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Conventional Casein Phosphopeptide-amorphous Calcium Phosphate Versus Casein Phosphopeptide-amorphous Calcium Phosphate with Nanosilver Particles On Enamel Microhardness of Primary Canines

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Objectives This study aimed to compare the effects of conventional casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) and CPP-ACP with 1% and 2% nanosilver particles on microhardness of primary canine enamel.
Methods The baseline surface microhardness of samples was measured using a Vickers hardness tester. Subsequently, they were immersed in a demineralizing solution for 24 hours, and their microhardness was measured. All samples were randomly divided into 4 groups (n=9): (1) Control group, (2) CPP-ACP, (3) CPP-ACP with 1% nanosilver, and (4) CPP-ACP with 2% nanosilver. Then they were subjected to pH cycles for 7 days. After the pH cycling, the surface microhardness of the samples was measured. Data analysis was performed using one-way ANOVA, paired samples T-test, Tukey,Box M and Leven analysis.
Results The mean enamel microhardness significantly decreased in all groups after demineralization ($P<0.05$). However, this reduction was significantly less pronounced in all three experimental groups compared to the control group ($P<0.05$). The rate of microhardness changes was 204.18±53.53 in the CPP-ACP group containing 1% silver nanoparticles, 59.77±175.70 in the CPP-ACP group containing 2% silver nanoparticles, and 208.77±27.42 in the conventional CPP-ACP group, with no significant difference observed among the three groups ($P=0.938$).
Conclusion Conventional CPP-ACP and CPP-ACP with 1% and 2% nanosilver are equally effective in preserving the enamel surface microhardness. Silver nanoparticles have no negative impact on enamel microhardness.
Keywords Casein phosphopeptide-amorphous calcium phosphate nanocomplex (CPP_ACP); Silver; Hardness; Dental caries

Introduction

Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) is a milk-derived nano-complex that exerts its remineralizing effect by increasing the concentrations of calcium and phosphorus in saliva. [1] CPP-ACP can function as a calcium phosphate reservoir, enhancing the buffering capacity of calcium and phosphate ions and creating a supersaturated condition of these ions, ultimately promoting the remineralization of demineralized enamel. [2]
CPP-ACP with nanosilver particles is a novel formulation containing nanosilver, chitosan, and fluoride, which offers caries prevention and antimicrobial effects without causing any change in tooth color. [3] Several studies have demonstrated that adding silver nanoparticles to dental materials can enhance their properties. [4-7] The antibacterial effect of nanosilver against Streptococcus mutans has been confirmed in in-vitro studies. Nanosilver exhibits antibacterial properties 25 times stronger than chlorhexidine, with lower cytotoxicity compared to silver diamine fluoride. [5] Haghgoo et al. recommended using nanosilver varnish beneath amalgam restorations to reduce microbial populations and prevent secondary caries. [8] Nozari et al. reported that nanosilver fluoride significantly impacted the remineralization of deciduous teeth enamel. [7] However, Akyildiz and Sonmez stated that nanosilver-fluoride is less effective against enamel caries lesions than sodium fluoride and silver diamine fluoride varnishes. [30]
Due to the importance of using More effective materials to prevent dental caries and the absence of a study on the effect of adding silver nanoparticles to CPP-ACP, the current study aimed to compare the effects of conventional CPP-ACP (GC Tooth Mousse, America) and CPP-ACP with 1% and 2% nanosilver particleson microhardness of human deciduous canine enamel.

Methods and Materials

This experimental study was conducted with the permission of the Shahid Beheshti University of Medical Sciences Ethics Committee (IR.SBMU.DRC.REC.1398.039).

Sample size

Based on Delbem et al.'s study in 2006, with an α error set at 0.05 and a study power of 0.8, the minimum required sample size for each group was calculated to be 8 samples. [5] Therefore, in the current study, each group consisted of 9 samples. A total of 36 intact human deciduous canines, extracted for orthodontic reasons, were collected to ensure they met the inclusion criteria. Inclusion criteria was stipulated that teeth should have no caries, previous fillings, cracks, or developmental anomalies.

Sample preparation

The teeth were stored in distilled water containing 0.1% thymol from extraction until the start of the test, to prevent dehydration and microbial growth. Before beginning of the experiment, the teeth were polished for 10 seconds using fluoride-free pumice paste and a low-speed Handpiece to remove calculus, plaque, and soft tissue residues.

The samples were then mounted in a special mold with green self-cure acrylic (Acropars, Iran), ensuring that the buccal windows were exposed. Each enamel block was polished using 200 , 400, 600, 800, 1000, 1200, 1500, and 2000 grit silicone carbide sandpaper sheets (Starcke, Matador, Waterest, Germany).

