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Effects of three commercial toothpastes incorporating "chitosan, casein phosphopeptide-amorphous calcium phosphate, sodium monofluorophosphate, and sodium fluoride" on remineralization of incipient enamel caries in the primary dentition: A preliminary *in vitro* study

Leila Basir[1], Raziye Meshki[1], Hilda Aghababa[1], Vahid Rakhshan[2]

[1]Department of Pediatric Dentistry, School of Dentistry, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, [2]Private Practice, Tehran, Iran

ABSTRACT

Background: Given the importance of primary dental caries, assessment of new preventive/therapeutic materials is necessary. In light of the scarcity of such studies on the role of new agents in primary dentition, this study assessed the efficacy of three commercial toothpastes with different ingredients on remineralization of early caries in the primary dentition.

Materials and Methods: Thirty-nine intact primary canines were used in this *in vitro* experimental study. The baseline microhardness of enamel was measured. Particular demineralizer was used for 96 h and then secondary microhardness was measured. Then, samples were divided into three groups treated by toothpastes with sodium fluoride, chitosan, and casein phosphopeptide-amorphous calcium phosphate and sodium monofluorophosphate (CPP-ACP+SMFP). Each group was incubated and pH-cycled. Subsequently, they were demineralized and remineralized using toothpastes. Eventually, tertiary microhardness was measured. Percent of enamel microhardness recovery (EMHR%) and efficacy of toothpastes in remineralization were established. Data were analyzed using paired *t*-test, repeated-measures test, Kruskal–Wallis, and Mann–Whitney U-test ($\alpha = 0.05, 0.017$).

Results: Demineralization significantly reduced microhardness from 316.2 to 248.5 Vickers hardness number (VHN) ($P = 0.000$). All toothpastes succeeded to remineralize the enamel significantly ($P = 0.000$). The efficacies of toothpastes differed significantly ($P < 0.05$). Mean EMHR percentages of toothpastes incorporating NaF, chitosan, and CPP-ACP+SMFP were 75.1%, 52.5%, and 55.8%, respectively. The highest increases in enamel microhardness were observed after using NaF-containing toothpaste (~53 VHN) which was significantly superior to other toothpastes ($P \leq 0.001$). However, there was no statistically significant difference between EMHR percentages of toothpastes including chitosan and CPP-ACP+SMFP ($P = 0.739$).

Conclusion: Although all three toothpastes could increase the microhardness of primary enamel, NaF toothpaste was superior to others. Toothpastes having chitosan and CPP-ACP+SMFP acted rather similarly.

Key Words: Casein phosphopeptide-amorphous calcium phosphate, chitosan, fluorophosphate, hardness tests, primary dentition, sodium fluoride, toothpastes

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Address for correspondence:
Dr. Hilda Aghababa,
Department of Pediatric Dentistry, School of Dentistry, Ahwaz Jundishapur University of Medical Sciences, Ahwaz, Iran.
E-mail: hildaaghababa001@gmail.com

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INTRODUCTION

Oral and dental health and function of teeth and their role in chewing, swallowing, speaking, and beauty are clear.[1,2] Dental caries is the most prevalent and expensive chronic disease.[1,2] Caries is the most common disease in childhood.[1-3] Studies indicate that this disease is caused by the host, food, and acidogenic bacteria such as *Streptococcus mutans* and Lactobacillus acidophilic.[1-3] Caries is started from enamel, and their progress is slow at first because the enamel surface is more resistant than deeper parts against demineralization.[1-3] This form of lesions is called white spot and is able to become remineralized by saliva and some medications before the progress of caries into the formation of any cavity.[1-3]

The most important method for prevention is through oral hygiene maintenance, which increases remineralization of the tooth surface and plaque pH, and decreases microorganisms that contribute to tooth caries so it prevents the process of caries formation.[2,4,5] There are some materials such as anti-plaque and anti-remineralization which are used at home: toothpastes, mouthwash, and tooth floss.[2,4,5] Due to some therapeutic properties, toothpastes have multiple functions in maintaining tooth and mouth health, which include creating anti-caries and remineralization properties.[1]

There are many studies focused on the role of fluoride in toothpastes (e.g., NaF and sodium monofluorophosphate [SMFP]) as an effective ingredient in stimulating remineralization of early lesions.[3-8] Fluoride has positive effects on remineralization and prevention of caries; however, high dosages can cause fluorosis or poisoning in children.[2,9] In addition, excessive fluoride dosages might become toxic for children. Safety and limitation issues in consumption dosage of fluoride calls for continued research on new caries-preventive medications with fewer adverse effects; newer useful materials include casein phosphopeptide–amorphous calcium phosphate (CPP-ACP) and chitosan.[3,7,8,10]

