initial caries in which no substantial defect is observable, from the morphological perspective by using elemental analysis and observations with a transmission electron microscope (TEM). We also considered the causes of crystal formation on the enamel surface. As a result, the outermost layer in the pre-eruptive enamel contained a large amount of Mg, which is a stabilizer of amorphous calcium phosphate, suggesting that the layer contains acidic calcium phosphates. It was suggested that tricalcium phosphate, dicalcium phosphate dihydrate, octacalcium phosphate, etc. form a layer on the surface of post-eruptive enamel with no caries and in post-eruptive enamel with caries exposed to saliva in the oral cavity and the layer containing calcium phosphate have higher acid resistance than HAp. The information from this study can be applied to basic clinical research on remineralization therapy and non-invasive treatments of initial caries.

Key words: Acidic calcium phosphates, Amorphous calcium phosphate, Enamel surface, Hydroxyapatite, Magnesium

Introduction

In 2013, the experts’ opinion\textsuperscript{1} that “Tooth brushing after 30 minutes or more after a meal is preferable to prevent tooth damage—dental erosion—due to tooth brushing” was reported by the media as “Tooth brushing should be avoided for 30 minutes after meal”. This report caused repercussions. Currently, the Specialist Committee of The Japanese Society of Conservative Dentistry, The Japanese Society of Pediatric Dentistry, and The Japanese Society for Oral Health of The Japanese Association for Dental Science publish their views on their web sites\textsuperscript{2-4} as to when to brush teeth after a meal. However, these are not based on Randomized Controlled Trial clinical studies with strong evidence. The scientific evidence for these opinions is derived mainly from in-vitro or in-situ experimental results. Apart from these statements, the International Dental Federation has published the opinion that Minimal Intervention should be the treatment policy for caries (since, in the case of mild caries, remineralization therapy should halt or suppress the progress of caries and a long-term recovery should be the goal)\textsuperscript{5}. Many studies\textsuperscript{6-10} have been conducted to evaluate the timing of brushing and methods to suppress or halt the progression of acid erosion and initial caries formation, and to restore tooth enamel. However, the analyses in these studies were done from a macroscopic point of view, such as evaluating the change in the hardness of the enamel surface and the change in X-ray permeability. In a study on the basic structure of the enamel surface, Yamashita et al.\textsuperscript{9,10} used transmission electron microscopy to confirm the existence of a second layer containing granular crystals on the surface of sound hydroxyapatite; however, the composition of the crystal was not mentioned. In other words, the chemical compositions of calcium phosphate and the crystal properties on the enamel surface have not been elucidated.

Therefore, we thought that it would be possible to judge the effects of timing of brushing and Minimal Intervention treatment from an unprecedented view point if changes in the crystalline deposits and the chemical compositions on the enamel surface were used as an evaluation index of remineralization. We considered that this could be an important method to help prevent enamel caries and dental disease. As a first step toward this goal, the purpose of this study was to investigate how crystals on the enamel surface differ when they are in contact with oral cavity liquids such as saliva versus no contact with these liquids. In addition, we assessed the condition of initial caries in which no substantial defect is observable, from the morphological perspective by using elemental analysis and observations with a transmission electron microscope (TEM). We also considered the causes of crystal formation on the enamel surface.

Materials and Methods

Sample preparation

The study specimens comprised five human permanent teeth with caries categorized according to the International Caries Detection and Assessment System (ICDAS) code 2 (Code 2: Distinct visual change in enamel visible when wet, lesion must be visible when dry)\textsuperscript{11,12}, obtained...
from the Department of Operative Dentistry, Tsurumi University School of Dental Medicine, and five partially impacted teeth extracted in the Oral Surgery outpatient clinic, Tsurumi University Dental Hospital. The permanent teeth with dental caries were immersed in 10% neutral buffered formalin solution, semi-impregnated in half-strength Karnovsky solution, and then washed with phosphate buffered solution. The present study was conducted following approval from the Ethical Committee of Tsurumi University, School of Dental Medicine (Approval No: 1041, 1401).

