The Effects of Nano-Hydroxyapatite and Casein Phosphopeptide-Amorphous Calcium Phosphate in Preventing Loss of Minerals from Teeth after Exposure to an Acidic Beverage (An *In vitro* Study)

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ABSTRACT

Background: This study aimed to evaluate the effect of antierosive agents (10% Nano-Hydroxyapatite (NHA), 10% Casein Phophopeptide-Amorphous Calcium Phosphate (CPP-ACP), and combination of 10% NHA and 10% CPP-ACP) on loss of minerals from enamel surface of permanent teeth treated with antierosive agents when exposed to an acidic beverage and investigate the morphological changes of treated enamel surface after demineralization with cola based beverage under Scanning Electron Microscope (SEM).

Materials and Methods: Sixty maxillary first premolars were randomly divided into four groups, 15 teeth for each group. Group I treated with 10% NHA, Group II treated with 10% CPP-ACP, Group III treated with 10% NHA and 10% CPP-ACP, and Group IV did not treat with any remineralizing agents. The teeth were immersed in the remineralizing solutions (10% NHA, 10% CPP-ACP, and combination of 10% NHA and 10% CPP-ACP) for 4 minutes twice daily for 28 days and then stored in the artificial saliva. The samples were immersed for 40 minutes in 20 ml Pepsi cola (PH=2.5). Atomic Absorption Spectrophotometer (AAS) was used to record the calcium and phosphorus concentrationsin Pepsi cola before and after demineralization with cola based beverage. SEM also usedto examine the morphological changes occurs in enamel surface of each group after demineralization with cola based beverage.

Results: Statistically, there is a highly significant increase in calcium concentration in Pepsi cola (mg/dl) after demineralization with cola based beverage. Group I showed the lowest changes in calcium concentration values among the three studied groups. Group II was the next, which also showed lower changes in calcium concentration values, then group III while the highest changes were recorded in group IV. There is a highly significant reduction in phosphorus concentration in Pepsi cola (mg/dl) after demineralization with cola based beverage. Group I showed the lowest changes in phosphorus concentration values among the three studied groups. Group II was the next, which also showed lower changes in phosphorus concentration values, then group III while the highest changes were recorded in group IV. Statistically, a highly significant difference was showed in calcium and phosphorus concentrations between the four studied groups after demineralization with cola based beverage. Group IV has a highly significant difference in comparison to group I, group II, and group III.

Conclusions: Both the remineralizing agents (NHA and CPP-ACP) were found to be effective in inhibiting the demineralization caused by cola based beverage.The combination of NHA and CPP-ACP had no synergistic effect on remineralization.

Key words: Nano-Hydroxyapatite, Casein Phophopeptide-Amorphous Calcium Phosphate, Erosion, Scanning Electron Microscope. (J Bagh Coll Dentistry 2015; 27(4):132-137).

INTRODUCTION

Dental erosion is defined as the irreversible loss of tooth substance by a chemical process not involving bacteria. The main cause of dental erosion is acid exposure [(1)] ; this may be caused by a sudden increase in consumption of soft drinks, diet cokes and fruit juices, which seems to be more significant compared to the other etiologic factors [(2)]. Considering the deteriorating effects of acidic beverages on the microhardness of dental enamel, it is essential to search for a material that induces remineralization of erosive lesions. The results of a large number of studies have shown that tooth pastes and mouth washes containing NHA have the potential to remineralize initial erosive lesions [(3)].

Nanotechnology is defined as the construction of materials, parts and useful systems with nanometer dimensions in longitudinal scale and subsequent application of the novel characteristics of this minimal scale technology [(4, 5)]. NHA is one of the byproduct of nanotechnology, with high tissue biocompatibility; therefore production of hydroxyapatite crystals in Nanosizes enables higher compatibility with enamel crystals [(6)]. NHA was used as a source of calcium and phosphorus in a sports drink. It has some advantages of high biocompatibility and chemical homogeneity with sound tooth enamel. Sports drink with NHA may prevent dental erosion [(7)].

CPP-ACP nanocomplex had been shown to localize at the tooth surface and prevent enamel demineralization in laboratory, animal and human in situ trials [(8)]. The CPP-ACP has also been shown to remineralize enamel subsurface lesions in situ when delivered in oral care products. The

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proposed anticariogenic mechanism for CPP-ACP is the localization of amorphous calcium phosphate (ACP) at the tooth surface which buffers the free calcium and phosphate ion activities, thereby helping to maintain a state of supersaturation with respect to tooth enamel, preventing demineralizationand enhancing remineralization[9]. In the present study, the effect of remineralizing agents was evaluated on preventing the loss of minerals from treated enamel surface of permanent teeth after exposure to an acidic beverage. As far as it is known there is no previous Iraqi study regarding the effect of NHA in preventing the loss of minerals from the enamel surface after exposure to an acidic beverage, for this reason this study was conducted.