CPP-ACP is a phosphorylated peptide derived from casein of milk which is connected to calcium and phosphate in enamel on one side and to tooth plaque on the other side, reducing the formation of white spot lesions.[3,5,11-15] In addition, it saves calcium, phosphate, and fluoride and maintains supersaturated condition of ions in the enamel surface.[3,5,11-15] Today, CPP-ACP is used in different forms such as gels, creams, and toothpastes.[3,5,12-14]

Chitosan is a copolymer of N-Acetyl-D-Glucose and D-glucosamine which is found in insect's skeleton and cellular wall of the fungus.[5,6,16-19] Evidence shows that adding chitosan to toothpastes improves antibacterial properties of toothpaste, also reduces adhesion of bacteria to the tooth, prevents decalcification of dental enamel, and prevents plaque formation and increases remineralization of decalcificated tissues.[5,6,17-19] These features make it a proper candidate for incorporation in toothpastes to control dental caries.[5,6,17,18]

Today, parents are more informed about and interested in using the best preventive and therapeutic products for their children. Therefore, identifying the efficacies of commercial products in the market is necessary. Moreover, the effects of chitosan on remineralization of incipient caries are not yet assessed, and studies on remineralization efficacy of CPP-ACP are scarce. In addition, most previous studies are on permanent teeth, while organic content of the primary enamel is higher than that of permanent teeth, and it causes their enamel to be more flexible and penetrable and more sensitive and susceptible to caries consequently.[20]

Thus, this research was conducted to comparatively assess the effects of three commercial toothpastes including chitosan, "CPP-ACP+SMFP," and sodium fluoride on remineralization of early caries of the primary dentition.

MATERIALS AND METHODS

Sample preparation

In this *in vitro* study, 39 primary human canine teeth extracted for orthodontic and pediatric treatment purposes were included. Study protocols and ethics were approved by the research committee of the university (#118-D/MP/36/105). The inclusion criteria were the existence of intact crowns without caries and pigments, hypoplasia, calcified spots, and enamel cracks. Before the experiment, teeth were kept in 2% formaldehyde solution at room temperature. After irrigation of enamel by distilled water and polishing it by oxide aluminum paper, the dental crown was separated from the root at the cement–enamel junction using diamond fissure burs attached to high-speed turbines. It was then mounted in a cube made of self-cure acrylic epoxy resin 1 cm in height. While mounting, the buccal surface of crown was parallel to the cross-section of the cube. Then, all surfaces of samples except a 2 mm × 2 mm window in the center of the buccal surface were covered with two layers of nail varnish resistant to acid (Max Factor, Paris, France).

Microhardness test

Baseline microhardness of enamel was measured at 4 points using a microhardness testing device (Buhler, Alzenau, Germany), exerting a 50-g force for 5 s through a diamond leverage. Afterward, the Vickers hardness number (VHN) was calculated for each point according to the formula (VHN = $F/A = 1.8544\ F/d^2$), in which F is the force on the diamond in kg, A is indentation area in mm^2 and d is mean of the diameter of the indenter in mm. The mean VHN was calculated for the 4 points and was considered as the main enamel microhardness (EMH) of samples. Teeth with initial microhardness values between 217.76 and 373.50 were selected for the study.

Incipient caries

A demineralization solution was used for creating incipient enamel lesions (0.05 M acetic acid, 2.2 mM $ClCa_2$, 2.2 Mm NaH_2PO_4, and 1 M KOH at pH = 4.5. Teeth were incubated in 1 liter of demineralizing solution for 96 h at 37°C (Innova, Boston, MA, USA). Then, teeth were irrigated with deionized water and placed in normal saline solution. Finally, they were sent to the laboratory for secondary testing of their microhardness, as stated above.

Experimental groups

After measuring secondary microhardness, samples were randomly divided into three groups treated with one of three toothpastes: Group 1: a toothpaste incorporating 500 ppm, 0.24% sodium fluoride (Crest for Children, Procter & Gamble, Cincinnati, Ohio, USA). Group 2: a fluoride-free toothpaste containing chitosan (Chitodent, Griesheim, Germany). Group 3: a toothpaste including CPP-ACP and SMFP (Misswake, Sheaffer, Switzerland). The authors declare that they are not by any means associated with any of the tested brands; they do not have any conflict of interest.

