Evaluation of CPP-ACP and Fluoride on Inhibition of Human Enamel demineralisation: Cross-sectional Hardness and MicroCT Studies

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Purpose: To evaluate the in vitro effect of different application frequencies of dentifrices containing CPP-ACP and fluoride on enamel demineralisation inhibition using a pH cycling model.

Materials and Methods: One hundred twenty blocks of human enamel were divided into 8 groups according to the treatment and number of times that the dentifrice slurry was applied (3 or 5 times). Control: dentifrice without fluoride (CO3 and CO5); fluoride dentifrice (FD3 and FD5, commercial dentifrice 1100 ppm as NaF); MI Paste (MP3 and MP5, Recaldent) and MI Paste Plus (MPP3 and MPP5, Recaldent 900 ppm as NaF). After pH cycling, cross-sectional microhardness (CSH) measurements were taken. The demineralised enamel changes were analyzed on three blocks per group by MicroCT. Data were analyzed by ANOVA and Tukey's HSD post-hoc test ($p = 0.05$).

Results: Statistically significantly higher CSH values ($p < 0.05$) were obtained for the surface layers (25 and 50 µm) for FD3, FD5, MP3 and MP5. The MP and FD groups showed similar results and had the least mineral loss. The MP increased the mineral density in enamel and decreased the depth of the lesion.

Conclusion: Inhibition of subsurface enamel demineralisation is possible with MP and FD, independent of the application frequencies. However, the MP and MPP groups had higher mineral density when five applications were performed.

Key words: casein, dentifrice, fluoride, prevention

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Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) complexes are anticariogenic and capable of remineralising the early stages of enamel lesions.[1,3,4,17,20,21,22] The use of fluoride is an effective measure in the prevention of dental decay, and the association of CPP-ACP and fluoride could increase remineralisation.[7,11,17] However, some authors suggest that CPP-ACP can be used as an effective adjunct to fluoride therapy but cannot be used as an alternative to fluoride.[24] Therefore, research using CPP-ACP and CPP-ACPF pastes with different approaches as preventive treatment is still needed. The mechanisms of demineralisation inhibition by CPP-ACP also need to be evaluated.

In a previous study, we concluded that the use of regular fluoride dentifrice or agents based on calcium and phosphate compounds could be useful to control the progression of carious lesions.[22] The results of that initial study showed that the highest frequency of CPP-ACP application (five times) was more effective in preventing demineralisation than was three times. The surface hardness test (SH) used in other studies was able to detecting early stages of enamel surface demineralisation, but this method cannot measure lesion depth. Thus, two methods were used in the present study to evaluate the depth of enamel changes: cross-sectional hardness (CSH) and micro-computed tomography (microCT). CSH can be used to measure lesion depth as a substitute for Transversal Micro Radiography (TMR) in

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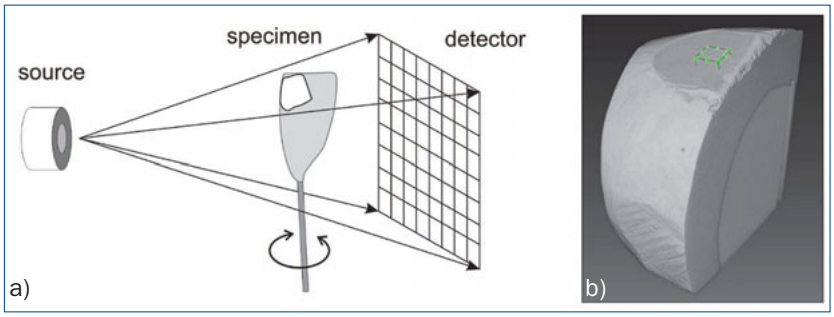

Fig 1 a) Setup of micro-CT scanner; b) volume reconstructed.

enamel studies. The technique is well established for enamel and can be used on enamel block lesions.[2,12,10] MicroCT can evaluate mineralised tissues non-destructively in three dimensions, which makes it a good choice to measure mineral changes in hard tissues.[8,15] Quantitative assessment using polychromatic microCT was useful for detecting mineral density changes.[7]

The null hypothesis was that the remineralisation treatments with fluoride dentifrice, CPP-ACP and CPP-ACP+F topical paste did not result in a greater reduction in enamel demineralisation compared to a placebo (dentifrice without fluoride) when applied at different frequencies.

