Casein phosphopeptide-amorphous calcium phosphate and glass ionomer show distinct effects in the remineralization of proximal artificial caries lesion in situ

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This study aimed to compare the ability of casein-phosphopeptide amorphous-calcium-phosphate (CPP-ACP) and glass-ionomer (GI) in remineralizing proximal artificial caries lesions (ACLs). Molar enamel-slabs were divided into: original-lesion control, intra-oral controls, and experimental (CPP-ACP or GI) groups. Specimens received ACLs and were bonded on subject maxillary first molars. After 4-weeks, mineral density (MD) was analyzed by μCT. Compared to control, CPP-ACP increased MD at 0–38/68–84 microns and the GI group had an increase at 0–68 microns, with a greater increase in MD compared to the CPP-ACP group from 0–53 microns. The mean percent remineralization (%R) showed differences between the GI, CPP-ACP groups and their paired controls. GI tended to increase remineralization more than CPP-ACP. In conclusion, CPP-ACP and GI demonstrated distinct remineralizing ability. GI induced greater remineralization in the superficial lesion, while CPP-ACP remineralized the lesion body. Their effects on percent remineralization and reducing lesion depth of proximal ACLs were similar.

Keywords: CPP-ACP, GI, Proximal caries lesion, Remineralization

INTRODUCTION

Dental caries is a dynamic process1), progressing from an initial subclinical lesion, through an early clinical detection phase, to cavitation2). The management of dental caries has evolved to early clinical detection of initial lesions and using products inducing remineralization to stop lesion progression and reverse surface demineralization before cavitation occurs3). Remineralizing products come in two types, professionally and self-applied. Professionally applied products include topical fluoride gels/foams and fluoride-containing restorative materials such as glass ionomers (GIs). Because of the outstanding properties of GIs, such as their ability to adhere to tooth structure by ion exchange4), long term fluoride release, and capability to absorb fluoride ions5), the use of glass ionomers to remineralize caries lesions has increased. In an in vitro study, GI reduced lesion area compared to fluoride vanish, fluoride sealant, and sealant6). When incipient proximal caries lesions were applied with GI, they were more likely to remain in or regress to the outer half of the enamel. The depth of these lesions was significantly less than that of the control group after 6, 12 months7). Other remineralizing products are self-applied, such as fluoride-containing dentifrices, the most commonly used self-applied topical fluoride, fluoride mouthrinses, and prescription fluoride gel or paste. However, there are concerns about the development of dental fluorosis with the use of high-concentration self-applied topical fluoride products, especially in young children who can easily swallow these products. This concern stimulated investigation into alternative agents to complement or substitute for fluoride. Milk-derived casein-phosphopeptide amorphous-calcium-phosphate (CPP-ACP) was introduced and researched as a topical agent. Casein-phosphopeptides (CPP) with the cluster sequence [-Ser(P)-Ser(P)-Ser(P)-Glu-Glu] can bind and localize amorphous-calcium-phosphate (ACP) in solution8). This maintains high concentrations of calcium and phosphate ions in subsurface lesions promoting enamel remineralization9). Self-applied CPP-ACP and professionally-applied GI have remineralizing ability as assayed by reduction in lesion area or depth10,11). However, their effect on mineral density is unclear. The aim of this study was to compare CPP-ACP and GI on remineralizing proximal artificial caries lesions (ACLs) in situ by evaluating the mean mineral density values at various lesion depths and percent remineralization values (%R) using micro-computed tomography (μCT) analysis.

MATERIALS AND METHODS

This study used a professionally applied material, GI (Fuji VII, GC Corporation, Tokyo, Japan), and a
self-applied material, CPP-ACP (Tooth Mousse, GC Corporation, Tokyo, Japan).

**Study design and subject recruitment**
We used a randomized, crossover, double-blind design, and was approved by the Ethics Committee, Faculty of Dentistry, Chulalongkorn University, Thailand (No. 1412011) in accordance with the World Medical Association Declaration of Helsinki. Six healthy volunteer orthodontic patients, aged 19–23 years, served as subjects. Intraoral examinations confirmed each volunteer had at least 22 natural teeth with no current caries activity, periodontal disease, or other oral pathology. All participants were deemed high caries risk patients (consuming sugary snacks or beverages more than 3 times/day between meals). Volunteers were not using antibiotics or medications affecting salivary flow rate. The stimulated and unstimulated salivary flow rates of each subject were >1.0 mL/min and >0.2 mL/min respectively. Following verbal and written explanations of the experimental protocol, informed consent was obtained.