CPP-ACP with 1% and 2% nanosilver preparation:

To prepare CPP-ACP with 1% and 2% nanosilver, 40 nm silver nanoparticles were added to fluoride varnish (measured with an accurate 0.01 mg A&D scale). The mixture was then hand-mixed in a plastic container with a plastic spatula for one minute to achieve a visually homogeneous consistency.

Group allocation

All prepared samples were randomly divided into 4 groups of 9 each. A randomization sequence in blocks of 4 was created by an independent person using random allocation software version 1.0.0 [7] to assign the teeth to one of the four main groups. The group allocation was written on a piece of paper, folded, and sealed in opaque envelopes. During the intervention, the group to which each tooth was assigned was revealed by the independent personnel. [8] The groups were labeled as follows: (1) control group (without therapy); (2) conventional CPP-ACP, (3) CPP-ACP with 1% nanosilver, and (4) CPP-ACP with 2% nanosilver.

Experiment

Initially, the microhardness of the samples was measured using a Vickers microhardness tester machine (Zwick Roell, Germany) with a 50 gr force for 20 seconds at Tehran University of Medical Sciences. Subsequently, to simulate caries, all enamel blocks were immersed in a demineralizing solution (acetic acid 0.05 M, NaH2PO4 2.2 mM, CaCl2 2.2 mM, pH adjusted to 4-4.5 with NaOH and HCL, 15 ml per tooth) for 24 hours. The microhardness of the samples was re-evaluated using the Vickers machine with a 50 gr force for 20 seconds at three different points on the enamel block. The average of these three measurements was calculated and reported as the microhardness of the sample.

Next, a layer of tooth mousse CPP-ACP was applied to the teeth in group B using a cotton swab, following the manufacturer's instructions. Similarly, CPP-ACP with 1% and 2% nanosilver were applied to the teeth in groups C and D, respectively. Teeth in group A did not receive any treatment.

After three minutes, all samples were placed in separate containers containing distilled water and in an incubator at 37°C for 24 hours. Subsequently, pH cycling was performed on the samples for 7 days using the following protocol:

The samples were immersed in a demineralizing solution for 3 hours, rinsed, and then immersed in distilled water for 30 minutes. Following this step, the teeth were immersed in a NaF-free remineralization solution for 20 hours (CaCl2 1.5 mM, NaHPO4 0.9 mM, KCl 0.15 mM, pH adjusted to 6.5-7, 15 ml per tooth). The teeth were then rinsed with distilled water and immersed in distilled water for 30 minutes. These 24-hour cycles were repeated for 7 days. The demineralizing and remineralizing solutions were replaced daily, and all procedures were conducted in an incubator at a temperature of 37±2°C.

At the end of the seventh day, the microhardness of the 4 groups was measured again at 3 different points using the Vickers microhardness tester machine with a 50 g force for 20 minutes, and the mean microhardness was calculated.

Data analysis was conducted using SPSS 22.0 statistical software. Mean and standard deviation values of the baseline and post-treatment microhardness were calculated for groups on the seventh day. The normality of the data was confirmed using the Shapiro-Wilk test. Paired samples t-test, One-way ANOVA, and the Tukey test, Box M variance, Levene analysis were employed to compare microhardness values at different times within and between the groups.

Results

The mean microhardness of the teeth was measured using a Vickers microhardness tester machine at three different time points: before testing (baseline), after immersion in the demineralizing solution (post-lesion), and after treatment with the desired toothpaste (post-treatment), as shown in Table 1.

The results of the ANOVA indicate that there was no significant difference in the average microhardness between the groups before the intervention. In other words, the microhardness values among the four groups were homogeneous before the intervention (Figure 1).

Table 1- Mean, standard deviation and rate of microhardness changes of samples enamel

Group	Baseline	Post-lesion	Post-treatment
A(control)	396.96±29.35	323.29±25.22	65.29±19.50
B(conventional CPP-ACP)	354.59±46.58	295.58±27.32	87.07±9.51
C (1%w nano-silver CPP-ACP)	326.18±35.45	290.64±64.68	86.48±18.3
D (2%w nano-silver CPP-ACP)	323.85±67.65	264.11±59.80	88.40±14.87

