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In Vitro Efficacy of Tricalcium Phosphate and Casein Phosphopeptide Amorphous Calcium Phosphate Fluoride for Remineralization of Enamel White Spot Lesions

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A B S T R A C T

Objectives: The main purpose of this study was to compare the remineralizing effects of casein phosphopeptide amorphous calcium phosphate fluoride (CPP-ACPF) and tricalcium phosphate (TCP) on artificially induced enamel white spot lesions (WSLs).

Materials and Methods: In this in vitro study, 45 sound extracted premolars were immersed in a demineralizing solution (pH=4.5) for 96 hours, and were randomly divided into 3 groups of TCP, MI Paste Plus, and control. They were exposed to the remineralizing agents for 5 minutes once a day for 30 days. After mounting the teeth in resin blocks and polishing, they underwent a microhardness test at 3 different depths from the enamel surface. Data were analyzed by Prism software, two-way ANOVA, and Tukey's test (α=0.05).

Results: The volume percentage of mineral content (VPM) was significantly different among the three groups at 30-, 60- and 90µm depths ($P<0.0001$). At 30µm depth, CPP-ACPF was significantly more effective than TCP ($P<0.0001$). At 60- and 90µm depths, there was no significant difference between CPP-ACPF and TCP ($P>0.05$).

Conclusion: Both CPP-ACPF and TCP had significant efficacy for remineralization of artificially induced enamel WSLs under in vitro conditions.

Keywords: Casein Phosphopeptide-Amorphous Calcium Phosphate Nanocomplex; Tricalcium Phosphate; Tooth Remineralization

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INTRODUCTION

The association of good oral hygiene and low rate of caries prevalence has been well documented [1]. Difficult oral hygiene practice in presence of orthodontic appliances can increase the rate of dental caries and periodontal problems in orthodontic patients due to the ecological environment created by orthodontic appliances, enhancing the proliferation and activity of cariogenic microorganisms such as *Streptococcus mutans* [2,3].

The outermost enamel layer is the most resistant to dissolution. This hypermineralized superficial layer is formed by two mechanisms. The first mechanism involves the deposition of fluoride and other ions available in the saliva, and the second mechanism is deposition of minerals by outward diffusion from subsurface lesions [4]. Normal remineralization occurs at near-neutral pH values by normal buffering capacity. Saliva can compensate early demineralization by deposition of calcium, phosphate, and fluoride, and the activity of buffering agents [5]. One suggested measure to enhance tooth remineralization is to increase the concentration of ions at the demineralized site. Topical fluoride is the most widely used material for this purpose, which has been proven to be effective in caries prevention [6]. The clinical efficacy of fluoride varies according to its application method and the compounds used in combination with it [7]. Although the remineralizing effect of fluoride has been well documented, it only affects the enamel superficial layers. Thus, attempts are ongoing to find synergistic and more effective compounds for this purpose [8]. These efforts led to the introduction of new materials containing calcium and phosphate ions [9]. Casein phosphopeptide amorphous calcium phosphate (CPP-ACP) and tricalcium phosphate (TCP) are among such materials [10]. Calcium-phosphate compounds exhibit remineralizing effects and have a synergistic effect with fluoride [11]. The polymorphic form of TCP $[Ca_3(PO4)_2]$ is the only form of calcium phosphate capable of necessary hydrolysis for the formation of calcium hydroxyapatite (HA) [12]. The hydrolysis process and formation of HA are accelerated in the presence of sodium fluoride. The HA formed by this method has a higher affinity for fluoride uptake than the conventional HA [12]. TCP with a 1:5 ratio of calcium/phosphate is available in both alpha and beta forms (beta form is less soluble), and is known as a remineralizing agent that acts by elevating the salivary concentration of calcium [13]. Also, the active type of TCP has been produced to enhance fluoride uptake by the enamel [13]. In fact, in this material, calcium phosphate does not interfere with the action of fluoride; instead, it enhances its effect [14]. Another advantage of this material is its optimal stability in humid environments [15].

Tooth Mousse (MI Paste) and Tooth Mousse Plus (MI Paste Plus) contain CPP-ACP and have been suggested for prevention or reversal of white spot lesions (WSLs) [16]. CPP is a protein derived from milk, which stabilizes calcium and phosphate ions in the amorphous form (ACP) by binding to them. This compound increases the salivary level of calcium and phosphate and results in remineralization [17]. Since the effect of these two substances at different depths has not been sufficiently investigated, and also controversy exists on the effect of these substances at different depths, the aim of this study was to assess the efficacy of CPP-ACP fluoride (CPP-ACPF) and TCP for remineralization of WSLs at different depths.