MATERIALS AND METHODS

Sample Preparation

Thirty-five human third molars, which had been extracted for surgical reasons, were used. This study was approved by the Ethics Committee of Medical Science, Fluminense Federal University (Protocol n. 393000/13). The teeth were free of caries and fluorotic or hypomineralised lesions and any other visible defects. The teeth were stored in thymol 0.1% during preparation. Four enamel blocks were obtained from each tooth. After embedding the blocks in acrylic resin (Classico Produtos Odontológicos; São Paulo, SP, Brazil), the buccal surfaces of the enamel specimens (2 mm x 2 mm x 2 mm) were ground with SiC paper (400-, 600-, 1200- and 2500-grit; Arotec; Cotia, SP, Brazil) in order to obtain flat surfaces. The specimens were then polished using a 1-µm diamond polishing suspension with a polishing cloth. The baseline surface microhardness (SH) of all specimens was measured using a microhardness tester (Micromet 5104, Buehler; Lake Bluff, IL, USA) with a Knoop diamond indenter under a 25-g load for 10 s. An average of five indentations, spaced 100 µm from each other, made on each specimen was used for the SH baseline value. After SH measurements, 120 enamel blocks were selected, with a Knoop hardness number (KHN) ranging from 297.32 to 388.78. The enamel blocks were randomly divided into 8 groups of 15 blocks each according to the dentifrice used and the number of times the dentifrice slurries were applied (3 or 5 times), as follows: control: silica-based dentifrice (Daudt; Rio de Janeiro, RJ, Brazil); silica-based fluoride dentifrice (Colgate-Palmolive; São Bernardo do Campo, SP, Brazil) FD3 and FD5; MI Paste (GC; Tokyo, Japan) MP3 and MP5; MI Paste Plus (GC) MPP3 and MPP5.

pH Cycling and Treatment with Dentifrices

After sonication and rinsing with distilled water, the specimens were immersed in 20 ml of the demineralising solution, consisting of 2 mM Ca [$Ca(NO_3)_2$], 2 mM PO_4 [KH_2PO_4], 75 mM acetic acid – at pH 4.8 for 6 h/day (6:00–7:00, 9:00–10:00, 12:00–13:00, 16:00–17:00, 20:00-21:00, 22:00-23:00). After each demineralisation challenge, the enamel specimens were rinsed in distilled water for 1 min, and then placed in 20 ml of the remineralising solution (1.5 mM Ca, 0.9 mM PO_4, 130–150 mM KCL, 100 mM cacodylate buffer, pH 7.0) for 18 h/day at 37°C. This solution approximates the mineral ion composition and supersaturation of saliva as originally reported by Ten Cate and Duijsters.[21] The dentifrice treatments were carried out after the first, third and fifth demineralisation cycles for the groups with three applications and after all cycles, except the last one, for the groups with five applications. The products were applied as a 1:3 slurry of toothpaste and deionized water, and a standardized volume (0.15 ml) was applied on each sample for 60 s. The solutions were changed after each cycle. After 5 days of pH cycling, the enamel specimens were rinsed in distilled water for 1 min, then immersed in 20 ml of remineralisation solution for 7 h, after which the post-treatment CSH measurements were performed.

Analysis of Enamel Hardness

The post-treatment CSH measurements were conducted with the same static load and time applied for the SH. The enamel disks were longitudinally sectioned through their center and embedded in acrylic resin with the cut face exposed and polished. Three rows of five indentations at 25, 50, 75, 100 and 125 µm were made. The values of the three measurements at each distance from the surface were averaged. To analyze the patterns of remineralisation, differential hardness profiles were calculated by subtracting the hardness values of the experimental groups (EG) from those of the control groups (EC) at each depth (ΔKHN = EG-EC). The area under the curve was calculated by GraphPad prism 6.0 (Graph Pad Software; La Jolla, CA, USA). The Knoop microhardness number was converted to vol% mineral following the methods described by Featherstone et al.[6]

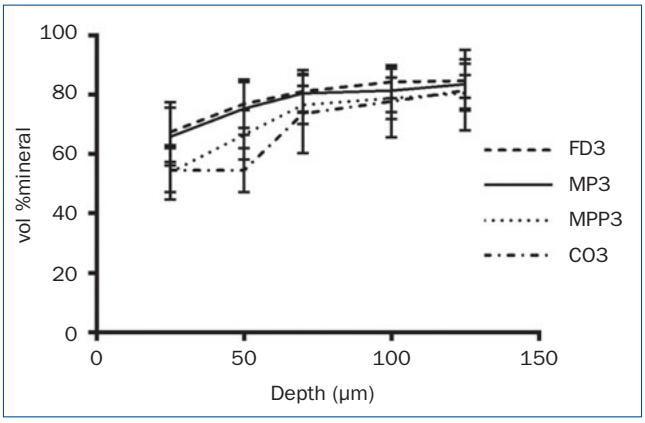

Fig 2 Mineral profile in the groups FD3, MP3, MPP3 and CO3.