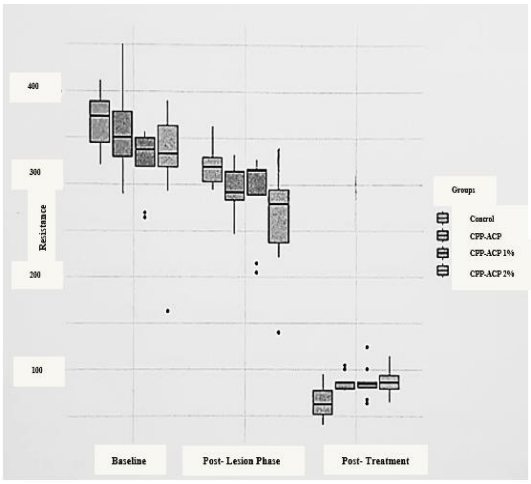

Figure 1: Descriptive chart of the examined data

The initial microhardness of all groups was similar at the beginning of the experiment. The results of the Box M variance analysis showed a significant difference between the three test times (P<0.0001). The Levene analysis demonstrated a significant difference between the baseline and post-lesion and post-treatment steps (P<0.01). There was also a significant difference between the samples' microhardness values baseline and after treatment (P<0.05), indicating that the microhardness of all treated groups was significantly higher than that of the control group. To investigate the effect of the interventions on the microhardness of the groups, paired t-tests were used to compare each dental group separately at each intervention stage.

According to Table 1 and figure 1, the microhardness in the conventional CPP-ACP, CPP-ACP with 1% nanosilver, and CPP-ACP with 2% nanosilver groups was significantly higher than the control group. However, there was no significant difference between the three intervention groups (Table 2).

Table 2- Rate of changes in surface microhardness in assessed groups- Paired t-test results.

Phase	Group 1	Group 2	n1	n 2	p-value
Baseline	Control	CPP-ACP	9	9	0.494
	Control	CPP-ACP+1%w silver nano particles	9	9	0.0572
	Control	CPP-ACP+2 %w silver nano particles	9	9	0.0458
	CPP-ACP	CPP-ACP+1%w silver nano particles	9	9	0.21
	CPP-ACP	CPP-ACP+2%w silver nano particles	9	9	0.176
	CPP-ACP+1%w silver nano particles	CPP-ACP+2%w silver nano particles	9	9	0.917
Post lesion	Control	CPP-ACP	9	9	0.178
	Control	CPP-ACP+1%w silver nano particles	9	9	0.111
	Control	CPP-ACP+2%w silver nano particles	9	9	0.00558
	CPP-ACP	CPP-ACP+1%w silver nano particles	9	9	0.796
	CPP-ACP	CPP-ACP+2 %w silver nano particles	9	9	0.121
	CPP-ACP+ 1%w silver nano particles	CPP-ACP+2 %w silver nano particles	9	9	0.192
Post treatment	Control	CPP-ACP	9	9	0.00675
	Control	CPP-ACP+1%w silver nano particles	9	9	0.00822
	Control	CPP-ACP+2%w silver nano particles	9	9	0.0043
	CPP-ACP	CPP-ACP+1%w silver nano particles	9	9	0.938
	CPP-ACP	CPP-ACP+2%w silver nano particles	9	9	0.86
	CPP-ACP+1%w silver nano particles	CPP-ACP+2%w silver nano particles	9	9	0.799

Discussion

Despite advances in caries prevention, tooth decay remains a significant public health issue. [4, 7] One of the recently introduced materials in this field is nanosilver particles. In this study, enamel surface microhardness was measured to compare the effectiveness of CPP-ACP in remineralizing enamel lesions. Surface microhardness evaluation is a reliable index for assessing the effectiveness of dental materials and is considered an effective and indirect measurement for assessing mineralization effects on teeth. [9, 10,11]

In recent years, CPP-ACP has been introduced as an effective non-invasive technique for preventing caries lesions. [12] CPP-ACP has shown successful results in remineralizing enamel lesions in several studies [14]. There is a reverse relationship between calcium and phosphor ion saturation and the occurrence of caries. [15]

Imani et al. reported that the effectiveness of CPP-ACPF (CPP-ACP containing fluoride) was significantly higher than fluoride-free CPP-ACP. [16] However, in Poureslami et al.'s study, the significantly higher effectiveness of CPP-ACPF was attributed only to fluoride. [13] In this study, to eliminate the significant effect of fluoride in increasing the efficiency of CPP-ACP, fluoride-free CPP-ACP (GC tooth mousse) was used. This allowed us to assess the degree of tooth demineralization prevention without the intervention of fluoride ions.

