Effects of Amorphous Calcium Phosphate Stabilized by Casein Phosphopeptides on Enamel De- and Remineralization in Primary Teeth: An In Vitro Study

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ABSTRACT

Purpose: Amorphous calcium phosphate, stabilized by casein phosphopeptides, has been found to enhance remineralization of subsurface lesions in permanent teeth. The purpose of the present in vitro study was to evaluate the potential of GC Tooth Mousse to enhance remineralization of initial demineralized enamel sites in primary teeth.
Methods: Forty-four demineralization sites were created in 22 extracted primary teeth. Samples were randomly assigned to 6 treatment groups (GC Tooth Mousse covering, GC Tooth Mousse covering and demineralization, and control groups). The mineral content of each sample was evaluated using energy dispersive X-ray analysis, performed from the enamel surface of each lesion inwards. The results were analyzed using analysis of variance, with a significance level $P<.05$.
Results: Samples treated with GC Tooth Mousse demonstrated an increase in the calcium-phosphate ratio by approximately 2% near the surface, a minimal increase of 1% at a depth over 60 μm, and no change at a depth from 40 to 60 μm, with no statistically significant differences ($P>.05$).
Conclusion: This study demonstrates a minimal increase in the subsurface calcium-phosphate ratio following GC Tooth Mousse treatment, especially in demineralized enamel tissue. (**J Dent Child 2012;79:9-14**)
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Dental caries is initiated by acid-producing bacteria (*Lactobacillus* and *Streptococcus mutans*), which cause carious lesions in the presence of fermentable carbohydrates. The process involves a series of demineralization and remineralization events in the enamel and dentin. If the demineralization process predominates, the initial caries lesion progresses into cavity formation.[1] Thus, enhancing remineralization of early carious lesions may be an effective noninvasive treatment of the disease.

Enamel and dentin are composed of biological apatite, which is not pure hydroxyapatite (**HA**), but rather a carbonate containing apatite together with other elements such as fluoride (**F**) and magnesium (**Mg**). The distribution of the different ions throughout the crystal matrix affects the physical diffusion and dissolution characteristics of the apatite. The calcium-phosphate (**Ca/P**) ratio is lower in enamel than in pure HA, implicating less calcium in the crystal.[2] When the pH at the surface of a tooth drops below 5.5, demineralization progresses more rapidly than remineralization. Thus, low pH causes dissolution of enamel crystal and consequent high concentrations of calcium, Mg, HPO4, PO4, CO3, and HCO3 in the proximity of the lesion.

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In time, depending on pH and temperature, new crystals are formed from the minerals in the solution around the lesion. In the presence of low levels of F ions, fluorhydroxyapatite can form directly, even under acidic conditions. Apatite formed in an environment containing more calcium and phosphate is expected to contain less CO_2 and less Mg, making it more acid resistant.

Dairy products have been shown to have anticariogenic properties in human and animal models.[3] In 1986, Harper concluded that the protective effect of cheese was best attributed to the casein phosphoprotein and Ca/P contents of the cheese.[4] Using an in situ model, Reynolds and his coworkers found phosphopeptides, which enfold Ca/P, to be responsible for the anticariogenic activity of casein. These casein phosphopeptides (**CPP**) contain a cluster of phosphoseryl residuals that increase the solubility of Ca/P by stabilizing amorphous calcium phosphate under neutral and alkaline conditions and form solutions that are supersaturated. Remineralization of subsurface lesions was observed at all pH values tested (4.5-7.0), with a maximum at pH 5.5.[5]

The purpose of the present study was to evaluate the capability of GC Tooth Mousse, a water-based, sugar free crème containing Recaldent casein phosphopeptide-amorphous calcium phosphate (**CPP-ACP**) in inducing remineralization of initial enamel caries, and in preventing demineralization of enamel in primary teeth in vitro.