The specimens were classified as follows: post-eruptive enamel with caries (Po-C) classified as ICDAS code 2, post-eruptive enamel with no caries (Po-N), and pre-eruptive enamel (Pr-E) defined as enamel found underneath the junctional epithelium (Fig. 1). Each sample was sectioned parallel to the long axis of the tooth at intervals of 500 to 700 µm to create a longitudinal section. Abbreviations used in this paper are listed in the abbreviation correspondence table (Table 1).

**Light microscopy**

Binocular microscopy was used to observe the enamel surface structure. The samples were polished to a thickness of 100 µm and observed under a polarizing microscope to examine optical and birefringence features.

**Backscattered electron image**

A backscattered electron image (BEI) was taken using an electron beam probe microanalyzer (EPMA; JXA-8900R, JEOL, Tokyo, Japan) under the following conditions: acceleration voltage, 15 kV; irradiation current, $1 \times 10^{-8}$ A; and image size, $1024 \times 1024$ pixels. The brightness of the BEI was reconverted from a 1024-grayscale to a 255-grayscale image.

**Ca and P measurements**

To confirm the Ca and P contents, elemental analysis of Ca and P with an acceleration voltage of 20 kV and an irradiation current of $5 \times 10^{-9}$ A, was carried out using the Wave Dispersive System (WDS). The quantitative values of Ca and P concentration were measured as count per second (CPS) at a depth of 0–1 µm, 10 µm, and 20 µm, respectively, from the tooth surface. Furthermore, the Ca/P ratio, Mg/P ratio, Mg/Ca ratio, and (Ca + Mg)/P ratio were calculated from the obtained CPS value. To correct the value of the Ca/P ratio obtained from the EPMA analysis, a natural apatite from Brazil with a known Ca and P concentration was used (1). The correction constant (K) was calculated—assuming the theoretical value of Ca/P ratio = 1.67—by measuring the characteristic x-ray CPS values of the natural apatite Ca and P under the same conditions as the analysis of each sample (2).

$$K = \text{theoretical value of Ca/P ratio (1.67)/natural apatite Ca/P ratio (1)}$$

$$\text{Ca/P ratio} = (K) (\text{Ca/P ratio of each sample}) (2)$$

**Mg measurements**

The analyses were based on the conditions of Ca and P. For measurement and correction of Mg concentration by EPMA, 99.9% pure Mg was used and calculated using the aforementioned correction equation.
Transmission electron microscopy (TEM)

Ultrathin sections of each sample were made with a diamond knife, and the crystal cross-sectional morphology of enamel was observed under TEM (JEM 1400, JEOL) at an acceleration voltage of 80 kV without dyeing. For some samples, ultrathin sections were prepared using Focused Ion Beam (FIB). In addition, electron beam diffraction was performed to simultaneously observe the crystals.

Statistical analysis

Data regarding the Ca/P ratio, Mg/P ratio, Mg/Ca ratio, and (Ca + Mg)/P ratio were statistically compared using a non-parametric multiple comparison test (Steel-Dwass method) with a significance level of 0.01.

Results

Light microscopy

In the Po-N group (Fig. 2A), a layer with a clear and high luminance, as compared to the deeper layer, was observed on the outermost enamel. In the Po-C group (Fig. 2C), a layer with high luminance was not clearly observed as it had been in the Po-N and Po-C groups. In the Po-C group (Fig. 2C), the layer which exhibited clear and high luminance was observed on the outermost enamel. In addition, there was a highly transparent layer exhibiting high calcification on the surface of the decalcified layer. The depths were measured with an electron microscope. Layers with different bright features were observed on the enamel surface in all groups using the polarizing microscope (Fig. 2).

Backscattered electron image

The grayscale concentrations of the three groups in the backscattered electron images were compared. A bright area with high backscattered electron density was observed on the enamel surface of the Po-N, Pr-E, and Po-C specimens, which measured approximately 3 µm (Fig. 3A), and 4 µm (Fig. 3B), 6 µm in width (Fig. 3C), respectively, when compared to the deeper layer.

Element analysis

Element analysis of Ca, P, and Mg in each sample showed that the
Figure 4. Element analysis of Ca, P, and Mg. A: Backscattered electron image of a Po-N specimen and elemental analysis of Ca, P, and Mg. B: Backscattered electron image of a Pr-E specimen and elemental analysis of Ca, P, and Mg. The amount of Mg in the Pr-E group was higher than other groups (indicated by arrows). C: Backscattered electron image (low magnification) of a Po-C specimen, large region of low electron density was observed (indicated by arrows). D: Backscattered electron image of a Po-C specimen and elemental analysis of Ca, P, and Mg.