MATERIALS AND METHODS

The Sample:

Sixty sound human upper first permanent premolars freshly extracted for orthodontic purposes were used in this study with age 12 years old.

Sample Preparation:

The extracted teeth were cleaned using conventional hand piece and rubber cup with non-fluoridated pumice and stored in deionized water with thymol crystals at room temperature. The teeth were examined by visible light cure machine, any tooth had a visible fracture or a crack was discarded [10]. A circle of 6mm in diameter were prepared on the buccal surface of each tooth by using adhesive tape. Then an acid resistant nail varnish was used to paint each tooth, the adhesive tape was removed leaving a circular window of 6 mm in diameter on the buccal surface of each tooth [11].

Preparation of Remineralizing Solutions:

Nano-Hydroxyapatite used in this study was hydroxyapatite Nanopowder Ca_{10} (PO_4) $_6$ (OH) $_2$ from mknano, Mississauga, Canada. 10% NHA suspension solution, using 2 M HCl, PH adjusted to 7 [6, 12]. 10% CPP-ACP is produced by hydrolysis of casein to form casein peptide, which is then complexed with amorphous calcium phosphate to form a casein phosphopeptide - amorphous calcium phosphate complex. The casein (10% w/v), trypsin (0.05% w/v), calcium chloride (100 mmol/L), disodium hydrogen phosphate (60 mmol/L) [13].

Preparation of Artificial Saliva:

To prepare one liter of artificial saliva, different materials in different concentrations were dissolved in (985.5) ml deionized water. These materials are: Sodium carboxymethyl cellulose (10 gm), Sodium chloride (1 gm), Sodium fluoride (0.0002 gm), Calcium chloride (0.05 gm), Potassium thiocyanate (0.01gm), Sorbitol (1 gm), Potassium chloride (1gm), Magnesium chloride (0.05 gm), Potassium phosphate (0.04 gm). Sodium carboxymethyl cellulose dissolved in 100 ml boiling water and after cooling, each other material was dissolved in deionized water and added to sodium carboxymethyl cellulose solution, pH was equal to 7 [14].

Sample Grouping:

Sixty teeth were divided into four groups according to the type of treatment.

Group I: 15 teeth treated with 10% NHA solution.

Group II: 15 teeth treated with 10% CPP-ACP solution.

Group III: 15 teeth treated with [10% NHA and 10% CPP-ACP] solution.

Group IV: 15 teeth left without treatment placed in artificial saliva.

The teeth were treated with remineralizing solutions for 4 minutes twice daily, once in the morning and once at night for 28 days and then stored in artificial saliva between exposure to remineralizing solutions and for the remaining 12 hours overnight. Each tooth was rinsed and dried with a piece of cotton before and after each immersion for 2 minutes and then stored in artificial saliva. Incubator was used to maintain the temperature at 37ºC. After the samples were remineralized for 28 days, they were immersed in 20 ml Pepsi cola (pH=2.5) for 40 minutes [15].

Biochemical Analysis of Pepsi Cola:

Calcium of Pepsi cola was analysed by using flame atomic absorption spectrophotometer following standardized procedure. Inorganic phosphorus of Pepsi cola measured by using a ready-made kit of (Biomagrhreb, Tunisia) and analyzed by UV visible recording spectrophotometry (Cecil CE 7200 UK) machine.

SEM Examination:

Representative specimens from all groups were randomly selected for SEM sample preparation. These were then examined by using Scanning electron microscope SEM (Tescan-Vega, USA) [6,12,15].

Statistical Analysis

Data were computerized and analyzed using SPSS 19.0 software. Student's paired t-test was used to compare the calcium and phosphorus concentrations before and after demineralization with cola based beverage in the four studied groupsand ANOVA was used to compare the calcium and phosphorus concentrations among groups after demineralization with cola based

beverage in the four studied groups, followed by LSD test.

RESULTS

Statistically, there is a highly significant increase in calcium concentration in Pepsi cola (mg/dl) after demineralization with cola based beverage. Group I showed the lowest changes in calcium concentration values among the three studied groups (1.72 ± 0.08). Group II was the next, which also showed lower changes in calcium concentration values (1.96 ± 0.12), then group III (2.31 ± 0.08) while the highest changes were recorded in group IV (3.72 ± 0.12) as demonstrated in Table (1).

There is a highly significant reduction in phosphorus concentration in Pepsi cola (mg/dl) after demineralization with cola based beverage. Group I showed the lowest changes in phosphorus concentration values among the three studied groups (9.56 ± 0.24). Group II was the next, which also showed lower changes in phosphorus concentration values (8.71 ± 0.18), then group III (7.68 ± 0.19) while the highest changes were recorded in group IV (5.59 ± 0.19) as shown in table (2).