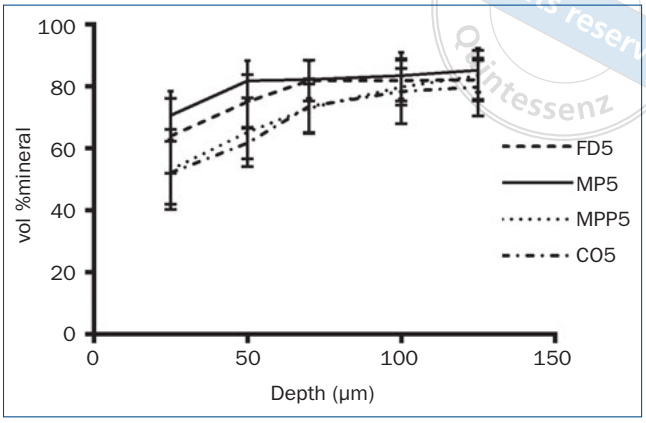

Fig 3 Mineral profile in the groups FD5, MP5, MPP5 and CO5.

Micro x-ray Computer Tomography (microCT)

After pH cycling, three enamel blocks from each group that had a mean CSH similar to the means of their respective groups were scanned by microCT as described below. Tomography scans were carried out using a high resolution microCT system (ZEISS Xradia 510 Versa, Carl Zeiss; Jena, Germany), which generates x-rays with cone-beam geometry. The distance from x-ray source to the sample was 30 mm, and that from x-ray source to the detector was 265 mm. A resulting dataset of 7.8 µm voxel size resolution was obtained. The LE #4 (low-energy) filter was installed in the beam path both to obtain optimized tomography and correct for beam hardening and ring artifacts. The source voltage was 60 kV at a current of ca 83 mA, with an exposure time of 6 s for each radiograph. The specimen was rotated at angular increments of 0.225 degrees, through 360 degrees.

Each projection was captured on a 1024 x 1024 16-bit detector array. The collected data comprised 1024^3 (1 billion) data points (voxels). The sample was mounted so that the x-ray beam was perpendicular to the treated enamel surface. In order to prevent the specimen from drying during scanning and to simplify sample mounting, the enamel-dentin blocks were embedded in acrylic resin. All specimens were scanned using the same setup and parameters.

A tomogram is the repetitive sectioning of a specimen with radiography; it captures the specimen from a large number of different angles and using mathematical algorithms to reconstruct it. The x-ray source and detector are stable while the specimen is rotating around its center (Fig 1a). From the set of acquired radiograms, the cross-sectional images of the specimen can be computed using tomographic reconstruction (Fig 1b). In x-ray micro computer-tomography, the dimensions of the reconstructed voxels (i.e. a volumetric pixel element) are in micrometers.

Following x-ray acquisition, the CT datasets were reconstructed to produce a stack of 2-D raw grey scale images (slices) of the specimen, forming the 3-D digital object.

The most basic method to explore the configuration of material (phases) is to transverse the data volume serially along user-defined two-dimensional cross sections using computer visualization. Although this is a simple method, it disregards the three-dimensional phase connectivity and fails when viewing large, complex sets. Hence, the data are explored using direct volume rendering. This method allows the study of details in the compositional variation in terms of connectivity, distribution and relative densities; the thickness of the demineralised region can be also be measured.

From the raw data sets, smaller volumes of interest consisting of 65 x 68 x 65 voxels were extracted. Three-D image analysis software (AVIZO, Thermo Fisher Scientific; Waltham, MA, USA) was used for visualization. A noise-reducing median filter was applied to the data after volume reconstruction.