METHODS

Buccal or lingual surfaces of 22 extracted primary teeth with at least 1 intact surface were coated with acid resistant nail varnish, leaving a rectangular area of 2 by 4 mm^2 exposed. To create artificial carious lesions, the White[5] protocol for the treatment of permanent teeth was implemented, using a synthetic polymer gel preparation.[5]

All teeth were immersed in a demineralization solution consisting of 0.1ml/L lactic acid, 500 mg/L HA, and 20 g/L polyacrylic acid average 450,000 Dalton at pH 4.8 and incubated for 4 days at 37°C. After 2 days, the solution was replaced with a fresh one of similar content. The demineralization process resulted in a subsurface lesion in the uncoated area of each tooth extending to approximately 100 µm toward the dentinoenamel junction (DEJ).

With the aid of an internal annulus saw microtome (Leitz 1600, Ernst Leitz Wetzlar, Germany), all teeth were cut perpendicular to the lesion's surface. Two areas were created from each tooth, resulting in 44 tooth surfaces available for the study. The nail varnish was scraped from the sawn parts of the teeth. One third of the teeth (N=7) were randomly assigned to a control group and not treated anymore. Each tooth provided an intact enamel site (Group 1) and a demineralized lesion site (Group 2). The teeth in the study group (N=15) were immersed in tooth mousse solution (GC Tooth Mousse, GC Corporation, Tokyo, Japan) for 10 consecutive days. Every day, the tooth mouse solution was replaced with a fresh one, creating: Group 3, intact enamel with tooth mousse; and Group 4, demineralized enamel with tooth mousse.

Half the study group sites (N=15) were then incubated again in the demineralization solution at 37°C for 4 consecutive days, creating: Group 5, intact enamel immersed in tooth mousse and then demineralized; and Group 6, demineralized enamel immersed in tooth mousse and then demineralized. Table 1 describes the 88 sites created from the 44 tooth surfaces half from the demineralized site and half from underneath the nail varnish—the intact enamel.

The mineral content (eg, calcium, phosphate) of all samples were assessed using scanning electron microscopy (SEM) and energy dispersive X-ray (**EDX**) tests. Since microanalysis requires a smooth surface of the tested area, all samples were gradually polished to 1 µm grit, using silicon carbide paper and diamond paste. Samples were then dehydrated under vacuum to remove any unbound water, as previously described.[6] To minimize charging during analysis, the samples were coated with a thin layer of carbon using a vacuum evaporator.

The samples were first photographed using SEM with an accelerating voltage of 15 keV and beam current of 10.0 nA. EDX analysis was then performed with a Norvar EDS X-ray detector capable of detecting elements with atomic weights as low as that of Beryllium (Be). The analysis was performed from the surface of each tooth towards the DEJ every 10 µm, to a depth of 100 µm.

STATISTICAL ANALYSIS

The analysis of variance method was used with repeated measures. The results were analyzed as a function of treatment type and tooth depth. Significance level was set at $P<.05$.

RESULTS

CA/P RATIO OF TOOTH ENAMEL BY EDX ANALYSIS

Figure 1 demonstrates the Ca/P molar ratios obtained from EDX analysis of enamel sampled from the surface ("0" µm) toward the DEJ. Results are represented for the nontreated intact samples (Figure 1a) and for each of the 5 treatment groups (Figures 1b-f). Mean averages and standard deviations are presented for the samples of each treatment group.

The Ca/P ratio of enamel samples ranged from 1.55 to 1.62. There was no statistically significant linear

Table 1. Description of the 6 groups of specimens creating 88 sites from the 44 tooth surfaces half from the demineralized site and half from underneath the nail varnish—the intact enamel.

	Control	Tooth mousse	Tooth mousse and then demineralization
Intact enamel (normal)	14 (Group 1)	15 (Group 3)	15 (Group 5)
Demineralized lesion (acid)	14 (Group 2)	15 (Goup 4)	15 (Group 6)

relation between the molar Ca/P ratio and tooth depth for any of the treatments or for the intact enamel samples (P=.45 and P=.61, respectively).