Statistically, a highly significant difference was showed in calcium and phosphorus concentrations between the four studied groups after demineralization with cola based beverage. Group IV has a highly significant difference in comparison to group I, group II, and group III (Table 3 and 4).

As shown in figure (1): (a) a smooth and intact surface was obtained in the normal anatomical enamel surface before demineralization with cola based beverage. (b) Specimen treated with 10% NHA and demineralized with cola based beverage show that acicular crystals of NHA sedimented on the enamel surface. This sedimented new layer will resist the demineralization. (c) Specimen treated with CPP-ACP and demineralized with cola based beverage, microscopic irregularities on the enamel surface were observed as adherent granules or globules. The deposited minerals on the enamel surface following mobilization of calcium and phosphate from the CPP-ACP will resist the demineralization. (d) specimen treated with 10% NHA and 10% CPP-ACP and demineralized with cola based beverage show cubes like crystals formed from CPP-ACP, were disorderly distributed on the enamel surface and filled with NHA crystals. The new layer which sedimented on the enamel surface of the tooth didn't resist the demineralization, so a honeycomb structure appeared in some regions on the surface of erosive lesions. (e) Specimen that not treated with any remineralizing agents and demineralized with cola based beverage show many micro pores and honeycomb structure were apparent on the enamel surface. This specimen can't resist the demineralization.

Table (1): Descriptive Statistics and State Difference of Calcium Concentration (mg/dl) in Pepsi Cola for the Four Studied Groups before and after Demineralization with Cola Based Beverage.

Groups	State	Descriptive Statistics				States' comparison (d.f.=14)		
		Mean	S.D.	Min.	Max.	t-test	P-value	Sig.
NHA	Before	1.50	0.00	1.50	1.50	-11.03	0.000	HS
	After	1.72	0.08	1.59	1.87			
CPP-ACP	Before	1.50	0.00	1.50	1.50	-14.45	0.000	HS
	After	1.96	0.12	1.81	2.13			
NHA+ CPP-ACP	Before	1.50	0.00	1.50	1.50	-38.22	0.000	HS
	After	2.31	0.08	2.16	2.46			
No treatment	Before	1.50	0.00	1.50	1.50	-71.82	0.000	HS
	After	3.72	0.12	3.51	3.89			

Table (2): Descriptive Statistics and State Difference of Phosphorus Concentration (mg/dl) in Pepsi Cola for the Four Studied Groups before and after Demineralization with Cola Based Beverage.

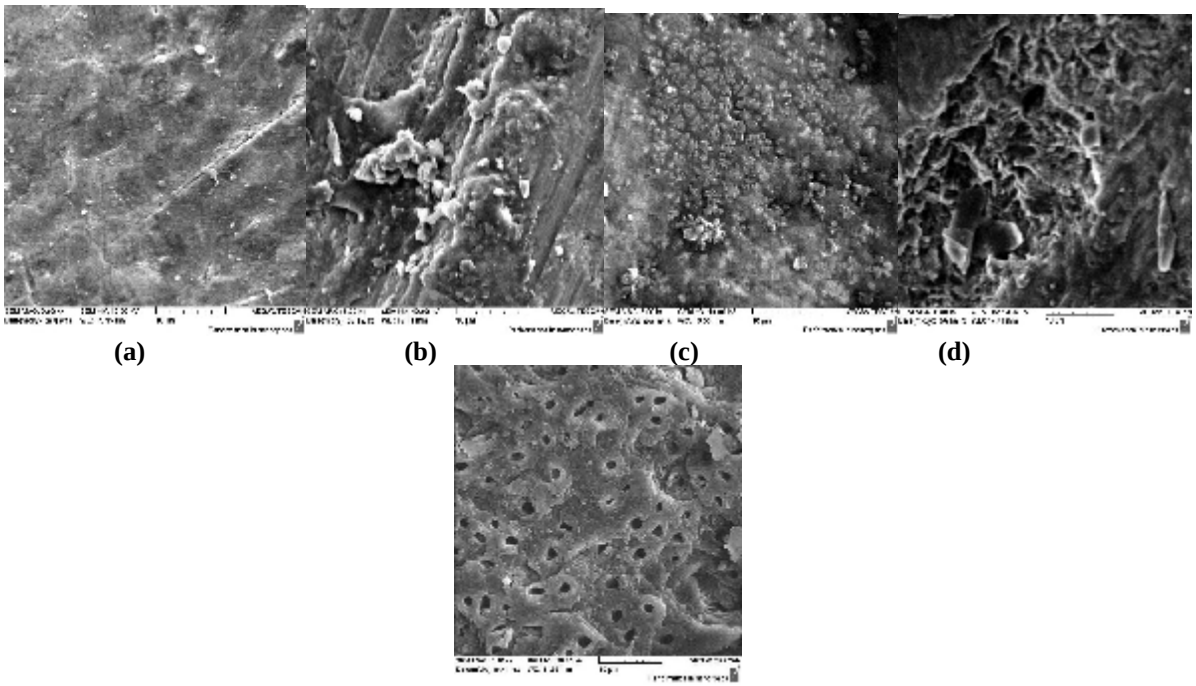
Groups	State	Descriptive Statistics				States' comparison (d.f.=14)		
		Mean	S.D.	Min.	Max.	T-test	P-value	Sig.
NHA	Before	11.50	0.00	11.5	11.5	30.92	0.000	HS
	After	9.56	0.24	9.24	9.96			
CPP-ACP	Before	11.50	0.00	11.5	11.5	59.62	0.000	HS
	After	8.71	0.18	8.28	8.97			
NHA+ CPP-ACP	Before	11.50	0.00	11.5	11.5	76.36	0.000	HS
	After	7.68	0.19	7.34	7.98			
No treatment	Before	11.50	0.00	11.5	11.5	117.86	0.000	HS
	After	5.59	0.19	5.25	5.84			