**Tooth preparation and selection, sectioning**
Six extracted human permanent molars without cracks, white spot lesions, or enamel hypoplasia were collected. The proximal enamel surfaces were polished with a polishing machine (DPS 3200, IMPETECH, South Africa) at 100 rpm for 45 s to remove the surface enamel layer with its fluoride rich zone. Flat surface areas of 3×6 mm² were cut from the same level of the mesial/distal tooth surfaces using a hard disc (273D, Intensiv, Grancia, Switzerland). Each of these sections was then cut into three 2×2×3 mm³ slabs, for a total to 6 slabs with only 5 slabs used by the assigned volunteer.

**Lesion formation**
All specimen surfaces were coated with nail varnish except for a center 1×1 mm² window and immersed in a demineralizing solution containing 20 g/L Carbopol 907, 500 mg/L hydroxyapatite and 0.1 mol/L lactic acid at 37°C for 96 h producing ACLs. The depth of the lesion was approximately 100 μm. The specimens were then washed in deionized water and sterilized for 12 h in ethylene oxide.

**Experimental protocol**
All subjects used a 1,450-ppm fluoride-containing dentifrice (Monofluorophosphate) (Fluocaril, Diethelm Trading Corporation, Bangkok, Thailand) 2×/day, before breakfast and bedtime, for one week before the study and during the test period. One week before the experiment period, volunteers were instructed not to use mouthwash, cigarettes, or consume products containing alcohol, Xylitol, or fluoride except the fluoride-containing dentifrice provided. They were instructed to keep to their regular diet and any changes shown in the daily diet record would be grounds for exclusion.

Five slabs from one tooth were randomly assigned to a volunteer as original lesion control, GI intra-oral control, CPP-ACP intra-oral control, GI test, and CPP-ACP test.

The 1×1-mm² area at the center of the test specimen was randomly applied with either CPP-ACP or GI. Each intra-oral specimen was inserted against the mesial wing of an orthodontic bracket to simulate proximal caries. The surface opposite the window surface was bonded to the bracket with flowable composite (Filtek Flow, 3M-ESPE, St. Paul, MN, USA) (Fig. 1). Each test specimen bracket was randomly bonded to the buccal surface of a maxillary first molar of each volunteer with a bonding agent (Transbond XT®, 3M Unitek, Monrovia, CA) while the intra-oral control specimen bracket was bonded on the contralateral tooth.

The application of each material was done per the manufacturer’s instructions. GI was applied on the entire 1×1 mm² lesion area at center of the specimen before bonding to the brackets. The subjects applied CPP-ACP 2×/day after morning/bedtime brushing. They were given instructions indicating steps and procedures during the test period.

The volunteers were randomly assigned for a material in the first session, and then alternated to the other material in the next session. The duration of the experiment was two sessions of a 4-week test period and a 1-week washout period in between.

**μCT scanning**
Specimens were assayed using a μCT system (InspeXio SMX-100CT; Shimadzu, Kyoto, Japan). A 0.5-mm-thick aluminum and 0.3-mm-thick copper filter was placed in the beam path to reduce beam-hardening effects. The specimen was mounted on a computer-controlled turntable, which synchronized the rotation and axial shift. The nominal isotropic resolution of the setup was 7.0 μm, with an integration time of 120 s. The tube voltage was 100 kV at a current of 165 μA.

For mineral density (MD) calibration, a series of mineral reference phantoms were scanned which included three hydroxyapatite (HAp) disks (Ratoc,
Tokyo, Japan) with different concentrations (0.20, 0.50 and 0.70 gHAp cm$^{-3}$) of HAp crystals embedded in epoxy resin, and a pure HAp disk (3.16 gHAp cm$^{-3}$) (Cellyard; Hoya, Tokyo, Japan).