No increase in enamel microhardness was observed in this study, similar to previous studies that reported a decrease in microhardness with similar procedures. [12, 18] To date, complete restoration of microhardness by any substance

has not been reported. [12] Tahmasbi et al. demonstrated increased enamel microhardness after using CPP-ACPF, NaF in a similar procedure. The effect of NaF in preventing enamel demineralization was significantly higher than other study groups. It should be noted that fluoride was present in all three materials in their study. Therefore, it can be concluded that the increase in enamel microhardness after demineralization was due to the effect of fluoride ions [18], which were absent in any of the experimental groups in this study. Savas et al. also showed increased enamel microhardness after demineralization due to CPP-ACPF treatment. [19] Oliveira et al. reported a significantly lower increase in enamel microhardness with CPP-ACP and CPP-ACPF than with conventional fluoride dentifrices. [12] The study by Oliveira GM et al. yielded similar results. [17]

In this study, the application time of CPP-ACP was 3 minutes, following the manufacturer's instructions. Pulido et al. suggested that longer application times may be required to detect and observe calcium and phosphorus deposition changes. [20] AL-Mullahi et al. concluded that applying CPP-ACP with an increased application time of up to 30 minutes and repeated applications could be more effective in remineralizing enamel lesions. [21] Since CPP-ACP creates supersaturation conditions by binding to the bacterial biofilm on the tooth surface, the absence of biofilm in in-vitro studies could be a limiting factor for CPP-ACP to exert its full effect. [17]

In this study, Demineralizing solutions were replaced after each cycle to eliminate the effect of calcium and phosphorus concentration in the environment. Additionally, teeth were rinsed after each treatment to ensure that the active solution ingredients only affected the bonding mechanism.

Recently, the effectiveness of adding silver compounds to fluoride in enamel lesion treatment has been investigated. Silver particles are available in two forms, with silver ions being one of these forms used in silver diamine fluoride (SDF) solutions. Silver diamine fluoride has successfully stopped and prevented dental caries in clinical trials through its direct antibacterial effect and its reaction with hydroxyapatite, leading to the creation of fluoroapatite. [23] However, Mohammadi and Farahmandfar's study showed that despite the lower reduction in enamel microhardness when using SDF compared to fluoride varnish, this difference was not significant, and both were equally effective in preventing the demineralization of anterior deciduous teeth. [24] In addition to several benefits, SDF has some disadvantages, including tooth discoloration, mild painful sores in the oral mucosa that usually disappear within 48 hours, and a metallic taste in the mouth when using this solution. [24, 25, 26] Nanosilver particles represent another available form of silver particles. [27] These particles are as effective as SDF solutions and do not cause tooth discoloration. These particles, with a size of 10 nm, can penetrate the bacterial matrix and interfere with cellular functions, especially in gram-negative bacteria. [22]

In this study, 1 and 2 wt% nanosilver particles were added to conventional CPP-ACP to assess their effect on enamel surface microhardness. Enamel microhardness of all three groups exhibited significantly less reduction after demineralization and placement in the pH cycles compared to the control group, which received no treatment. There was no significant difference in microhardness changes between the three intervention groups. Conventional CPP-ACP, 1 wt% nanosilver CPP-ACP, and 2 wt% nanosilver CPP-ACP were equally effective in remineralizing primary enamel lesions. Therefore, adding nanosilver particles to CPP-ACP does not increase enamel microhardness.

Nanosilver fluoride solution comprises nanosilver particles, chitosan, and fluoride. Several studies have shown that the synergistic use of chitosan, nanosilver particles, and fluoride compounds can effectively arrest dentin caries. [4] In this study, only nanosilver particles were added to the CPP-ACP paste, which may have made nanosilver particles less effective. Chitosan is naturally antibacterial and acts as a stabilizing agent for nanosilver particles and silver ions. [26, 27]

Mares-Garcia et al. reported that remineralization with fluoride varnish containing nanosilver particles in teeth with white-spot lesions In patients with Down syndrome was much more effective than Nano-silver-free fluoride varnish. [29] The presence of oral bacteria and differences between in-vitro and in-vivo studies can affect the difference in results between these two studies. Nozari et al. stated that after nanosilver fluoride application, enamel surface microhardness was significantly higher than fluoride varnish and nano-hydroxyapatite groups. [7] Akyildiz and Sonmez reported that enamel surface microhardness after demineralization in NaF and SDF groups was higher than nanosilver group, and nanosilver fluoride was not as effective as NaF and SDF on enamel caries lesions. [30] The present study demonstrated no significant difference between CPP-ACP containing nanosilver particles and conventional CPP-ACP.

It is essential to note that the expected effect of nanosilver particles is related to their antimicrobial properties. [28] Furthermore, this study demonstrated that nanosilver particles do not deteriorate the enamel microhardness. Additionally, because CPP-ACP is water-soluble, it can help release silver into the oral environment from CPP-ACP.

In this study, an attempt was made to simulate the oral cavity conditions. However, one limitation of this study was the excessive demineralization in in-vitro conditions compared to the oral environment.

Conclusion

Conventional CPP-ACP and CPP-ACP with 1 wt% and 2 wt% nanosilver particles were equally effective in increasing the enamel surface microhardness of human deciduous canines. Silver nanoparticles did not have a negative effect on enamel microhardness.

Conflict of Interest

No Conflict of Interest Declared ■

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