Effect of CPP-ACP on remineralization of artificial caries-like lesion: an in situ study

Abstract: The purpose of this double-blind, randomized, crossover in situ study is to compare remineralization of preformed enamel lesions by casein phosphopeptide-stabilized amorphous calcium phosphate (CPP-ACP) and fluoride dentifrice products. During each of four 10-day experimental legs, 10 participants wore intraoral removable palatal acrylic appliances with four human enamel slabs with preformed lesions. A 0.03-mL treatment paste was dripped extraorally onto the enamel blocks once a day for 3 min. The four randomly allocated treatments were as follows: CO- Control: silica dentifrice without fluoride; MP: MI Paste; MPP: MI Paste Plus and FD: Fluoride dentifrice - 1100 ppm F as NaF). Knoop surface hardness (SH) test was performed in three stages (T0 - sound enamel, T1 - after preformed lesion, and T2 - after treatment) and the cross-sectional hardness (CSH) test was performed after treatment using a 50-gram Knoop load for 15 s. Knoop hardness number (KHN) was similar between treatments. %SHr was significantly higher in the MP, FD, and MPP when compared to CO group (Kruskal-Wallis and Mann-Whitney tests, p < 0.05). Harder enamel was found in MP (75 μm) and FD groups at 75 to 175 μm. Treatment with DF, MP, and MPP promoted an increase of 20.27%, 19.24%, and 14.71%, respectively, in Integral Hardness Change (ΔIHC) when compared to CO (p<0.05). Remineralizing agents (MP, MPP, and DF) were able to inhibit demineralization of human enamel subjected to high cariogenic challenge in situ. DF had the greatest preventive potential against the progression of carious lesions.

Keywords: Dental Enamel; Fluorides; Hardness Tests; Tooth Remineralization.

Introduction

Casein phosphopeptide-stabilized amorphous calcium phosphate (CPP-ACP) is reported to have topical anticariogenic effects owing to its ability to stabilize calcium and phosphate in an amorphous state. Reynolds et al. show that not only does CPP increase fluoride incorporation into biofilm, but it also increases the incorporation of fluoride into subsurface enamel and substantially increases remineralization of enamel subsurface lesions when compared with fluoride alone. In a recent study, bioavailable calcium levels have been significantly correlated with enhanced
remineralization of enamel subsurface lesions. The presence of saliva and biofilm is indispensable for the mechanism of action of CPP-ACP. In an acidic environment, ACP separates from CPP, thereby increasing salivary calcium and phosphate levels. CPP can stabilize the level of ACP in the saliva by preventing precipitation of calcium and phosphate, and can stabilize calcium levels. CPP-ACP localizes ACP in the dental biofilm, buffering free calcium and phosphate ion activities and helping stabilize the level of ACP in the saliva, maintaining an amorphous state of supersaturation with respect to tooth enamel, depressing demineralization and enhancing remineralization. Lower adherence of microorganisms has been observed after incorporation of CPP into the salivary pellicle, in addition to delayed oral biofilm formation and maturation.

In vitro enamel remineralization models have been widely used for predicting the anticaries efficacy of CPP-ACP, but several questions about the remineralizing effects of CPP-ACP remain unanswered. The preventive potential of CPP-ACP has been observed in in situ human models, showing significant remineralization of enamel subsurface lesions. In situ models are more realistic, but they must be very carefully designed to assess the ability of remineralizing agents so as to minimize the confounding effects of many variables involved. Thus, fluoride availability from the brushing of natural teeth can be a confounding factor. This residual fluoride present in the saliva after brushing probably reflects the slow elimination of fluoride from intraoral reservoirs, such as biofilms.

Further studies are needed to obtain more information about the remineralization process when fluoride and CPP-ACP are used in a randomized, controlled, double-blind trial. The aim of the present study is to evaluate the remineralization potential of CPP-ACP crèmes and to compare it with that of fluoride dentifrice products in artificial lesions on enamel surfaces using surface microhardness (SH) and cross-sectional hardness (CSH) as parameters in a randomized, controlled, double-blind, crossover in situ model. The null hypothesis was that remineralizing agents such as fluoride dentifrice (FD), MI Paste (MP), and MI Paste Plus (MPP) would not result in greater remineralization in artificial caries compared to a placebo (dentifrice without fluoride) in an in situ model.