**μCT image analysis**

Three-D image analysis software (TRI/3D-BON; Ratoc) was used for visualization and quantitative measurements. Grayscale values were converted into MD values (gHAp cm$^{-3}$) using a linear calibration curve based on the grayscale values obtained from the mineral phantoms (linear regression, $R^2=0.98–0.99$). Subsequently, a $3\times3$ median filter was applied to suppress image noise$^{14,15}$. Mean MD values were calculated and plotted against depth in a volume of interest of $210\times210\times350$ μm$^3$ (thickness×width×depth). Lesion depth (μm) and mineral loss ($\Delta Z$; vol%μm) were calculated from the MD profiles; with the reference point of the depth axis (0 μm) set at the axial position of the apparent enamel lesion surface beneath the varnish. $\Delta Z$ on each sample was calculated from the profiles by subtracting the area under the line of sound enamel. Lesion depth was recorded as the depth of the region where the mineral content reached 95% of the maximum MD$^{16}$.

The vol% mineral profile of each enamel specimen’s demineralized and remineralized lesion was compared with the median sound enamel vol% mineral profile of the same section. The percent remineralization values $\%R=(\Delta Z_d−\Delta Z_r)/\Delta Z_d\times100$ was calculated by trapezoidal integration, where $\Delta Z_d$ represents the difference between the area under the sound enamel profile and the demineralized enamel profile, and $\Delta Z_r$ represents the difference between the area under the sound enamel profile and the remineralized enamel profile$^{17}$.

**Statistical analysis**

Statistical analyses were performed using SPSS 16.0 (SPSS Inc, Chicago, IL, USA). The mineral density, lesion depth of each group, were described and the differences of these outcome variables between the two test groups were tested by the independent $t$-test, and further comparison with their controls were tested by paired $t$-test, except the difference of percent remineralization ($\%R$) was tested by Mann-Whitney U test. Furthermore, to compare the remineralizing ability between the two intra-oral control groups and extra-oral control group, we used one way ANOVA, following by post-hoc tests (Bonferroni) for multiple comparisons. The significance-level was set at $p<0.05$.

**RESULTS**

Mean MD values (g/cm$^3$) at various lesion depths of the 5 sample groups are seen in Fig. 2. There were no significant differences in the mean MD values at various lesion depths between the two intra-oral control groups, implying that the oral environment was similar in the
two test sessions (p>0.05). Thus, results between the two test materials and control groups could be compared.

After 4 weeks, the mean MD of the GI test group was significantly higher than that of the CPP-ACP test group from 0–53 microns in depth (p<0.05). The CPP-ACP test group had a significant increase in mean MD values from 0–38/68–84 microns while the GI test group showed a significant increase from 0–68 microns, compared with their intra-oral controls (p<0.05). Moreover, we found that comparing the original lesion control and the two intra-oral controls, there was no significant difference between the mean MD values throughout the lesion suggesting that using fluoride-containing dentifrice alone in these high caries-risk subjects was not sufficient to increase the mineral density of the intra-oral control specimens (p>0.05).

Comparison of the mean lesion depths showed a significant difference between the GI and CPP-ACP groups and their paired intra-oral controls (p<0.05) (Table 1). Moreover, the 4-week remineralization of the enamel lesion only showed significant differences in the mean percent remineralization values between the GI group (70.6±13.7%) and their paired intra-oral controls (72.6±15.3%) (p<0.05) (Table 1). However, there was no significant difference between the two test groups. The GI group yielded a 36.6% higher remineralization efficacy than the CPP-ACP group. Exposure to GI and CPP-ACP resulted in a 149.5% and 86.6% increase in percent remineralization, respectively compared to the use of dentifrice alone (paired intra-oral controls).

**DISCUSSION**

We assayed our samples using μCT because this allowed us to evaluate MD at different depth intervals compared to the analysis of lesion depth or area derived using polarized light microscopy. Another major advantage is it allows for 3-D analysis. μCT is also non-invasive, non-destructive, and less time consuming. There is no need for specimen sectioning, resulting in no specimen loss from the sectioning process, making it a good choice as a substitute to more conventional techniques.