MATERIALS AND METHODS

This in vitro study was ethically approved by the ethics committee of the university (IR.SSU.REC. 1397.005). The sample size was calculated using the following formula:

$$(n=\frac{(z-\frac{\alpha}{2})+(z-\beta\ )2s^2}{d^2}) \text{ and } N=n\sqrt{1-k}$$

Where $\alpha = 0.05$; $\beta = 0.10$; $d = 2$; $z-\frac{\alpha}{2} = 1.96$; $z-\beta = 1.23$; s = standard deviation = 1.76; n=sample size for two groups; N= sample size for three groups; and K= number of groups.

A total of 45 sound premolars extracted for orthodontic reasons were collected and stored in 0.1% thymol solution. Subsequently, the teeth were cleaned with fluoride-free pumice powder and a rubber cup. Finally, the debris was rinsed with saline. To protect tooth crowns from complete demineralization, all tooth surfaces except for a window (4mm × 5mm) in the middle third of the buccal surface were coated with acid-resistant nail varnish (Colorama Express®; Colorama/CEIL, Sao Paulo, Brazil).

The teeth were randomly divided into three groups of 15, including the control group, TCP (Clinpro Tooth Creme 0.21% NAF Anti Cavity Toothpaste; 3M ESPE, St. Paul, MN, USA), and MI Paste Plus (GC America Inc., Alsip, IL, USA).

Demineralization process:

Before applying the aforementioned remineralizing agents, the teeth were immersed in 15mL of a demineralizing solution with the following composition to artificially induce WSLs: 0.05mM/L lactic acid, 2.2Mm/L NaH_2PO_4, 2.2Mm/L $CaCl_2$, and 0.2mM/L fluoride [18].

The solution was refreshed daily. After 96 hours of storage in the demineralizing solution, they were rinsed with thymol solution and stored in thymol until the end of the process [19].

Control group: The teeth were not treated with any material and were only immersed in the demineralizing solution.

MI Paste Plus group: MI Paste Plus (GC America Inc. Alsip, IL, USA) was prepared. The teeth were dried, and the paste was applied on the exposed window. According to the manufacturer's instructions, the paste was left in place for 5 minutes; excess paste was cleaned with a linen cloth and the rest was left on the tooth surface for another 2 minutes and finally rinsed with saline (Table 1). This treatment was performed once daily for 30 days.

TCP group: Treatment in this group was also performed using the commercially available TCP paste (Clinpro Tooth Creme 0.21% NAF Anti Cavity Toothpaste; 3M ESPE, St. Paul, MN, USA) similar to the MI Paste Plus group (Table 1).

Microhardness measurement:

After demineralization, the teeth were cut buccolingually at the exposed enamel window, and the microhardness of enamel at the exposed window was measured. The specimens were mounted in resin blocks (Megadental, Büdingen, Germany) and polished by a 1000-grit abrasive paper. To achieve a completely glossy surface, they were polished by a polishing paste. Next, the microhardness profile of each lesion was measured at 30-, 90-, and 60µm depths along the cutting surface using a hardness tester (MHV-1000z; SCTMC Company, China) with a Knoop diamond indenter under 10g force for 5 seconds. The first entry point was at 30µm depth from the outer enamel surface. Indentations were made perpendicular to the surface and along the dentinoenamel junction. Three indentations were made at each depth, and the mean Knoop hardness number (KHN) of the three indentations was calculated for each depth. The KHN was then converted to the volume percentage of mineral content (VPM) using the equation used by Hu and Featherstone [20]. The mean VPM of sound enamel (cut-off point) was considered to be 90%. It means that enamel with a mineral content higher than 90% of intact enamel at higher depths would be considered identical to intact enamel. The VPM of each group was used to calculate the relative loss of minerals (ΔZ) using the Simpson's rule. The ΔZ was calculated by multiplying the volume by the distance [20]. The ΔZ values were transferred to a graph in terms of distance and the values under the curve were calculated by integral using the Simpson's rule. The mineral loss integral was calculated for each group. In fact, by subtracting the integral values of the demineralized enamel from the intact enamel, the mineral loss values were calculated.

KHN equation:

In this equation K is the force in grams (g), and L is the penetration depth in micrometers (µm).

$$KHN = 14.230 \, (F/D^2)$$

KHN to VPM converting equation:

$$\sqrt{KHN} = 0.197[VPM]-0.24; \; r=0.916$$

Statistical analysis:

Normal distribution of data was analyzed by the Kolmogorov-Smirnov test. Comparisons of VPM of the three groups at different depths were performed by the Prism software, ANOVA, and Tukey's test at 0.05 level of significance.