Methodology

Experimental design
This study was approved by the local Human Ethical Committee (process 411.762). The study was conducted in accordance with the Declaration of Helsinki. This was a double-blind crossover in situ study performed in four phases with a length of 10 days each.

Sample preparation
Enamel blocks (from human molar teeth) were sequentially polished and selected according to the SH test results. Blocks were demineralized and subjected to post-treatment in the in situ model (Figure 1). In this study, human third molars, which had been extracted for surgical reasons, were used. The samples were free of caries, of fluorotic or hypomineralized lesions, and of any other visible defects. The teeth were stored in 0.1% thymol during the sample preparation process. A total of 160 enamel blocks were obtained from the buccal and lingual surfaces of 80 third human molars. After fixing the blocks in acrylic resin, the buccal surfaces of the enamel specimens (3 x 3 x 3 mm) were ground with SiC paper (400, 600, and 1,200 grit; Struers, Copenhagen, Denmark) to obtain flat surfaces. The specimens were then polished using a 1-µm diamond polishing suspension with a polishing cloth (Arotec Ind & Com, Cotia, Brazil). The baseline SH of all specimens was measured using a microhardness tester (Micromet 2001, Buehler, USA) with a Knoop diamond indenter under a 50-gram load for 15 s. Five indentations, spaced 50 µm apart, were made, and the mean values of enamel surface hardness were calculated (baseline SH = T0). A total of 160 specimens with a mean SH value of 326.32 ± 38.12 was chosen. The enamel blocks were distributed into four groups of 40 blocks each: MI- MI Paste (Recaldent™, GC Corporation, Tokyo, Japan); MPP - MI Paste Plus (900 ppmF; Recaldent™, GC Corporation, Tokyo, Japan); FD - Crest™ (1,100 ppmF; Colgate-Palmolive Ltda., São Bernardo do Campo, Brazil); and CO - Dentifrice without fluoride (Daudt Ltda., Rio de Janeiro, Brazil).
Enamel subsurface demineralization

Enamel blocks were covered with a protective acid-resistant nail varnish (Colorama; Brazil) applied on the sides (cut surfaces) and on the bottom of each block, except for the enamel surface. After sonication and rinsing with distilled water, the specimens were immersed separately in 10 mL of unagitated demineralizing solution [2 Mm Ca (Ca(NO$_3$)$_2$), 2 Mm PO$_4$ (KH$_2$PO$_4$), and 75 Mm of acetate at 4.3 pH]. The demineralizing solution was not replaced during the demineralization period (72 h). After demineralization, the specimens were rinsed under a steady stream of deionized water. Five indentations separated by a distance of 100 μm were made in the central region of each enamel slabs. The average of the five indentations made on each specimen was used as the SH preformed lesion value (SH = T1). After SH measurements, the prepared specimens were then stored in 100% relative humidity at 4°C until later use.