CHANGE IN CA/P RATIO IN SUBSURFACE AREA

Figure 2 demonstrates the change in Ca/P ratio from the enamel surface to approximately 100 µm into the enamel, comparing the Ca/P ratio between Groups 2 and 4 (Figure 2a) and between Groups 1 and 3 (Figure 2b). Under the enamel surface, and up to 25 µm, the Ca/P ratio increased to approximately 2% in the samples that were demineralized and then immersed in tooth mousse, compared to those that were only demineralized. At a depth of over 60 µm, a more modified increase was detected (less than 1%). At the subsurface lesion depth, from 40 to 60 µm, the ratios in these 2 sample groups were similar.

Comparing the intact samples immersed in tooth mousse with the nontreated samples, an increase in the Ca/P ratio of approximately 2% was evident near the enamel surface, and a more minimal increase of less than 1%, in tooth depth over 60 µm (Figure 2b). Again, as in Figure 2a, at the subsurface lesion depth from 40 to 60 µm, the ratio was almost unchanged between the 2 treatment groups.

TOTAL WEIGHT PERCENTAGE OF CALCIUM AND PHOSPHATE FOR TOOTH ENAMEL

Figure 3 presents the total weight percentage of calcium and phosphate, resulting from the EDX analysis of enamel, sampled from the surface ("0" µm) toward the DEJ. Results are represented for the nontreated intact and for each of the 5 differently treated enamel samples. Each line represents the average of samples for each of the enamel treatments. For all enamel samples, the total calcium