Statistical Analysis

Data were analysed using Statgraphics Centurion XVI software (Statpoint Technologies; Warrenton, VA, USA). Initially, all data were checked using the Shapiro-Wilk and Levene tests. Based on these preliminary analyses, the CSH data were submitted to one-way ANOVA and Tukey's HSD post-hoc test. The ΔKHN and vol% mineral data were analyzed using Kruskal-Wallis and Mann-Whitney tests. All analyses were performed at a significance level of $\alpha = 0.05$.

RESULTS

Cross-sectional Hardness

The results differed statistically significantly for the group and distance factors as well as the interaction between group and distance, which indicates that the effect of the treatments differed depending on the depth of the enamel surface. When the effect of the treatments was compared with the negative control group (CO) at each distance from the surface, the treatments with MP ($p < 0.01$) and FD ($p < 0.05$) were more effective in reducing enamel demineralisation at the 25- and 50-µm depths when compared

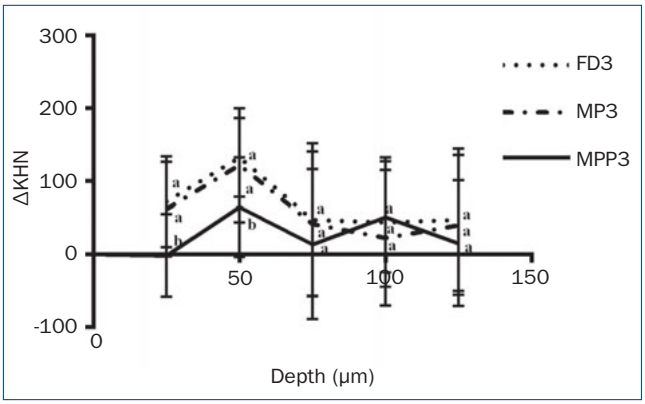

Fig 4 Differential hardness (hardness vs depth) calculated by subtracting experimental group (FD3, MP3 and MPP3) profiles from placebo (CO3). Values in the same column followed by different lower-case letters indicate statically significant differences ($p < 0.05$).

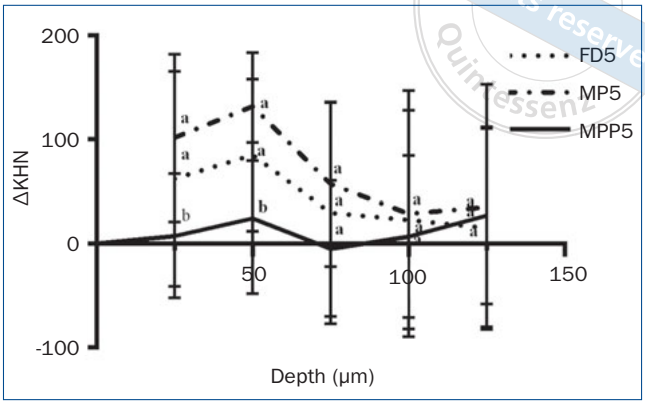

Fig 5 Differential hardness (hardness vs depth) calculated by subtracting experimental group (FD5, MP5 and MPP5) profiles from placebo (CO5). Values in the same column followed by different lower-case letters indicate statically significant differences ($p < 0.05$).

with the control group. MPP3 and MPP5 did not significantly reduce demineralisation compared with CO groups ($p > 0.05$), but were different from the FD and MP groups ($p < 0.05$). The vol% mineral vs depth from the surface for all groups is shown in Figs 2 and 3. Vol% mineral was statistically significant in FD and MP when compared to MPP and CO (up to 50 μm). In addition, only MP5 and FD5 showed a difference up to 75 μm when compared to CO5. None of the groups differed significantly at the other distances from the surface (Figs 2 and 3, $p > 0.05$).

Differential hardness profiles (ΔKHN = EG-EC) provided evidence of different subsurface lesion patterns among the FD, MP and MPP groups. Inhibition of the demineralisation process was more pronounced at 25 and 50 μm (Figs 4 and 5) than at other distances in the MP and FD groups ($p < 0.05$). The frequency of application did not influence the results ($p > 0.05$).

The area under the curve for MP3 was 5918, for FD3 7071, MPP3 3385, MP5 7149, FD5 4406, and for MPP5 it was 1129. FD3, FD5, MP3 and MP5 all showed one peak at 50 μm (Fig 2). A second peak was found in MPP5 at 125 μm (Fig 5).

MicroCT Analysis

A grey scale was assigned, with the densest structures appearing white (sound enamel) and the least dense appearing black (lesion).[23] Representative microCT cross-section images of the surface lesions post-product use showed inhibited demineralisation when compared to control (Fig 6).