Slurry solution of each toothpaste was produced by adding deionized water to them with a weight ratio of 3:1 (17 g of each toothpaste blended with 52 ml deionized water using a vibrator [Vortech, Seoul, Korea]).

Toothpaste application and pH-cycling

A remineralization solution with following combinations was produced: (1.5 mM) $CaCl_2$ with pH = 7 and (0.15 M) KCl and (0.9 mM) NaH_2PO_4 and (1 M) KOH. Each group was in an incubator for 10 days under pH-cycling. Samples were incubated and pH-cycled at 37°C for 10 days: Each sample was placed in 10 ml demineralizer for 3 h twice and placed in 10 ml remineralized solution for 2 h once. Samples of each group were floated in toothpaste solution (4 ml for each sample for 60 s) before the first demineralization and after the second demineralization. Then, all samples were kept overnight in remineralization solution in the incubator at 37°C. After each time of pH-cycling, samples were irrigated by deionized water for 30 s to prevent interactions between solutions. Toothpaste solutions were renewed each time. Solution pH was measured daily by a pH meter (model 3310, Jenway, Staffordshire, UK).

After 10 days of pH-cycling and tooth irrigation, samples were transferred to the laboratory for the final EMH assessment. The following equation was used for calculating the percentage of EMH recovery (EMHR) of samples (Increase of EMHR percentage illustrates greater therapeutic effects of the material: EMHR% = $EMH_R - EMH_D/EMH_S - EMH_D \times 100$ where EMH_R is enamel microhardness after remineralization, EMH_D is enamel microhardness after demineralization, and EMH_S is enamel microhardness of sound teeth.

Statistical analysis

Based on the results presented by Memarpour *et al.*,[3] the sample size was predetermined as 39 specimens (13 per group), to obtain powers >90%. Before and after changes were assessed using paired *t*-test and repeated-measures test. Differences between groups were assessed using the repeated-measures test, Kruskal–Wallis, and Mann–Whitney U-test. The software in use was SPSS version 21 (IBM, Armonk, USA). The level of statistical significance was 0.05 for all tests except Mann–Whitney for which it was adjusted to 0.017.

RESULTS

The average baseline microhardness was higher than its value after 96 h for teeth demineralization, and *t*-test identified a significant reduction in microhardness after demineralization [P = 0.000, Table 1].

According to Table 2, there was a significant difference between the second and third experiment phases (after demineralization and after using toothpaste) in each of three toothpastes, showing that each brand succeeded to increase EMH (repeated measures $P = 0.000$).

The efficacies of toothpastes differed significantly [Kruskal–Wallis $P < 0.05$, Table 3]. The highest increases in EMH were observed after using fluoride. Mann–Whitney indicated that there was a statistically significant difference between EMHR% of fluoride-incorporating toothpaste with the toothpastes including CPP-ACP+SMFP ($P = 0.001$) and chitosan ($P = 0.000$). However, there was no significant difference between toothpastes containing chitosan and CPP-ACP+SMFP ($P = 0.739$).

DISCUSSION

This study indicated that all tested toothpastes were capable of remineralizing dental enamel and recovering EMH. Among them, fluoride-containing toothpaste was more effective than the other toothpastes. However, there was no significant difference between toothpastes containing CPP-ACP+SMFP and chitosan. In this study, demineralization and remineralization pH were equal to oral pH during tooth brushing.[21] In addition, to prevent reaching the saturation threshold, fresh demineralization and remineralization solution were used, and their pH was measured.[21]

Our findings indicated the superiority of sodium fluoride in regaining enamel mineralization. This was consistent with the study of Jabarifar *et al*.[22] who showed that Crest for Adults with 1100 ppm fluoride increased the hardness of enamel in primary dentition more than did Crest for Children (having 500 ppm NaF) and Pooneh with 500 ppm NaF. This indicates that there is a direct relationship between fluoride and an increase of remineralization of carious lesions.[22]