The presence of either CPP-ACP or GI (Fuji VII) on the test specimen on one side of the mouth did not affect the other side of the mouth, as shown by MD at various depths of the intraoral controls in the two sessions. This finding is similar to that of a previous study.[18]

The remineralizing mechanism of GI occurs from the acid-base reaction when the glass powder is mixed with poly-acrylic acid. In the setting reaction, the hydrogen ion from acid attacks the glass particles causing metal ions such as calcium, strontium and aluminum to be released from the material. Moreover, the poly-aryllic acid will act as a self-etching agent to dissolve the calcium and phosphate ions from the tooth. Fluoride is then released from GI restorations to the tooth and saliva. The evidence of ion exchange between GI material and the tooth structure was demonstrated, showing fluoride and strontium created hypermineralized zone between GI and dentine.[19] Hypermineralized zone was also found in the enamel lesion after GI was placed adjacent to it.[20]

Even though GI was applied to the tooth lesion surface, there is still microleakage from thermo-cycling in the oral cavity between the tooth surface and GI which allowed oral fluid to seep through.[21] As a result, GI could release or uptake more fluoride, calcium and phosphate still could reach the lesion, thus remineralization process continues.

The GI group demonstrated greater MD at a depth of 0–53 microns compared to the CPP-ACP group. This may have resulted from GI creating a hypermineralized zone[20] at the interface of enamel and GI which may inhibit remineralization in the deeper region of the lesion. Moreover, the GI group (Fuji VII) had a higher mean %R value than did the CPP-ACP group. This may be because Fuji VII has the highest fluoride release and uptake among other glass ionomer (Fuji) products.[22] This mechanism is a continuous process, with the fluoride...
level release highest on the first day of application and gradually decreasing on subsequent days until the fluoride release level stabilizes after 1 week\textsuperscript{20}. In our study, all volunteers used fluoride toothpaste 2×/day; hence, there was a continuous fluoride release and uptake by Fuji VII.

The group of volunteers in our study was a high-caries-risk group due to their consuming sugar more than 3×/day between meals. This behavior resulted in the samples being repeatedly exposed to acidic conditions, favoring the release of fluoride from GI and calcium and phosphate ions from CPP-ACP\textsuperscript{24}. A study has shown that at low pH, the surface degradation of GI generated higher fluoride release\textsuperscript{25}.

The CPP-ACP group yielded greater MD at depths of 0–38 microns and 68–84 microns than was seen in the CPP-ACP intra-oral control group. This indicated that CPP-ACP remineralized the deeper lesion region better than Fuji VII. CPP-ACP is electroneutral and can penetrate the subsurface lesion unaffected by the electric charge of enamel\textsuperscript{26}. In addition, due to the small size of the CPP-ACP nanocomplex, it can enter the porosities of an enamel subsurface lesion and diffuse along its concentration gradient into the body of the lesion. The CPP-ACP can then release its weakly bound calcium and phosphate ions, which would deposit into crystal voids. The crystal growth during remineralization has the same pattern as crystal growth during amelogenesis\textsuperscript{27,28}. Microradiography has shown that CPP-ACP tends to remineralize throughout the lesion even in the presence of fluoride\textsuperscript{29}.

The porosities between enamel rods present after subsurface demineralization alters the light refractive index of the tooth surface, making it dull or opaque in color, resulting in white spot lesions\textsuperscript{30}. CPP-ACP applied prior to orthodontic bracket and adhesive removal in vitro was efficient in remineralization of white spot lesions, restoring the translucency of the sound enamel\textsuperscript{31}.

The results from our study have clinical applications. Fuji VII should be used to remineralize caries lesions in posterior teeth, which do not have high esthetic requirements but need remineralization of high MD. CPP-ACP, which tended to remineralize the lesion subsurface, may be used in anterior teeth, which have greater esthetic requirements.

Although this in situ study has limited number of sample size, further clinical trial could use the important findings of this present study to confirm the significant findings.

CONCLUSION

In this study, Fuji VII could remineralize the superficial region of caries lesions more than CPP-ACP, while CPP-ACP induced remineralization tended to occur in the body of the lesion. Fuji VII could promote more remineralization than CPP-ACP; however, they were similar in their reduction of lesion depth and percent remineralization of proximal caries lesions.

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