The surface lesion was smaller and denser on the specimens treated with MP5 than on the other groups. In sound enamel, the mineral density and hence grey level should be homogeneous. In Fig 6, the lesion is the slightly darker surface region of the image (arrow). The mineral density in the lesions increased with 5 applications in the MP and MPP groups. The lesion depth formed was around 50 μm and was similar to the results found by CSH.

DISCUSSION

The purpose of the sound enamel blocks was to assess the ability of products or conditions in vitro to enhance or inhibit demineralisation rather than remineralisation. Under these conditions, the specimens demonstrated a statistically significant difference in CSH after treatment and lesion formation. The enamel demineralisation process is related to the pH value and the ionic content of calcium, phosphate and fluoride, which determines the degree of tooth mineral saturation.[25] In the cyclic model used here, the demineralisation and remineralisation solutions were changed after each cycle, so that the concentration of calcium and phosphate ions in the solutions would not affect the results.[22] The subsaturation condition can lead to the dissolution of hydroxyapatite and the diffusion of calcium and phosphate ions towards the enamel surface, reducing the CSH after pH cycling. With respect to CSH, at the distance of 70 μm from surface, there was a change in the hardness.

The present study showed that MP and FD (1100 ppm F^-) can reduce mineral loss in an in vitro caries model. In previous research by our group, we observed a change in SMH related to different application frequencies of CPP-ACP dentifrice, in which 5 applications of MP were more effective in improving surface hardness; the subsurface effects of fluoride and CPP-ACP were not available.[22] Continuing to build on this research, we believed that the cross-sectional microhardness (CSH) and MicroCT could give more information about mineral distribution. Microhardness has been used as a direct measure of changes in mineral content in studies on demineralisation/remineralisation.[1,9,10,17,22] CSH measurements at depths between 25 and 125 μm below the enamel surface provided quantitative insights into the extent of de- and remineralisation. Accordingly, MP and FD reduced the dental demineralisation using this model; therefore, the null hypothesis was rejected. MP5 and FD5 were the only treatments able to reduce the sub-

