Effects of Calcium Phosphate Precipitation Method on Acid Resistance to Apatite Powder and Bovine Tooth

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The aims of this study were to evaluate the effects of CPP method on the crystallinity of apatite powder and on the acid resistance of bovine enamel. Crystallinity degrees of apatite powder before and after CPP treatment were measured by powder X-ray diffraction analysis. Polished bovine enamel specimens treated with CPP method or NaF were immersed in a lactic acid solution for up to five days. The demineralized depth of enamel was measured with a surface roughness analyzer. XRD peaks became sharper after the CPP treatment, indicating an increased crystallinity of the apatite powder. The demineralized depth of bovine enamel treated with CPP method was shallower than that of enamel treated with NaF. Results of this study revealed that the CPP method increased the crystallinity of apatite powder and the acid resistance of enamel. Therefore, the CPP method would be useful not only for treating dentin hypersensitivity, but also for the prevention of dental caries.

Key words: Hydroxyapatites, Calcium Phosphates, Crystallinity

INTRODUCTION

Calcium phosphate precipitation (CPP), a treatment method for dentin hypersensitivity, fulfills its objective and function by occluding open dentinal tubules with apatitic minerals⁴. This method entails treating the dentin surface with an acidic calcium phosphate precipitation (CPP) solution that contains both calcium and phosphate (1 M CaHPO₄ • 2H₂O dissolved in 2 M H₃PO₄), followed by neutralization with a basic post-treatment solution (1 M NaOH with 0.1 M NaF or 1 M NaHCO₃ with 0.3 M NaF). On use of a post-treatment solution with lower concentrations of NaF (<50 mmol/L) or without NaF, the precipitate formed by the CPP method would be composed of dicalcium phosphate dihydrate (DCPD; CaHPO₄ • 2H₂O)⁵. Therefore, 0.1 mol/L NaF was added to the post-treatment solution to transfer the composition of the precipitate to apatitic minerals (HAP; Ca₃₀₋ₓ(HPO₄)ₓ(PO₄)₆₋ₓ(OH)₂₋ₓ). Treatment of dentin surface with the CPP method resulted in the occlusion of dentinal tubules with apatitic minerals to a depth of approximately 10–15 μm from the dentin surface. In conjunction, dentin permeability was greatly reduced to approximately 1.2–5.8%. The precipitate in the dentinal tubules formed by the CPP method was stable in synthetic saliva, since saliva is supersaturated with respect to HAP⁶. In light of these findings, the CPP method is thought to be ideal for the occlusion of dentinal tubules, hence a potential good treatment method for dentin hypersensitivity.

In clinical application, not only the dentinal tubules but that the enamel and dentin surfaces are similarly exposed to the CPP solution and post-treatment solution. The CPP solution is thought to have no capacity to dissolve tooth substance, since it is in equilibrium with respect to DCPD and supersaturated with respect to HAP. However, it is unclear how the CPP method affects tooth components such as enamel and dentin.

The aims of this investigation were to evaluate the effects of CPP method on the crystallinity of synthetic HAP powder and on the acid resistance of bovine enamel. Synthetic HAP powder was used for the investigation of crystallinity because of two key reasons: (1) large surface area; and (2) reproducible constant data. A large surface area makes measurements easier with conventional analytical equipment. In addition, reproducible constant data can be obtained with HAP powder because it is synthesized—and hence free from various ions which can influence the properties of hydroxyapatite.

MATERIALS AND METHODS

Preparation of HAP powder

HAP powder was prepared as described previously⁷.
In brief, a solution of calcium chloride (CaCl₂, 2.0 L of 1.0 mol/L) and disodium hydrogen phosphate (Na₂HPO₄, 2.0 L of 0.6 mol/L) containing 0.2 mol/L of NaOH was added dropwise into a three-necked flask containing 1.0 L of double-distilled water at 20 or 100°C using a roller pump (RP-NE1, Furue Science, Tokyo, Japan) at a speed of 1.0 mL/min for each reactant. The resultant white precipitate was kept in the vessel for one week to allow maturation. After which, it was filtered and thoroughly washed with double-distilled water. The products were dried under vacuum for 24 hours and stored under vacuum. The products were identified as HAP by powder X-ray diffraction (XRD) analysis and Fourier transform infrared (FT-IR) spectroscopy (FTS-40, Bio-Rad, Cambridge, MA, USA).