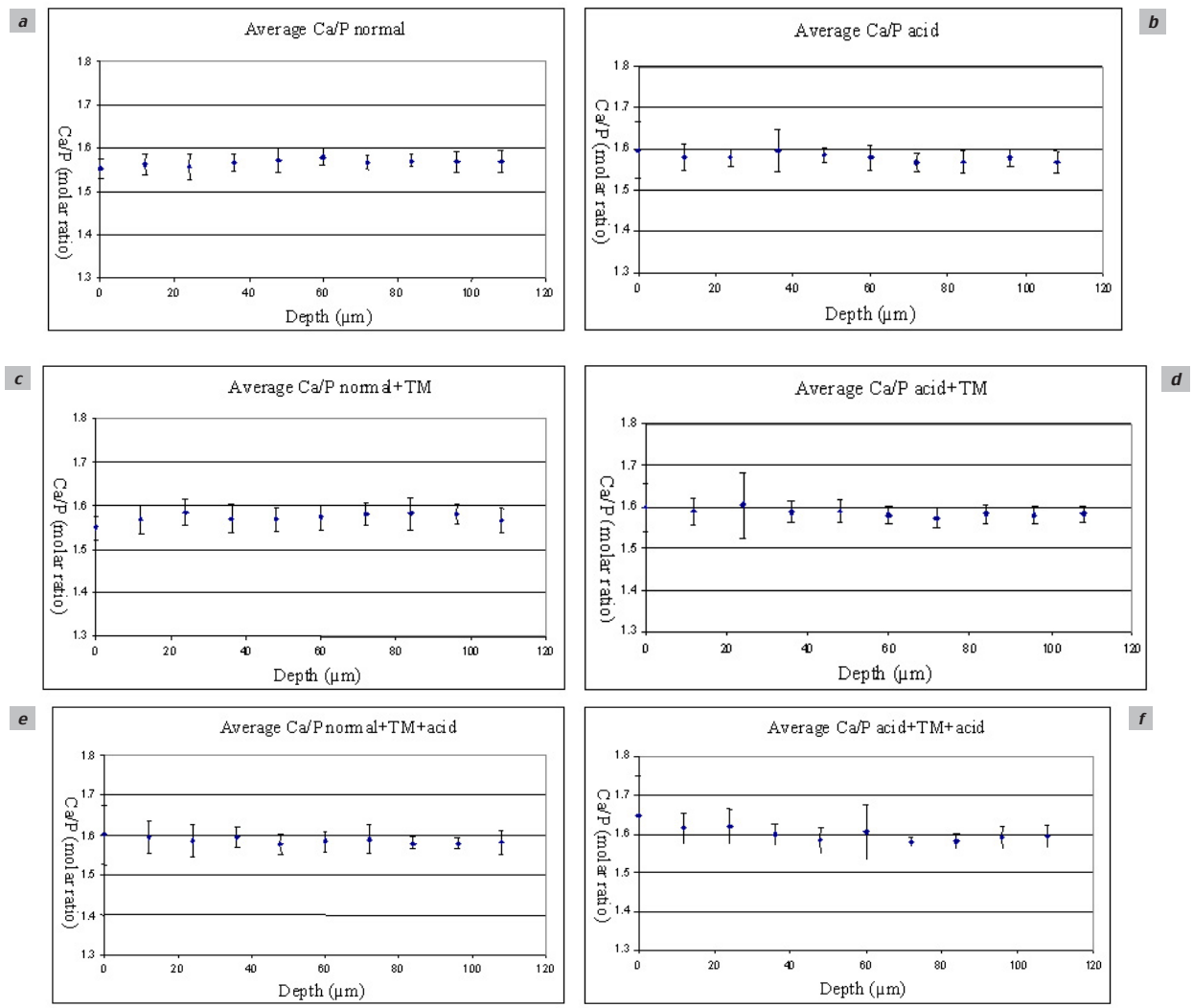

Figure 1. Energy dispersive X-ray analysis of the calcium–phosphate ratio of enamel samples along a line scan from the surface toward the dentinoenamel junction in: (a) intact enamel (N=14); (b) demineralized enamel (N=14); (c) intact enamel immersed in GC Tooth Mousse (N=15); (d) demineralized enamel immersed in GC Tooth Mousse (N=15); (e) intact enamel immersed in GC Tooth Mousse and then demineralized (N=15); (f) and demineralized enamel immersed in GC Tooth Mousse and demineralized again (N=15).

and phosphate weight percentage ranged from 52% to 55%, with a decrease of approximately 4% to 7% in the mineral weight at the upper 10 µm of enamel surface.

A decrease in the total weight percentage of calcium and phosphate was observed in the demineralized enamel samples, both in those immersed and not immersed in tooth mousse. Following an additional demineralization treatment (brown lines), a decrease in mineral weight percentage was observed at a depth of 40 to 60 µm, with no statistically significant difference (P=.47).

No difference in total mineral weight was found between the intact enamel immersed in tooth mousse (solid pink line) and the intact untreated enamel (solid blue line; P=.25). In contrast, the total mineral weight of the demineralized enamel was considerably less in the samples immersed in tooth mousse (dotted pink line) than in those untreated (dotted blue line) at a surface depth up to 60 µm, but without any statistically significant difference (P=.82).

Following an additional round of demineralization (dotted brown line), the weight percentage decreased further, mainly in the 40 to 60 µm depth area with no statistically significant difference (P=.42).

DISCUSSION

In this in vitro study, application of CPP-ACP (**GC Tooth Mousse**) to primary teeth enamel resulted in a minimal increase in the subsurface Ca/P molar ratio compared to demineralized teeth that were not immersed in GC Tooth Mousse.

EDX analysis was used to demonstrate both Ca/P ratios and total weight percentages of calcium and phosphate following GC Tooth Mousse treatment, before and after demineralization of enamel in primary teeth. EDX analysis, used in conjunction with SEM, is a sensitive method of qualitative and quantitative compositional analysis. A finely focused electron beam (electron probe) is bombarded on the surface of a conducting sample. The wavelength and intensities of the characteristic X-ray emitted from the material and the intensities of secondary electrons and backscattered electrons are measured.[7] The X-rays are generated in a region approximately 2 µm in depth. The lithium drifted silicon detector provides collection efficiency with a resolution of 133eV (Mn) and 65eV (F). Typical operating conditions are an acceleration voltage of 15.0 keV and a beam current of 10.0 nA. Minimal grain size can be measured at approximately 2 to 5 µm (for spot size 1 µm).