However, other tested toothpastes were as well successful agents in this study. For dental structure, it is necessary that calcium and phosphate ions penetrate into the tissue to restore deep defects. A newer material for remineralization of caries uses phosphopeptide derived from casein protein of milk, which includes various phosphoric clusters and can create CPP-ACP and cause sustainability and durability of calcium and phosphate ions.[3,5,11-15] This chemical combination can save these ions and maintain their supersaturation status and might finally increase enamel remineralization.[3,5,11-15] In addition, CPP-ACP can attach to enamel, bacterial plaque, and oral soft tissue in response to changes of the oral environment which is created by bacterial plaque and acidic food; afterward, it releases calcium and phosphate which can recover early caries.[3,5,11-15] Using products with high-density CPP-ACP may remineralize dental tissues and recover their caries.[23-26] The literature is consistent with our findings which showed improved remineralization after using CPP-ACP toothpaste. In a series of articles, CPP-ACP was not as effective as fluoride on remineralization of incipient caries and also fluoride plus CPP-ACP may have no potential for additional remineralization in comparison to fluoride.[27-29] According to Meyer-Lueckel *et al*.,[13] additional CPP-ACP application after fluoride 1400 ppm has less effect than using fluoride 1400 ppm in long term on remineralization of caries.[13] These results

Table 1: Comparing mean of incipient microhardness and microhardness (unit: Vickers hardness number) after 96 h in all samples

Microhardness	Mean±SD	Minimum	Maximum	*t*	*P*
Baseline	316.18±27.33	254.00	371.00	32.27	0.000
After 96 h	248.49±27.13	173.00	313.00		

SD: Standard deviation

Table 2: Descriptive statistics of enamel microhardness (unit: Vickers hardness number) according to toothpaste type and experiment phase

Toothpaste	Experimental phase	Mean±SD	Minimum	Maximum
Fluoride	Baseline	310.69±27.33	254.00	361.00
	After demineralization	240.46±26.83	181.00	289.00
	After using toothpaste	293.46±32.52	231.00	351.00
Chitosan	Baseline	315.61±25.12	278.00	357.00
	After demineralization	253.00±26.23	212.00	273.00
	After using toothpaste	285.61±19.81	249.00	320.00
CPP-ACP+ SMFP	Baseline	298.23±29.14	279.00	371.00
	After demineralization	251.92±28.67	214.00	281.00
	After using toothpaste	290.46±30.19	249.00	320.00

CPP-ACP+SMFP: Casein phosphopeptide-amorphous calcium phosphate and sodium monofluorophosphate; SD: Standard deviation

Table 3: Comparing mean of descriptive indices of enamel microhardness recovery (unit: %) in three toothpastes

Toothpaste	Mean±SD	Minimum	Maximum
CPP-ACP+SMFP	55.79±14.06	27.94	78.57
Chitosan	52.47±15.89	24.36	71.93
Fluoride	75.08±8.83	53.97	86.11

CPP-ACP+SMFP: Casein phosphopeptide-amorphous calcium phosphate and sodium monofluorophosphate; SD: Standard deviation

are compatible with our results. On the other hand, Vyavhare *et al*.[30] showed that nanohydroxyapatite and fluoride have the potential for remineralization of incipient enamel caries and CPP-ACP can be used as an effective therapeutic material with fluoride.[30]

Chitosan as well was shown to be effective in remineralization. Chitosan and its derivatives are identified as a therapeutic combination in dentistry because their connection to the cellular wall and bacterial membrane creates bacteriostatic and bactericidal effects.[16,19,31] Arnaud *et al*.[17] suggested that chitosan interfere with the enamel demineralization process and prevents phosphor release and it acts as an inhibitor for acid penetration.[17] Visveswaraiah and Prasad[32] showed that both solutions of carboxymethyl chitosan and chitosan lactate can protect enamel against caries initiation.[32] The final results of our research and these studies indicated that chitosan has a positive role in preventing caries.

When pH-cycling is used for assessing anti-caries effects of therapeutic combinations, limitations of generalizing experimental results to clinical conditions need to be recognized. Although this model is superior to other models, there are difference between this model and dynamic and ever-changing environment of the oral cavity. A pH-cycling model cannot simulate different food habits, various health status, and quality of saliva content the same as real mouth condition. For example, fluoride is diluted by saliva. Therefore, *in vitro* studies cannot completely simulate the oral environment, and further clinical studies are warranted.

CONCLUSION

All toothpastes incorporating sodium fluoride, CPP-ACP+SMFP, and chitosan were capable of remineralizing the primary enamel. However, the toothpaste containing NaF acted more effectively compared to the other two; whereas, chitosan and CPP-ACP+SMFP had rather close results.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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