Preparation of CPP and post-treatment solutions
The CPP solution was prepared as described previously.¹ Reagent grade 1 mol/L CaHPO₄·2H₂O (Nacalai Tesque, Kyoto, Japan) was dissolved in a 2 mol/L H₃PO₄ solution at room temperature to prepare a DCPD-saturated CPP solution (pH=1.4). Clear solutions ([Ca]=1 mol/L; [PO₄]=3 mol/L) were filtered through 0.22-μm Millex-GS filter assemblies (Millipore Corp., Bedford, MA, USA) and kept in closed bottles at room temperature. Post-treatment solution I was prepared so that the concentration of NaF (Nacalai Tesque, Kyoto, Japan) in a 1.0 mol/L NaOH solution (Nacalai Tesque, Kyoto, Japan) was 0.1 mol/L (pH=12.4). Post-treatment solution II was prepared so that the concentration of NaF in 1.0 mol/L NaHCO₃ was 0.3 mol/L (pH=8.8). In addition, 0.1 mol/L NaF and 0.3 mol/L NaF solutions were prepared from reagent grade chemicals.

Treatment of HAP powder with CPP method
In a typical procedure, 1 g of HAP powder was suspended in 5 mL of the CPP solution. HAP powder was ultrasonicated in the CPP solution for up to five minutes. Subsequently, the suspension was filtered and washed at least five times with a large amount of double-distilled water. The washed powder was dried under vacuum and immersed in 5 mL of post-treatment solution (I or II) with ultrasonication for prescribed periods (1−10 minutes). Thereafter, the HAP powder was washed in the same manner and lyophilized.

Powder X-ray diffraction (XRD) analysis
XRD patterns of the lyophilized specimens were recorded with a vertically mounted diffractometer system (ADG-301, Toshiba, Tokyo, Japan), using Ni-filtered monochromatized CuKα radiation (λ=0.1540 nm) generated at 30 kV and 10 mA. The HAP powder obtained above was first scanned from 3 to 60° 2θ (where θ is the Bragg angle) in a continuous mode (1.0° 2θ/min, time constant of 2 seconds), then the HAP 002 peaks at 2θ=25.9° were recorded separately at a minimum of five times from 24 to 27° (0.125° 2θ/min, time constant of 10 seconds) to obtain crystallinity information and for quantitative analysis. Angular width of the (002) diffraction peak was measured at half the height of maximum intensity above background. The half width, “β₁/₂”, was corrected for instrumental broadening, “b₁/₂”, by Warren’s method using the equation B₁/₂⁺b₁/₂ = β₁/₂, where β₁/₂ is the corrected peak width. Commercially obtained, highly crystalline synthetic hydroxyapatite (Tomita Pharmaceutical Co., Tokushima, Japan) was used as a reference to determine the value of b₁/₂. As peak width inversely correlates with crystal size and lattice perfection (i.e., the smaller the width, the larger and/or less strained the crystals), the reciprocal of the β₁/₂ value (1/β₁/₂) was therefore used as a means to directly correlate...
the XRD data to these crystal parameters. The \(1/\beta_{1/2}\) value was the average of at least five independently treated specimens.

**Preparation of enamel blocks**

Freshly extracted bovine mandibular incisors were used immediately. Each tooth was sectioned horizontally below the cementoenamel junction using a low-speed water-cooled diamond saw (Buehler Ltd., Evanston, IL, USA). The crown was embedded in a self-curing acrylic resin (Tray Resin®, Shofu Inc. Kyoto, Japan), leaving only the labial side exposed. The exposed surface of the enamel was polished with SiC up to #1500 using an automatic polisher (ECOMET3, Buehler, IL, USA) to obtain a flat mirror surface. Polished enamel surface was sectioned into two halves at the center using a low-speed water-cooled diamond saw (Buehler Ltd., Evanston, IL, USA), as shown in Fig. 1. Margins of the polished enamel were covered with a paraffin wax. Width was approximately 1 mm.