Figure 1. Schematic illustration of the experimental design.
Intraoral phase

Ten healthy adult volunteers (seven females and three males), aged 21 to 42 years, were chosen to participate in the study according to the criterion of Shen et al. The data were collected from March to December 2017 at the Dental School of Fluminense Federal University. Patients with systemic diseases, pregnant women or breastfeeding mothers, patients undergoing orthodontic intervention, and those using medication that could affect saliva quality and flow rate were excluded from the study. All subjects were given oral and written information about the aim of the study and about the test dentifrice. All volunteers gave their informed written consent. Participants lived in a city with a fluoridated water supply (0.7 mgF/l). Participants maintained their normal diet and oral hygiene procedures throughout the study. An in situ pilot study with five volunteers was performed to test the efficacy of the in situ model. SH was measured before and after in situ treatment. After that, the sample size was calculated assuming an 80% power for detecting a 20% difference between the means of the control and treatment groups at a 5% significance level. A sample size of 10 participants was required. For the intraoral phase, removable acrylic palatal appliances covering the palate from the first premolar to the last tooth in the arch were manufactured for each participant. The appliances were designed with bilateral troughs (13 mm long, 6 mm wide, 4 mm deep) cut into the base and designed to house the enamel slabs. Before that, the enamel slabs were washed with distilled water and sterilized by exposure to ethylene oxide vapor for 12 h. The enamel slabs were retained by sticky wax to produce a 1-mm-deep trough above the enamel surface, allowing plaque to accumulate and to be retained. A plastic mesh (Plásticos Gonçalves Ltda., São Paulo, Brazil) was put over the block, leaving 1-mm space for plaque accumulation. The participants were instructed not to brush the intraoral device where the samples were placed, nor to take any type of medication containing fluoride, to use only fluoride-free toothpaste (starting 1 week before the study and kept throughout the investigation period), to perform their oral hygiene as they usually did, to wear the intraoral device from 8:00 a.m. to 8:00 p.m. every day, and to remove it only at meal times (when the appliances were removed, they were stored in a sealed moist plastic bag at room temperature). A syringe containing the treatment paste (without identification) was given to the volunteers during the test period. All participants wore the device for 12 h. Every day at 2:00 p.m., the participants had to draw 0.03 mL into a syringe and apply the solution on the enamel slabs in the acrylic device, rubbing it with their finger and spreading it to allow for its penetration through the plastic mesh. After waiting for 3 min, they were instructed not to remove their appliances for at least 30 min after each treatment. While wearing the appliance, the participants were instructed not to eat or drink anything. The appliances were cleaned with distilled water only and not over the inset enamel slabs. The participants crossed over to each of the four treatments after at least a 1-week washout period. The participants were supplied with a dentifrice without fluoride. After completion of each treatment period, the enamel slabs were removed from the appliances, rinsed with deionized water, and stored in a humidified environment prior to analysis. The volunteers received intraoral devices with four demineralized enamel slabs in each group. The participants were randomly assigned to one control and three experimental procedures. The randomization was performed through a table generated by a computer program (www.randomizer.org).

Surface hardness

The sound enamel (T0), subsurface lesion (T1), and post-treatment (T2) measurements were conducted using the same static load and time. The first author performed all measurements (without identification). Five indentations with a 100-µm space between T0, T1, and T2 indentations were made with a Knoop diamond indenter under a 50-gram load for 15 s. The percentage recovery of SHr (%SH) was calculated \[\%SHr = \frac{T2 - T1}{T2} \times 100\].

Cross-sectional hardness

At the end of the SH test, the enamel specimens were embedded in acrylic resin with the outer enamel surfaces perpendicular to the surface of resin blocks,
polished as described earlier, and evaluated by cross-sectional microhardness measurements. Two rows of five indentations each were made, one at the center of the exposed dental enamel and one at 100 µm from the central row of indentations using a 50-gram load for 15 s. The indentations were made at 25, 50, 75, 100, 125, 150, 175, 200, 225, and 250 µm from the outer enamel surface. The mean values of the two measurement points were calculated at each distance from the surface. For the analysis of inhibit demineralization or remineralization patterns, hardness profiles were calculated at each depth by the hardness values for the experimental groups and for the control group. Integrated hardness (KHN x μm) for the lesion in the treatment groups was calculated by the trapezoidal rule and subtracted from the integrated hardness for the control group to obtain the integrated area of subsurface regions in enamel, referred to as integrated change of subsurface hardness (ΔIH). These values were then converted to % change [%ΔIH = (IH_ treatment - IH_ control)/IH_ treatment *100].

**Statistical analysis**

The data and graph were prepared using GraphPad Prism, version 8.0 (GraphPad Software, Inc, San Diego, USA). Initially, all the data were checked by the Shapiro-Wilk test. Based on these preliminary analyses, the data (KHN and %SHr) were subjected to the Kruskal-Wallis and Mann-Whitney tests. KHN before and after treatment in the same groups was analyzed using Wilcoxon’s matched-pairs signed-rank test. Multiple comparisons using the Holm-Sidak test were used to evaluate CSH. All analyses were performed at a significance level of α = 0.05.