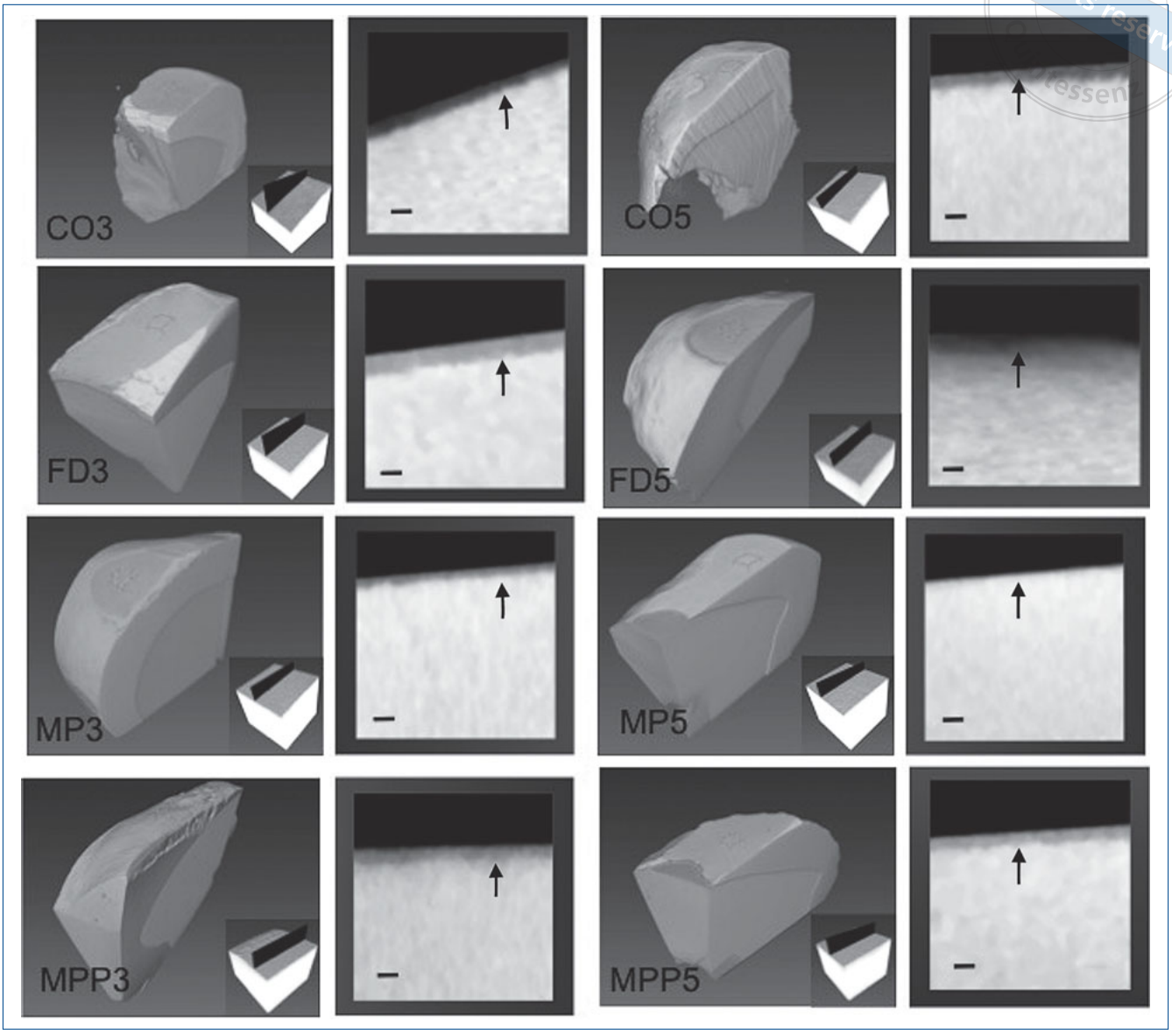

Fig 6 MicroCT cross-sectional images. CO3: control 3x; CO5: control 5x; FD3: fluoride dentifrice 3x; FD5: fluoride dentifrice 5x; MP3: MI Paste 3x; MP5: MI Paste 5x; MPP3: MI Paste Plus 3x; MI paste 5x. Demineralized area is indicated by the arrow (bar: 50 µm).

surface enamel demineralisation compared with CO at 75 µm. There was no difference between 3 and 5 applications; however, the inhibition of demineralisation at this depth was improved with 5 applications (Fig 5). Both products resulted in statistically significant improvements in vol% mineral compared to the control (Fig 3).

The MPP in this model produced only a minor inhibitory effect on lesion formation in enamel, which was not statistically significantly different from the control (CO). Thus, the MPP was not able to reduce the dental demineralisation using this model; therefore, the null hypothesis was accepted. It was an interesting result, since the MPP with 900 ppm/F^- should be more effective than MP, perhaps indicating that during the pH cycling, the F^- in MPP is not fully available to inhibit demineralisation and/or enhance remineralisation.[5,22] On the other hand, previous studies have shown that CPP-ACPF provides superior remineralisation effects[9,16,17] and greater resistance to acid softening compared to artificial saliva[5] and distilled water.[7] Oliveira et al[16] also reported that MI Paste Plus under laboratory conditions reduced carious lesions in demineralised enamel in pH cycling.[16] The formula of the artificial saliva used in these studies contains different concentrations of calcium and phosphorous ions than the remineralisation solution used in the present study; thus, this could explain the difference found in this study regarding the ability for remineralisation by the CPP-ACPF group.[16] Hamba et al[7] also demonstrated a protective effect against demineralisation when

CPP-ACPF paste was applied before a demineralisation solution. The application of CPP-ACPF on the enamel was performed for 30 min daily for 7 days. At other times during the treatment period, the specimens were stored in deionized water without any acid challenge during that time.

Although several studies have shown that casein phosphopeptide–amorphous calcium phosphate can remineralise subsurface enamel lesions,[11,14,21] it performs similar to or even worse than saliva for mean fluorescence loss and for lesion area.[15]