One half of the untreated polished surface served as a control. For the other half of the polished surface, it was treated with one of the following: CPP method I (1 M CaHPO₄ • 2H₂O in 2 M H₃PO₄; post-treatment solution: 1 M NaOH with 0.1 M NaF), CPP method II (1 M CaHPO₄ • 2H₂O in 2 M H₃PO₄; post-treatment solution: 1 M NaHCO₃ with 0.3 M NaF), or fluoride solution (0.1 M NaF or 0.3 M NaF). In brief, the CPP solution was applied to the enamel surface with a cotton swab for one minute, and then the post-treatment solution applied with a cotton swab for two minutes. For NaF treatment, the fluoride solution was applied to the enamel surface with a cotton swab for two minutes. Then, any remaining solution on the tooth surface was wiped away with a cotton swab. All specimens were gently washed with distilled water for one minute.

**SEM observation**

Morphologies of the enamel surface before and after the CPP or NaF treatment were observed with a scanning electron microscope (Hitachi Co., Tokyo, Japan). The specimens obtained following the above procedures were dried with a critical point dryer (Hitachi Co., Tokyo, Japan). After the specimens were coated with gold, their surfaces were observed using SEM.

**Demineralization**

Enamel blocks obtained above were immersed in 0.06 M lactic acid solution (pH 5.0) at 37°C for 24, 72, and 120 hours. Following each demineralization period, the specimens were rinsed with distilled water for one minute and then the paraffin wax washed away gently with a steamer. After washing the specimens again with distilled water, the demineralized depths of the enamel surfaces were measured in the mesio-distal direction at a constant speed of 0.5 mm/second using a surface roughness analyzer (or surface texture measuring instrument) (Surfcom 300A, Tokyo Seimitsu, Tokyo, Japan). Each average value of demineralized depth was obtained from 20 measurements of at least five independently treated enamel blocks.

**Statistical analysis**

The mean and standard error (SE) for the \(1/\beta_{1/2}\) value of HAP powder and for the demineralized depth of enamel were calculated. Significant differences between mean values were evaluated by ANOVA with the level of significance set at 0.05.

**RESULTS**

Figure 2 shows the typical X-ray diffraction (XRD) patterns of apatite powder before and after treatment with the CPP method. In addition, the XRD patterns of the commercially obtained DCPD and HAP are shown for comparison. (a) Before treatment; (b) After treatment with the CPP solution for 1 min; (c) After treatment with the CPP method (CPP solution for 1 min and then the post-treatment solution for 2 min); (d) DCPD; and (e) HAP.
Fig. 3 Effects of varied treatment periods of CPP solution on the crystallinity of HAP powder when the post-treatment period was fixed at 1 min. (○) Post-treatment solution of CPP method was 1 mol/L NaOH with 0.1 mol/L NaF; (●) Post-treatment solution of CPP method was 1 mol/L NaHCO₃ with 0.3 mol/L NaF.

Fig. 4 Effects of varied post-treatment periods on the crystallinity of HAP powder when the treatment period of the CPP solution was fixed at 1 min. (○) Post-treatment solution of CPP method was 1 mol/L NaOH with 0.1 mol/L NaF; (●) Post-treatment solution of CPP method was 1 mol/L NaHCO₃ with 0.3 mol/L NaF.

Fig. 5 SEM images of enamel surface before and after the CPP or NaF treatment. (a) Untreated polished enamel surface; (b) After treatment with CPP method I (1 M CaHPO₄·2H₂O in 2 M H₃PO₄, post-treatment solution: 1 M NaOH with 0.1 M NaF); (c) After treatment with CPP method II (1 M CaHPO₄·2H₂O in 2 M H₃PO₄, post-treatment solution: 1 M NaHCO₃ with 0.3 M NaF); (d) After treatment with 0.1 mol/L NaF; (e) After treatment with 0.3 mol/L NaF. Bar represents 30 μm.
of commercially obtained DCPD and HAP were shown for comparison. An XRD peak corresponding to DCPD (2θ=11.7) was observed after treatment with the CPP solution (Fig. 2b). On application of the post-treatment solution, the peak corresponding to DCPD disappeared and all the peaks corresponding to HAP were observed (Fig. 2c). Also, poorly crystallized apatite powder changed to a highly crystallized apatite powder with sharp XRD peaks after treatment with the CPP method. No significant differences in XRD pattern were observed between the two post-treatment solutions.