Figure 5. Ca/P molar ratio in each sample (measured to a depth of 20 µm from the surface). No significant difference was observed with regard to each sample type or depth (p > 0.01). However, the value of the outermost surface of the enamel (0–1 µm) tended to be lower than those measured at 10 µm and 20 µm in the Po-N and Pr-E groups. Data are provided as mean ± s.d. (n = 5).

Figure 6. Mg/P molar ratio in each sample (measured to a depth of 20 µm from the surface). Notice the high Mg value of Pr-E on the enamel surface (0–1 µm). A significant difference was observed between depths of 0–1 µm and 10 µm in the Pr-E group. Data are provided as mean ± s.d. (n = 5). **p < 0.01.
amount of Mg in the Pr-E group (Fig. 4B) was higher than that in the Po-N (Fig. 4A) and Po-C (Fig. 4D) groups. Based on the surface analysis, we measured and compared the values of the Ca/P ratio, Mg/P ratio, Mg/Ca ratio, and (Ca + Mg)/P ratio.

**Ca/P value**

The Ca/P ratio of enamel was determined from the correction value using natural apatite as the standard sample. In the Po-N group, the Ca/P ratio was 1.42 ± 0.22 at a depth of 0–1 µm, 1.52 ± 0.28 at 10 µm, and
1.51 ± 0.27 at 20 µm. In the Pr-E group, the Ca/P ratio was 1.36 ± 0.20 at a depth of 0–1 µm, 1.46 ± 0.24 at 10 µm, and 1.45 ± 0.20 at 20 µm. In the Po-C group, the Ca/P ratio was 1.43 ± 0.45 at 0–1 µm, 1.37 ± 0.32 at 10 µm, and 1.37 ± 0.44 at 20 µm. A statistically significant difference was not found with regard to the Ca/P ratio; however, there was a tendency for the value of the outermost surface of enamel (0–1 µm) to be lower than that measured at 10 µm and 20 µm in the Po-N and Pr-E groups. The Ca/P ratio was particularly low, with a value of 1.35, where high levels of Mg were detected in the Po-C specimens (Fig. 5).

The measured values of the Ca/P ratio were specific to dicalcium phosphate dihydrate (DCPD; CaHPO₄·2H₂O, Ca/P ratio = 1.0) and octacalcium phosphate (OCP; Ca₈H₂(PO₄)₆·5H₂O, Ca/P ratio = 1.3). They did not necessarily agree with the theoretical value of tricalcium phosphate (TCP;Ca₁₀(PO₄)₆(OH)₂, Ca/P ratio = 1.5).

Mg value

The values of Mg/P ratio, Mg/Ca ratio and (Ca + Mg)/P ratio were determined from the correction value, with 99.9% Mg metal used as a standard sample. In the Pr-E group, the Mg/P ratio from the dentin interface at 0–1 µm was higher when compared to those measured at 10 µm and 20 µm (Fig. 6). The Mg/Ca ratio showed the same results with the Mg/P ratio (Fig. 7). Significant differences were observed between the depths of 0–1 µm and 10 µm in the Pr-E group with regard to the Mg/P ratio and in Mg/Ca ratio (p < 0.01). In terms of the (Ca + Mg)/P ratio (Fig. 8), no significant differences were revealed among the values, which was inconsistent with the results found when analyzing the Mg/P ratio (Fig. 6) and Mg/Ca ratio (Fig. 7).

Crystal identification

There were no cross sections containing quadrangular or polygonal crystals typical for the acidic calcium phosphate (TCP, DCPD, or OCP), and HAp crystals with a central perforation were not observed on the outermost layer of the enamel of the Pr-E group (Fig. 9A, 9a). For this group, the crystal size that was generally smaller than that observed in the other two groups. In the Po-N group, a cross section of quadrangular or polygonal crystals was detected (Fig. 9B). When the outermost surface of the enamel in the Po-C group (Fig. 9C, 9c-1) was observed in TEM, a cross section of the quadrangular or polygonal crystal typical for the acidic calcium phosphate—TCP, DCPD, or OCP—was observed (Fig. 9c-1). Furthermore, hydroxyapatite (HAp;Ca₁₀(PO₄)₆(OH)_2) crystal with a central perforation was observed on the inner layer (Fig. 9c-2). Underneath that was a layer of high-density crystals that were hexagonal in shape. The electron beam diffraction image confirmed that the crystals were different from the HAp crystals found on the outermost layer (Fig. 9c-3). Additionally, the enamel rod structure was observed at levels extending to the surface layer in the Po-C group (Fig. 9D, 9d) but not in the Pr-E group.