The X-rays, generated in a region approximately 2 µm in depth, quantitatively produce only concentration ratios, because the penetration of the particles depends on the density of the material. EDX analysis is very useful for measuring relative concentrations of trace elements. The Ca/P ratio in a carious lesion can be determined, but because the probe does not measure H_2 or O_2, the absolute concentration of either Ca or P cannot be determined.[8] Ngo et al.,[8] however, found a high correlation between the total weight percentage of calcium and phosphate measured by EDX analysis and microradiography of enamel caries lesions.

CPP-ACP maintains calcium and phosphate at a super-saturated status compared to calcium and phosphate in the saliva and preserves them in close proximity to the enamel lesion, thereby decreasing demineralization and enhancing remineralization of enamel lesions.[9] A number of studies have documented an inverse association of plaque calcium and inorganic phosphate levels with caries.[10,11] Reynolds reported that the CPP-ACP effect is additive to that of F[12] and causes remineralization of subsurface lesions in human enamel in vitro[13] and in situ.[14-16]

Zhao and Cai showed in vivo that 2% solution of CPP-amorphous calcium fluoride phosphate complexes caused remineralization of enamel subsurface lesions.[17] Use of CPP-ACP solution demonstrated effectiveness in the prevention of demineralization of teeth.[18]

Studies of CPP-ACP's effects on enamel have been performed only on permanent teeth. To the best of our knowledge, this is the first in vitro study on the effect of CPP-ACP on primary teeth. Primary teeth enamel is

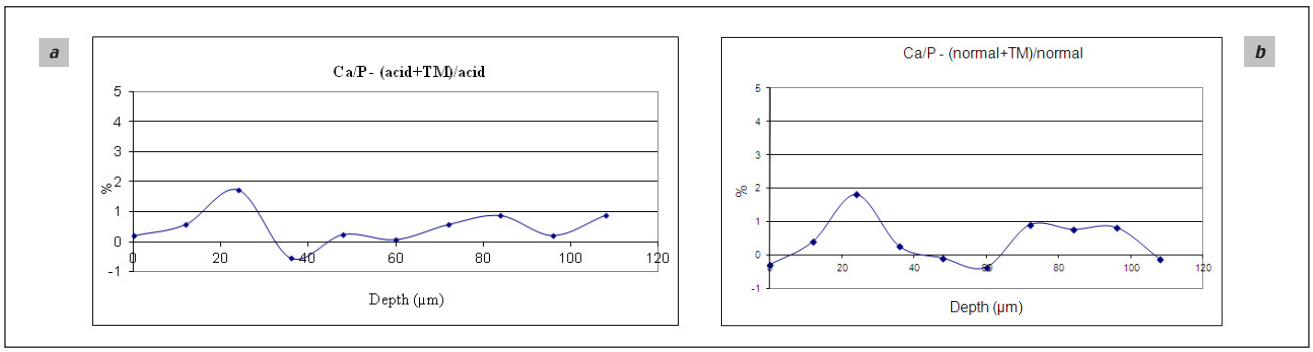

Figure 2. (a) Comparison of the average calcium–phosphate ratios (CPR) of samples that were demineralized and then immersed in Tooth Mousse and those that were only demineralized. The CPR of the group treated with demineralization and then with GC Tooth Mousse, divided by the CPR of the group that just underwent demineralization, was subtracted from 1 and multiplied by 100 to obtain a percentage. The negative values indicate that the results for the group that only underwent demineralization were higher than those for the group treated with GC Tooth Mousse. (b) Comparison of the CPR between the intact samples immersed in tooth mousse and those that were not treated, calculated and shown in percentage.