For a quantitative comparison of crystallinity of HAP powder, 1/β_{1/2} values were calculated according to Warren’s method (see MATERIALS AND METHODS section). Results are shown in Figs. 3 and 4. Figure 3 shows the 1/β_{1/2} values of HAP powder as the period of treatment with the CPP solution increased from 0 to 5 minutes, while post-treatment time was fixed at 1 minute. The 1/β_{1/2} value increased from 3.5±0.1 (mean±SD) to 4.0±0.2 with increase in treatment time (CPP solution treatment for 1 minute followed by post-treatment for 1 minute using 1 M NaHCO₃ with 0.3 M NaF). However, the 1/β_{1/2} value became almost stable when the CPP treatment time exceeded 1 minute.

Figure 4 shows the 1/β_{1/2} values of HAP powder when treatment time with the CPP solution was fixed at 1 minute and the post-treatment solution was applied for various time periods. The 1/β_{1/2} value of HAP powder increased in proportion to the post-treatment time. The 1/β_{1/2} value increased from 3.5±0.1 to 4.0±0.2 after treatment with the CPP method (CPP solution for 1 minute followed by post-treatment for 1 minute using 1 M NaHCO₃ with 0.3 M NaF). No significant differences were observed between the type or treatment period of the post-treatment solution (1–10 minutes). On the other hand, the 1/β_{1/2} value of the HAP powder treated with 0.3 mol/L NaF for 1 minute was 3.7±0.1.

Figure 5 shows the SEM images of the enamel surface before and after the CPP or NaF treatment. The enamel surface was completely covered with the precipitate after treatment with both CPP methods (Figs. 5b and 5c). In contrast, there was little precipitate on the enamel surface after 0.1 or 0.3 mol/L NaF treatment (Figs. 5d and 5e).

Table 1 shows the demineralized depths of bovine enamel surface after immersion in lactic acid. It is noteworthy that acid susceptibility differs among teeth. Therefore, two adjacent areas of the same tooth were used to the end of measuring the demineralized depths accurately and symmetrically. One served as the control, while the other was used as a test surface. It should also be noted that the demineralized depth was represented as a percentage compared with the adjacent control depth. Depth of the control was 257.2±20.5 μm (mean±SD) after demineralization for 24 hours. This value is represented as 1.0 in Table 1. The depths were significantly shallower for the CPP method than for non-treatment. Furthermore, the demineralized depth of bovine enamel treated with CPP method I was shallower than that treated with 0.1 M NaF, and CPP method II resulted in a shallower depth than 0.3 M NaF in all immersion periods. No significant differences were observed between 0.1 M NaF and 0.3 M NaF.

**DISCUSSION**

This study showed that the crystallinity of apatite powder increased with CPP treatment. In addition, the acid resistance of bovine enamel was significantly increased by the CPP method compared with 0.1 or 0.3 mol/L NaF solution, despite the same concentrations being used in the two post-treatment solutions in the CPP method. The reason for the increase in
the crystallinity of apatite powder could be explained from the XRD pattern (Fig. 2). After treatment with the CPP solution for one minute, a DCPD peak at 2θ = 11.7 was observed (Fig. 2b). It meant that some apatite powder was converted into DCPD after the CPP solution treatment. Following application of the post-treatment solution, the DCPD peak disappeared and a XRD pattern corresponding to HAP post-treatment solution, the DCPD peak disappeared and a XRD pattern corresponding to HAP was observed. However, when treatment time with the CPP solution was 30 seconds, the DCPD peak did not appear and the crystallinity of apatite powder showed no significant differences before and after treatment with the CPP method. These findings clearly suggested that the formation of DCPD played an important role in increasing the crystallinity of apatite powder.

DCPD is known to react with fluoride more rapidly than does hydroxyapatite, Ca₅(PO₄)₃OH, or tooth enamel. As a result, transformation from DCPD to FAP occurred more easily than the transformation from HAP to FAP. Fluoride could be taken up into the tooth component more easily after DCPD formation, and so the crystallinity of the tooth increased significantly. In light of these phenomena, the two-treatment procedure proposed by Chow and Brown has been proven to be particularly efficient in increasing the level of enamel-bound fluoride. First, an acidic calcium phosphate solution (0.7 mol/L Ca²⁺, 1.9 mol/L PO₄³⁻), which was saturated with respect to dicalcium phosphate dihydrate and fluoroapatite, was applied to the enamel. DCPD formed in the outer enamel. Following treatment with acidulated phosphate fluoride (APF), DCPD was converted into fluoroapatite.