Figure 10. Scale to a depth of 30 µm from the surface. The outermost layer—approximately 500 nm in depth—contains acidic calcium phosphate in Po-C and Po-N.

Figure 11. Explanatory and theoretical flow chart for the HAp crystal formation process. Mg and Ca ions aggregate in the solution to form ACP. ACP is then transformed into the most stable HAp crystal via acidic calcium phosphates, such as OCP or DCPD. However, Mg ions are also incorporated during crystallization process. As HAp crystallization progresses, Mg ions are replaced by Ca ions, which have a larger ion radius, and the Mg concentration in the HAp crystal decreases.

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**Discussion**

**Recognition of the layers containing acidic calcium phosphate at the interface between post-eruptive enamel and the saliva in the oral environment**

The results of this experiment suggest that the outermost layer—approximately 500 nm in depth—contains acidic calcium phosphate of Po-C and Po-N (Fig. 10).

The Ca/P ratio showed a low value for the surface of the Po-N specimens. If we assume that the proportion of HAp on the outer surface of the enamel is high, the Ca/P ratio should be close to 1.67. This suggests that crystals different from HAp, specifically OCP, TCP, and DCPD, exist on the surface of teeth classified as Po-N. Thus, the presence of a cross-sectional layer of quadrilateral or polygonal crystals characteristic of OCP, TCP, or DCPD on the outer surface observed by TEM is the morphological finding that supports this theory.

There was no significant difference between the Ca / P ratio, Mg / P ratio and Mg / Ca ratio in Po-C. It is thought that the degree of dental caries was different for each sample of Po-C. Although the cross section of quadrilateral or polygonal crystals typical for acidic calcium phosphate in TEM was observed on the enamel surface and was similar to that observed in Po-N specimens, it was confirmed by electron beam diffraction that the crystals on the outermost surface were different from HAp. Thus, this strongly suggests that crystals such as OCP, TCP, and DCPD exist on the surface of Po-C specimens.

It is suggested that the layer containing acidic calcium phosphate on the enamel surface observed in the Po-N and Po-C groups was formed by a mechanism similar to that responsible for initial caries formation. When the enamel demineralizes due to a decrease in pH, Ca, and PO₄ ions contained in the decalcified enamel, saliva, and dental plaque undergo crystallization again, which results in the deposition of minerals (remineralization) on the enamel surface and the formation of a highly calcified layer (remineralization layer)⁴,⁷,¹². The deposition of acidic calcium phosphate crystals, such as OCP, TCP, and DCPD, which are not considered to be present in mature enamel, was confirmed along with the presence of HAp crystals, which are typically found in highly calcified layers. Depending on the acidity of the oral cavity, the highly calcified layer becomes thicker or the acidic calcium phosphate type crystals change to HAp. In demineralized layers containing H⁺ ions, circulation occurs due to the concentration gradient of anions and cations in a closed system; thus, when a decline in H⁺ concentration does not occur, demineralization progresses⁴,¹⁴. In Hayashi’s⁵ in-vitro acid-erosion model experiment on human enamel, most of the minerals were eluted from the crystal due to the demineralization of enamel. At the same time, the study confirmed that a portion of the minerals was re-deposited as a thin layer on the outermost surface of the tooth. It was also mentioned that the redeposited crystals were sparingly soluble in acid and that the presence of acid calcium phosphates, other than hydroxyapatite, was possible. The crystals on the surface of the tooth were found to be acid-resistant. Based on the above findings, the layer contained acidic calcium phosphates, such as TCP, DCPD, and OCP, which were strongly suggested to form on the outermost layer of Po-N and Po-C specimens.