**Results**

All participants completed the study with 100% compliance. The participants reported no adverse events. Demineralized lesions (all groups) had similar initial SH prior to exposure to the oral environment. After formation of the preformed lesion (T1), the median KHN was lower and significantly different as compared to T0 in all groups (Figure 2; Wilcoxon’s paired t-test; p < 0.05). After treatment (T2), the median hardness was high in all groups except for CO (p < 0.05). SH of experimental groups was significantly higher in MPP (median=175.5; 95%CI = 142–211.1), MP (median = 175.4; 95%CI = 154–195.2) and DF (median = 168.9; 95%CI = 106.4–190.1) groups when compared to CO (median = 127.4; 95%CI = 104–222.1), respectively (Figure 2). Figure 3 shows that MP, MPP, and FD groups had a gain of %SHr after the in situ phase; however, CO had loss of %SHr (Kruskal-Wallis and Mann-Whitney tests; p>0.05). Results showed statistical significance for the group and distance factors and for the interaction between group and distance, indicating that the effect of the treatments was different, depending on the depth of the enamel surface. There were significant differences (p < 0.05) in lesion depth changes (CSH). When the effect of the treatments was compared with the negative control group (CO) at each distance from the surface, the treatments with MP (p < 0.02) and FD (p < 0.01) were more effective in reducing enamel demineralization at the 75 and 75–175 µm depths, respectively, when compared with CO. The MPP group did not significantly reduce demineralization compared with the CO group (p > 0.05) and was not different from the FD and MP groups (p > 0.05). KHN vs. depth from the surface for all groups is shown in Figure 4. Treatment with DF, MP, and MPP reduced the lesion by 20.27%, 19.24%, and 14.71% when compared to CO (Table).

**Discussion**

In the present study, demineralized enamel specimens were attached to removable acrylic palatal appliances to investigate the remineralization potential of CPP-ACP/CPP-ACFP-based crèmes in an in situ model. Human enamel blocks with preformed lesions were used to assess remineralization or the inhibition of demineralization. In situ caries models have a vital role in the elucidation of the caries process and in the screening of products containing remineralizing agents. In the current study, MP, FD, and MPP were better at reducing demineralization (caries progression) when compared to the fluoride-free dentifrice (placebo control), thus validating the in situ model used. Therefore, the null hypothesis was rejected. In the in situ model, enamel
blocks with preformed lesions were pulled back 1 mm from the border of the acrylic appliance and covered with a plastic mesh, allowing for biofilm accumulation and demineralization. Our findings indicate that 10 days of study were enough to curb demineralization, but not enough to allow for total remineralization in experimental groups. Other study has demonstrated remineralization at the same time of exposure and with other method of evaluation. The Knoop hardness method is effective, sensitive, and able to detect mineral losses and gains in both enamel and dentin. In the present study, surface and cross-sectional hardness tests were used to assess changes in demineralized enamel both on its surface and at depth.

In the present study, the application of MP, MMP, and FD significantly prevented demineralization. Therefore, while saliva played a role in remineralization, hardness did not increase in preformed lesions when placebo dentifrice was used. The findings are in line with those of some other studies. However, in some studies, remineralization of the lesion treated with saliva increased with the length of treatment. This suggests that a larger length of study could increase remineralization of the demineralized enamel when exposed to saliva. In the experimental...
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groups, MPP was the remineralizing agent with the lowest surface hardness gain. This finding is at odds with that of other studies. According to Shen et al., larger levels of inorganic phosphate and calcium and, consequently, larger remineralization were found in products containing CPP-ACP when compared to similar products. Shen et al. also observed higher concentrations of salivary calcium, inorganic phosphate, and fluoride ions when tooth mousse (TM) and tooth mousse + 900 ppm F (TMP)

Figure 4. KHN (median and coefficient of variation) at different depths. (*) denotes statistically significant difference between the experimental and control groups (p < 0.05).

Table. Integrated hardness (KHN x μm) and integrated change of subsurface hardness (ΔIHC).

| Groups | ΔIH (KHN x μm) | % ΔIHC (KHN x μm) |
|--------|----------------|-------------------|
| CO     | 1959 (1731 to 2188) | -                  |
| MP     | 24 (2213 to 2639)   | 19.24             |
| MPP    | 2297 (2041 to 2552) | 14.71             |
| FD     | 2457 (2229 to 2686) | 20.27             |