The difference between the procedure proposed by Chow and Brown and the CPP method described in this study lay in the concentrations of calcium and phosphate used. In the CPP solution, a concentration of 1 mol/L of calcium was needed to occlude the open dentinal tubules completely for the treatment of dentin hypersensitivity. To prepare the DCPD-saturated CPP solution, 2 mol/L of phosphate was needed. Another difference was the second treatment solution (post-treatment solution). The CPP method employed a basic post-treatment solution containing NaF to neutralize the CPP solution.

The principal of the CPP method depends on the solubility capacity of calcium phosphate as a function of the pH of the solution. Large amounts of calcium phosphate dissolve in acidic solutions. In contrast, a low amount of calcium phosphate dissolves in neutral and basic solutions. Against this backdrop, the concentration of NaF in the post-treatment solution of the CPP method significantly determines the composition of the precipitate that forms in the dentinal tubules. In our previous study, the precipitate formed by the CPP method was DCPD when the concentration of NaF in the post-treatment solution was below 50 mmol/L. On the other hand, an apatitic precipitate was formed when the concentration of NaF was above 50 mmol/L. In addition, when a higher NaF concentration (0.3 mol/L) was used in the post-treatment solution, CaF₂ was formed as a by-product.

Chow and Brown studied the effects of varied amounts of F⁻ and PO₄³⁻ ions on the composition of solid products. The apatite materials formed in solutions with higher concentrations of F⁻ and PO₄³⁻ ions were generally in poor crystalline form than those formed from dilute solutions. Further, the precipitate formed under alkaline conditions showed poor crystallinity. With the CPP method, apatitic precipitate was produced under acidic or neutral conditions, although the concentrations of F⁻ and PO₄³⁻ in the CPP method were higher. Therefore, the precipitate formed by the CPP method might have poorer crystallinity than that of Chow and Brown’s method. Nonetheless, the CPP method caused a large amount of apatitic minerals to be formed on the tooth surface and in dentinal tubules. In light of this result, the CPP method seemed to be useful for preventing dentin caries, especially root caries. Generally, dentin caries proceeds through dentinal tubules and the incremental lines in dentin. Therefore, by occluding the open dentinal tubules with apatitic minerals, the CPP method would block or delay bacterial invasion through the dentinal tubules.

Results of this study also showed that the CPP treatment increased the acid resistance of teeth. This meant that demineralization by plaque and plaque products would decrease. An increase in acid resistance of teeth is important not only for caries prevention, but also for hypersensitive teeth. In hypersensitive teeth, plaque control plays an important role in either preventing or aggravating the state of dentin hypersensitivity. With good plaque control, open dentinal tubules were gradually occluded by deposition of calcium phosphate minerals from saliva. On the other hand, in non-plaque control teeth, peri- and intertubular dentin was demineralized by plaque and plaque products. This resulted in an increase in the diameter of dentinal tubules with experimental time, causing severe dentin hypersensitivity. Increasing acid resistance after the CPP treatment would reduce the demineralization of peri- and intertubular dentin. Nonetheless, even with good leverage on the present study’s encouraging results, it remains to be validated the resistance of the CPP method against plaque demineralization in vivo settings.

On the treatment time required for CPP solution and post-treatment solution, the CPP solution was...
applied to teeth for 30 seconds, and then the post-treatment solution applied for one minute for treatment of hypersensitive teeth. However, considering the degree of crystallinity of teeth, application of the CPP solution would need at least one minute and the post-treatment solution five minutes — based on the time-course changes of the $1/\beta_{1/2}$ value (Figs. 3 and 4).

Prior to clinical use, it is mandatory that the safety and stability of the CPP method be unequivocally established. In fact, it may seem hazardous to use 1 mol/L NaOH as the post-treatment solution. NaHCO$_3$ has already been used in obstetrics and gynecology after the mucous membrane is washed. Therefore, for clinical use evaluation, NaHCO$_3$ was employed as the post-treatment solution in the CPP method in this study. Based on the favorable results obtained in this study, it thus warrants further clinical evaluation with a view for practical applications.

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