**Formation of Mg present in the interface between pre-eruptive enamel and the saliva in the oral environment**

A cross section of quadrilateral or polygonal crystals, typical for acidic calcium phosphate, was not observed in TEM even though the Ca/P ratio showed a lower value at the surface than those measured at 10 μm and 20 μm of the Pr-E specimens, as the crystal size of the Pr-E specimens was very small compared to those of the Po-N and Po-C groups. The outermost layer of the Pr-E specimens contained a large amount of Mg, which is a precursor of HAp⁶-¹⁸ and a stabilizer of amorphous calcium phosphate (ACP)¹⁹, suggesting that the layer contains acidic calcium phosphates.

A large amount of Mg was detected in the outermost enamel surface of Pr-E—immature enamel and in newly calcified areas—due to the following HAp crystal formation process. First, Mg ions and Ca ions aggregate in the solution to form ACP. ACP (Ca/P = 1.5 to 1.73) is then transformed into the most stable HAp crystal (Ca/P = 1.67) via acidic calcium phosphates, such as OCP or DCPD²⁰,²¹. However, it is not only Ca ions that are incorporated into the crystal; Mg ions are also incorporated during the crystallization process. As crystallization of HAp progresses, Mg ions are replaced by Ca ions, which have a larger ion radius²², and the concentration of Mg in the HAp decreases (Fig. 11). It is suggested that some of the processes mentioned above, such as those involved in HAp crystal formation, also occur during remineralization—the process of forming a layer containing acidic calcium phosphate on the outermost enamel surface of Po-N and Po-C specimens (Fig. 11). The low values of Mg found on the surface of Po-N specimens might be due to the small amount of Mg ions found in the saliva and/or the amount of Mg ions replaced by Ca ions over time during the ACP-to-HAp transition.

It has been reported that Mg ions are present in the newly formed calcified area²³,²⁴; therefore, remodeling always occurs in bones and calcification occurs in dentin as a result of odontoblast activity—especially in the tubular dentin where Mg is present in large amounts. It is known that Mg becomes a cofactor for about 300 enzyme catalysts involved in HAp transition.

Although the present study did not directly demonstrate the presence of ACP in the outermost layer of enamel, its presence is suggested based on the findings that Mg ions are involved in the stability of ACP and that the Mg value was highest at the outermost layer of the Pr-E specimens. This finding is supported by the in-vitro study conducted by Iijima et al.²⁵, which showed the transformation of ACP to HAp via OCP.

Based on the above findings, it is conceivable that ACP exists in the outermost surface of pre-eruptive enamel because Mg, which acts as a stabilizer of ACP, is contained there. Eventually, when the enamel’s outermost surface comes in contact with saliva, its structure composition transitions from ACP to HAp via OCP. Usually in the oral cavity, a thin layer containing acidic calcium phosphate is formed on the outermost surface of the tooth due to variations of pH in the saliva.

The interface between the enamel and demineralized layer using human-extracted teeth and acid solution have been experimentally observed. Hayashi et al.²⁶ reported that a layer of acidic calcium phosphate is formed and that this layer maintains a metastable state, enabling the reversible reaction of demineralization and remineralization. The layer containing acidic calcium phosphate found in this study is considered to be the same as the layer reported by Hayashi et al. That is, this study revealed that the layer containing acidic calcium phosphate maintained the metastable state, and that this was present on the enamel surface of teeth within the oral cavity. Since acidic calcium phosphate precipitates at a pH lower than that of HAp crystals, the layer containing calcium phosphate present on the enamel surface is considered to have higher acid resistance than HAp. Although the acid resistance and strength of this layer need to be verified in detail in the future, information from this study can be applied to basic clinical research on remineralization.
therapy and non-invasive treatments of initial caries.

Acknowledgments

We would like to express our gratitude to Dr. Koji Inoue and to the doctors of the Department of Oral and Maxillofacial Surgery, Department of Oral Anatomy and Department of Operative Dentistry, Tsurumi University School of Dental Medicine. A part of this work was conducted at the Advanced Characterization Nanotechnology Platform of the University of Tokyo, supported by the “Nanotechnology Platform” of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. The Ministry of Education, Culture, Sports, Science and Technology Supported Program for the Strategic Research Foundation at Private Universities. We would like to thank Editage (www.editage.jp) for English language editing.

Conflicts of Interest

The authors have declared that no conflicts of interest exist.

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