Table 1. Materials used in this study and their application method according to the manufacturers' instructions

Material	Manufacturer	Manufacturer's instructions
MI Paste Plus	GC America Inc., Alsip, IL, USA	First cleaning the teeth and then smearing a small amount of MI Paste Plus across the tooth surfaces with a clean finger or cotton-tipped applicator.
TCP	Clinpro Tooth Creme 0.21% NAF Anti Cavity Toothpaste, 3M ESPE, St. Paul, MN, USA	Place a pea-sized amount of Clinpro Tooth Crème on a soft-bristle toothbrush and brush teeth for at least 2 minutes. After brushing, spit out. Rinsing is optional.

Table 2. Volume percentage of mineral content at different depths based on group

Groups	30µm depth			60µm depth			90µm depth		
	Mean	SD	P	Mean	SD	P	Mean	SD	P
Control	70.75	3.76	<0.0001*	69.75	4.68	<0.0001*	70.94	4.78	<0.0001*
MI Paste	94.5	9.99	<0.0001*	90.09	8.35	<0.0001*	88.35	8.005	<0.0001*
TCP	84.9	6.72	<0.0001*	87.12	5.68	<0.0001*	90.8	6.68	<0.0001*

*Significant based on two-way ANOVA and Tukey's multiple comparisons test. The P-values are related to the difference of volume percentage of mineral content of the two treatment groups with the control group at each depth. SD: Standard deviation

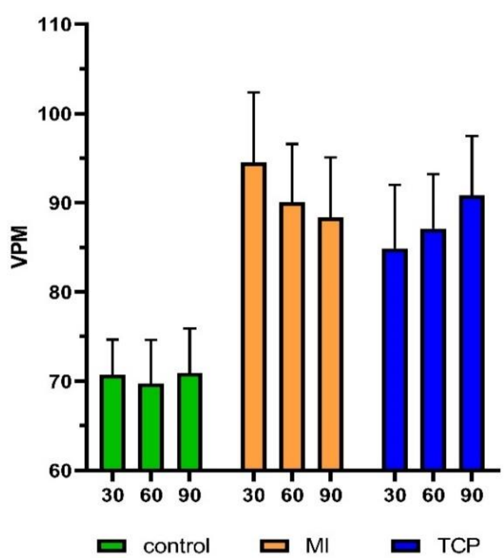

Fig. 1: Volume percentage of mineral content of the three groups at different depths of 30-, 60-, and 90µm

RESULTS

Figure 1 and Table 2 show the VPM of the three groups at different depths of 30-, 60-, and 90µm. Two-way ANOVA showed a significant difference among the groups in VPM at 30- to 90µm depths (P<0.0001). Thus, pairwise comparisons were performed by the Tukey's test.

At 30µm depth, the highest VPM belonged to the MI Paste Plus group (94.5), and the lowest to the control group (70.75). ANOVA found a significant difference between the mean VPM in the control group and the other two groups at this depth (P<0.0001). Comparison of MI Paste Plus and TCP groups also showed a significant difference at this depth (P<0.0001). It means that at this depth, both treatments were significantly more effective for enamel remineralization than the control group, and MI Paste Plus was significantly more effective than TCP.

At 60µm depth, the highest VPM was recorded in the MI Paste Plus group (90.09) and the lowest in the control group (69.75). Two-way ANOVA showed significant differences between the two treatment groups with the control group (P<0.0001), and no significant difference was found between MI Paste Plus and TCP at this depth (P=0.9382). It means that both groups were significantly and equally effective for remineralization at this depth.

The mean VPN at 90µm depth was in the range of 103.9±59.6 and unlike the other two depths, the highest mean VPM belonged to the TCP group (90.8±6.6). Pairwise comparisons of the groups at this depth showed a significant difference between the control group and the two treatment groups (P<0.0001), and no significant difference between the two remineralizing groups (P= 0.9803). At this depth, the two treatment groups were equally effective with no significant difference.

DISCUSSION

Risk factors known to cause enamel WSLs include poor oral hygiene before and during orthodontic treatment, age (treatment initiation during the pre-puberty period), high decayed, missed, and filled teeth index, duration and location of enamel etching, and first molar status in terms of caries [21]. The prevalence of WSLs in the literature ranges from 2% to 96% [22]. At early stages, these lesions are easily detectable and reversible, but they become irreversible in case of progression into active caries [23]. The present study investigated the effects of TCP and CPP-ACPF on enamel WSLs. Also, microhardness was measured by measuring the KHN to determine the effect of remineralizing pastes. The KHN and the Vickers hardness number are commonly measured for assessment of hardness of different tooth parts due to their optimal reliability [24]. The error rate of KHN is less than 5%, and the repeatability of this test is also very high [25]. A correlation coefficient of 0.91 between KHN and mineral content is another reason for using this method [26]. The method used for induction of enamel WSLs in the present study is a reliable method with easy implementation that yields optimal results [19].