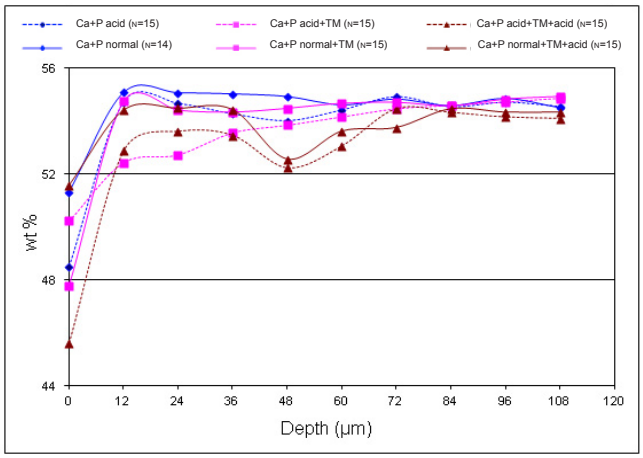

Figure 3. Weight percentage of calcium and phosphate in enamel, analyzed by energy dispersive X-ray, along a line-scan from enamel surface toward the dentinoenamel junction. Each line represents the average of the samples in the group. Intact enamel is shown by a full blue line.

more prone to demineralization due to: lower resistance to acid[19-21]; a low level of mineralization compared to permanent teeth enamel; and 4 µm diameter prisms compared to 6 µm in the enamel of permanent dentition.[22,23]

In the current study, the Ca/P molar ratio (1.57±0.02) in intact enamel of primary teeth, which remained constant across enamel depths, was lower than the theoretical value of 1.66 for pure hydroxyapatite. This observation is congruent with the literature, since the enamel contains defect-apatites that are slightly calcium deficient and remnants of other elements.[24]

The Ca/P molar ratio was almost unaffected by tooth depth in the different experimental groups, implying that the pattern of decrystalization due to demineralization of calcium and phosphate in the apatite crystal (10 to 6) is similar along the enamel rods. In enamel samples that were immersed in GC Tooth Mousse following demineralization, a higher ratio was observed vs samples that were only demineralized. This was found mainly at the enamel subsurface (Figure 2) and might be due to precipitation of more calcium than phosphate from the tooth mousse environment into the enamel. In a similar study in the permanent dentition, Reynolds et al.[13] found the Ca/P molar ratio of sound enamel to be 1.62±0.06. This ratio dropped to 1.5±0.06 in demineralized enamel, suggesting precipitation of an acidic calcium phosphate phase in the lesion during demineralization. Remineralization of the lesions with CPP-ACP resulted in an increase in the Ca/P molar ratio to 1.58±0.04. In our study, the changes of Ca/P ratio values following demineralization and Tooth Mouse treatments were more minimal, probably due to the variation in enamel microstructure between primary and permanent teeth and differences in study design.

To create an environment similar to that of the mouth, we left the enamel surface untouched, without the grinding or polishing performed in other studies.[13,25] This may explain the higher value of mineral weight percentage we obtained, compared to that measured by Ngo et al.[8] We did not find the mineral weight percentage to increase after immersion of intact enamel in tooth mousse. Unexpectedly, the mineral weight percentage after immersion of demineralized enamel in tooth mousse declined slightly. This finding had no statistical significance and could be due to normal variation.

The present study has the obvious limitations of an in vitro study, namely that application of the CPP-ACP does not exactly mimic the action of the GC Tooth Mousse in the mouth. While the study supports the existing evidence of the remineralization effect of CPP-ACP on demineralized enamel surfaces, further in situ studies are required to establish clinical evidence for its actual capability in enhancing enamel lesion remineralization in primary teeth. A 1% to 2% change in the Ca/P ratio is a small but persistent finding. Studies are needed that will assess the absolute concentration of calcium and phosphate and the creation of new HA crystals under experimental conditions.

Application of GC Tooth Mousse to primary teeth enamel in an in vitro study resulted in a minimal increase in subsurface Ca/P molar ratio compared to demineralized teeth that were not immersed in GC Tooth Mousse. It is possible that a combined F and tooth mousse treatment will yield better results. Additional in situ studies are needed to evaluate the influence of tooth mousse on teeth within the oral cavity.

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