The present study also showed that MPP was less effective in reducing mineral loss than fluoride dentifrice (FD), as shown by the ΔKHN data from cross-section analysis. Similarly, MI Paste Plus did not demonstrate a remineralisation effect compared to the other NaF system,[16,17] given the relatively low level of available fluoride.[9] Hamba et al[7] observed that enamel treatment with CPP-ACPF and CPP-ACP showed a similar inhibitory effect on enamel demineralisation, however, the 900 and 9000 ppm F solutions showed shallower lesions and less mineral loss after 120 h. In addition, the results of our study disagree with a previous in situ study conducted by Shen et al,[21] where CPP-ACPF induced statistically significantly more remineralisation compared to 0.1% fluoride dentifrice. Moreover, the participants in the Shen et al[21] study received fluoride from their regular oral hygiene and this may have influence the results of their study. Furthermore, according to those authors, the products contained high levels of calcium and phosphate ions that together with the fluoride ion in Tooth Mousse Plus were released into saliva to substantially increase the salivary concentrations of calcium, inorganic phosphate and fluoride.[21] The mixing of calcium ions with phosphate ions to produce an ion activity product for amorphous calcium phosphate that exceeds its solubility product results in the immediate precipitation of ACP or, in the presence of fluoride ions, amorphous calcium fluoride phosphate (ACFP). In the intraoral environment, these phases (ACP and ACFP) are potentially very unstable and may rapidly transform into a more thermodynamically stable, crystalline phase (i.e. hydroxyapatite and fluorhydroxyapatite). However, before phase transformation, calcium and phosphate ions should be transiently bioavailable to promote enamel subsurface lesion remineralisation.[4]

The most characteristic microCT difference between EG and CO was the density and width of the outer surface. EG produced a more homogeneous, higher density when compared with CO. The images were obtained from 3 samples of each group, which were selected based on means being similar to that of their respective group; therefore, it is representative of the final value obtained in the CSH. In other studies using microCT, the quantification of MD gradients was performed on demineralised enamel using pure hydroxyapatite phantoms.[7,8] It can reliably quantify MD of natural lesions and is suitable for quantitative demineralisation and remineralisation.[8] In the present study, the mineral profile was constructed using the formula proposed by Featherstone et al[6] (Figs 2 and 3).

Although the FD groups showed lower density, the depth appears to be greater than that of CO. The MP groups in the area of the lesion show a slight alteration of grey color when compared to the FD and CO groups (Fig 6). Overall, these results agree with those of Reynolds[18] and Shen et al.[21] According to Shen et al,[21] CPP-ACP increased remineralisation throughout the body of the lesion, whereas the fluoride-alone products tended to predominantly remineralise the surface layer. From the size and electroneutrality of the nanocomplexes, one would expect these to enter the pores of an enamel subsurface lesion and diffuse down concentration gradients into the body of the subsurface lesion.[18] In the oral environment, the CPP-ACPF product increased the concentration of calcium, phosphate and fluoride ions in saliva, which prevents spontaneous precipitation and allows the ions to penetrate deeply into subsurface lesions.[20,21]

In this study, the MP5 group showed the shallowest lesions, being different from all the other treatment groups (Fig 3). In contrast, Pulido et al[17] observed that MI Paste did not show any effect on reducing the progression of the lesion. These results might be different due to shorter treatment applications (2 min) and the in vitro model used. Hamba et al[7] also found no difference in the degree of demineralisation when enamel was treatment with CPP-ACP and CPP-ACPF. The 900 and 9000 ppm F^- groups showed significantly different lesion depths when compared with 90 ppm F^-, CPP-ACP, CPP-ACPF and deionized water. On the other hand, Hamba et al[7] assessed the resistance to demineralisation of the surface of bovine enamel treated with CPP-ACP, CPP-ACPF and NaF in deionized water. It differs from human enamel primarily in that it is more porous and forms lesions more rapidly.[13] Another difference was that in pH cycling, the demineralisation period shorter in the present study than in the Hamba et al[7] study. Thus, as mentioned above, these differences can influence the mineral loss and gain from enamel or inhibit demineralisation. pH cycling is the most advanced test method for assessing de- and remineralisation, but neither the microbiological induction of caries nor important specific actions of saliva can be adequately simulated in vitro.[23] As shown, the amount of remineralisation or inhibition of demineralisation potentiated by CPP-ACPF varies significantly depending upon the protocol. There is also considerable variation among studies and methods; this lack of standardisation makes comparisons difficult.

MicroCT was used to show the degree of demineralisation between the groups; however, further studies are needed to determine the composition of the minerals and depth of demineralisation in the lesions after application CPP-ACP crèmes and fluoride.

CONCLUSION

The results obtained from this study showed inhibition effects of MI Paste and FD on subsurface enamel lesions. The highest frequency of application (5 times) was not more effective in preventing demineralisation at depth; however, the MP and MPP groups had higher mineral density when 5 applications were performed.

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