*H (KHN x μm) - Integrated of subsurface hardness (total area-95%CI); **% ΔIHC (KHN x μm) represents the % change in ΔIH values. Lowercase letters denote statistical difference (p < 0.05).
were used, significantly increasing the salivary levels of calcium and inorganic phosphate. TMP had higher saturation than fluoroapatite (FA) because of higher salivary levels of calcium and of inorganic phosphate, as well as of fluoride. The release of calcium and phosphate ions by CPP depends on pH, with an increase in the release of the ions as pH decreases.\textsuperscript{15} In the presence of fluoride ions, however, CPP releases larger amounts of calcium and phosphate ions at all pH levels\textsuperscript{22-24}. Hence, the presence of calcium, phosphate, and fluorine in the biofilm was expected to improve the efficacy of MPP as compared to MP, but according to SH analysis, this association – fluoride and CPP-ACP – was not found in the present study. These findings had already been reported in \textit{in vitro} studies.\textsuperscript{10,11,13} The lower concentration of fluoride\textsuperscript{22-24} or the lower concentration of available calcium could be the cause for the poor efficiency of ACP-CCPF in remineralization described in \textit{in vitro} studies.\textsuperscript{11} We then speculate that the presence of phosphate, which may either compete with fluoride for calcium bonds or precipitate, might have influenced these results. Other studies have shown that another system containing calcium and phosphate and with higher fluoride levels has been more effective in remineralization.\textsuperscript{14,23,24} The effect of fluoride ions on remineralization depends on the availability of calcium and phosphate and, thus, products with higher fluoride availability could promote higher remineralization. In the current study, to minimize methodological differences, all products were applied for 3 min once a day, following the MP and MPP manufacturer’s instructions. Earlier, we had observed that the higher frequency of application could improve the efficacy of CPP-ACP products.\textsuperscript{9,10} In addition, MP and FD were statistically different from the control when five 1-minute applications were compared to three 1-minute applications\textsuperscript{8} or to an experimental protocol with a longer duration.\textsuperscript{22} This suggests that the increase in frequency of application\textsuperscript{8} or duration\textsuperscript{15,22} of treatment could prove more advantageous for remineralization.

In our study, all remineralizing agents showed remineralization after treatment, but remineralization was less prominent in the outer enamel layer, regardless of the group. The specimens with preformed lesions were subjected to large cariogenic challenges. Note that the volunteers did not make use of fluoridated dentifrice and did not brush the specimens, which could have contributed to higher remineralization. The presence of fluoride from other sources, such as toothpaste used by the volunteer for toothbrushing, could have had a remineralizing effect and been a confounding factor.\textsuperscript{16,27,28} The volunteers were living in a town supplied with fluoridated water and the release of fluorine by the saliva could have also contributed to the outcomes obtained. However, CSH, FD, and MP were able to inhibit demineralization and/or promote remineralization at a depth of 175 μm and 75 μm, respectively. MP and MPP showed similar results in both analyses. Reynolds\textsuperscript{1} utilized microradiography to assess remineralization and found that CPP-ACP is better than a dentifrice with higher fluoride levels at promoting remineralization of subsurface lesions. These findings differ from those obtained in the present study.

In this study model, we observed that preformed lesions should be treated with a remineralizing agent to inhibit their progression. The control group (no treatment) could not inhibit deep demineralization after 10 days. This is the most important finding of this study. After the \textit{in situ} phase, there was mild remineralization in SH in the control group, but the values were lower than those observed in the experimental groups (progression) at all depths. Shen et al.\textsuperscript{4} reported better remineralization of enamel subsurface lesion \textit{in situ} after Tooth Mousse Plus (TMP) and Tooth Mousse (TM) application than after the use of the fluoride products. According to the authors, the product provided high concentrations of stabilized and bioavailable calcium, phosphate, and fluoride ions in the saliva, promoting remineralization in the body of the lesion. Our results indicate that all products were effective in decreasing demineralization during \textit{in situ} treatment, but FD was more effective than when no treatment was performed. Although fluoride was present in both fluoride products, the results suggest that an amount of fluoride might be inactive in MPP.\textsuperscript{13} The amount of fluoride release would depend on the differences in solubility and release of fluoride according to the fluoride products.\textsuperscript{23} For products containing CPP-ACP agents, longer periods may be necessary for a more uniform remineralization challenge.
In the present study, a cross-sectional design and oral hygiene with fluoride-free toothpaste were used to reduce variability, especially regarding individuals and the fluoride available in the oral cavity. In brief, this study took extra care to minimize variability among specimens, and the obtained results are important for understanding the mechanism of action of CPP-ACP. The outcome measures for MI Paste Plus compared to those of the MI Paste group indicate that fluoride application does enhance remineralization, but from a clinical point of view, it was of interest to find that this association, despite the limitations and short duration of this study, was not superior to fluoride toothpaste alone. Remineralizing agents (MP, MPP, and DF) were able to inhibit demineralization of human enamel subjected to high cariogenic challenge in situ. DF had the greatest potential against the progression of carious lesions.

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