In the present study, the VPM of the teeth in MI Paste Plus group at all three depths of 60-, 30- and 90µm was significantly higher than that of the control group, indicating effective remineralization of WSLs. Zenouz et al. [27] stated the same results; they measured the hardness and compared the effects of CPP-ACP alone and CPP-ACP with sodium fluoride on superficial enamel, and confirmed the effectiveness of these materials in hardening of superficial enamel but reported that CPP-ACP and CPP-ACPF were less effective than fluoride. Hegde and Moany [19] examined the effect of CPP-ACP on artificial enamel lesions using electron microscopy and observed that after 35 days, there was a significant difference in the amount of minerals following its application, and its effect was dose-dependent. They also found that keeping the specimens in the remineralizing agent for a longer period of time increased their rate of remineralization. They showed the effect of CPP-ACP on superficial enamel. Thus, less effects at 60- and 90µm depths may be expected.

The present findings were also in agreement with the results of Rajan et al [28]. They applied several remineralizing agents, including CPP-ACPF containing fluoride on artificial enamel lesions, and observed the specimens under a microscope to measure the lesion depth. They reported that the SHY-NM toothpaste was the most effective remineralizing agent.

TCP has anti-caries properties [29], and like other compounds containing calcium and phosphate, it promotes natural remineralization by the saliva. TCP is a biomaterial with high potential for bio-applications [30]. This substance is available in 1:5 calcium to phosphate ratio and in both alpha and beta forms. It increases the concentration of calcium in the saliva, and is one of the substances that is known to improve the remineralization process [31]. In a previous study, addition of beta form to toothpastes containing 5000ppm fluoride resulted in 30% increase in remineralization and an increase in enamel microhardness [32]. Patil et al. [33] performed a similar study by comparing three substances of CPP-ACP, CPP-ACPF, and TCP with artificial saliva as the control group. Similar to the present study, they showed that TCP, MI Paste Plus, and MI Paste were significantly more effective than the control group.

In the present study, the ranking obtained for MI Paste Plus and TCP was similar to that in the study by Patil et al, [33] only at 90µm depth. At 30- and 60µm depths, MI Paste Plus was more effective, even though this difference was only significant at 30µm depth. The greater effect of TCP at deeper parts may be due to the greater stability of this product in wet environment, compared with other products containing calcium and phosphate [34], which contributed to the penetration of this substance into enamel under in vitro

conditions.
In the present study, high remineralization by TCP was consistent with the results of Shen et al, [35] who found this material to be less effective than others due to its low solubility in water and large molecules. However, the difference between in vivo and in vitro settings, and also dose and time of application of remineralizing agents should be taken into account when comparing the results. A higher dose of TCP was used in the present study. Also, Shen et al. [35] used remineralizing agents for 60 seconds, 4 times daily for a period of 10 days. Bajaj et al. [36] compared the efficacy of CPP-ACP, TCP, and HA for enamel remineralization with the control group. They observed the changes with a polarizing microscope and reported results similar to the present findings. HA was more effective than the other two substances in their study. Chokshi et al. [29] compared the efficacy of fluoride varnish, CPP-ACP, and TCPF. They found that fluoride varnish was more effective. Also, the effect of TCPF and CPP-ACP significantly increased after 40 days of treatment compared with 20 days, which confirms the time-dependent efficacy of these substances at high doses. The present study also showed the same results regarding the efficacy of these materials in high doses.
Since the magnitude of change in enamel VPM was high at all three examined depths, and none of the tested three depths were in intact enamel, the intact enamel mineral could not be calculated for the purpose of comparison and calculation of mineral change (ΔZ) in the present study. Therefore, hardness measurement at higher depths (intact enamel) is recommended to calculate ΔZ in future investigations. Considering the limitations of in vitro studies, further clinical studies are also required on remineralizing agents especially TCP.

CONCLUSION

The present results clearly showed that the products containing CPP-ACPF and TCP had a significant effect on remineralization of enamel WSLs. Thus, MI Paste Plus may be recommended for remineralization of WSLs.

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CONFLICT OF INTEREST STATEMENT

None declared.

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