Table (3): Descriptive Statistics and Group Difference for Calcium Concentration (mg/dl) in Pepsi Cola after Demineralization with Cola Based Beverage.

Groups	Descriptive Statistics				Groups' comparison (d.f.=59)		
	Mean	S.D.	Min.	Max.	F-test	p-value	Sig.
NHA	1.72	0.08	1.59	1.87	1148.77	0.000	HS
CPP-ACP	1.96	0.12	1.81	2.13			
NHA+CPP-ACP	2.31	0.08	2.16	2.46			
No treatment	3.72	0.12	3.51	3.89			

Table (4): Descriptive Statistics and Group Difference for Phosphorus Concentration (mg/dl) in Pepsi Cola after Demineralization with Cola Based Beverage.

Groups	Descriptive Statistics				Groups' comparison (d.f.=59)		
	Mean	S.D.	Min.	Max.	F-test	p-value	Sig.
NHA	9.56	0.24	9.24	9.96	1053.09	0.000	HS
CPP-ACP	8.71	0.18	8.28	8.97			
NHA+CPP-ACP	7.68	0.19	7.34	7.98			
No treatment	5.59	0.19	5.25	5.84			



(a) (b) (c) (d)

(e)

Figure (1): SEM Images of a Representative Specimen in Different Groups after Demineralization with Cola Based Beverage: *(a)* Sound Enamel Surface, *(b)* Group I (Treated with NHA, *(c)* Group II (Treated with CPP-ACP), *(d)* Group III (Treated with NHA and CPP-ACP), *(e)* Group IV (No Treatment).

DISCUSSION

Dental erosion was effectively prevented by the increase in PH and the degree of saturation with respect to dental enamel (DS_{En}) which was caused by abundant release of calcium, phosphate and hydroxide ions from remineralizing agents, and the incapability of phosphoric acid to chelate with enamel calcium. Most calcium compounds are known to have low solubility and it makes them less manageable. However, in the present study the use of nanotechnology allowed the release of ions from hydroxyapatite in great quantities. The solubility was reported to dramatically increase per unit time due to the higher specific surface area and low crystallinity of the nano-sized crystals. Therefore more active constituents can be released [16-18].

Due to low solubility of pure hydroxyapatite, no enough calcium and phosphorus were available to increase the stability of hydroxyapatite in the enamel and to prevent dissolution of the dental enamel. Since the surface area and proportion of atomicity increase with decreasing particle size, NHA has bioactive and biocompatible properties to be used in this study [19].

Pepsi cola was used in this study to induce artificial erosive effect as in other studies [20,21]. Pepsi cola is the most popular and most widely drinks used in the country. When the changes in calcium concentrations in Pepsi cola after demineralization with cola based beverage were measured, group I showed the lowest changes in calcium concentrations values among the three studied groups. Group II came the next, which also showed lower changes in calcium concentrations values, then group III while the highest changes were recorded in group IV. There is a highly significant increase in calcium concentrations before and after demineralization of all groups. This increase in calcium concentrations in Pepsi cola means that there was some loss of calcium from the enamel surface of the teeth after demineralization with cola based beverage.

When also the changes in phosphorus concentrations in Pepsi cola after demineralization with cola based beverage were measured, group I showed the lowest changes in phosphorus concentrations values among the three studied groups. Group II came the next, which also showed lower changes in phosphorus concentrations values, then group III while the highest changes were recorded in group IV. There is a highly significant reduction in phosphorus concentration before and after demineralization of all groups. This reduction in phosphorus concentration in Pepsi cola means that the acidic nature of phosphoric acid had the ability to chelate calcium in saliva and on tooth surface, so their potential to cause erosion is strongly dependent on this effect [22].

A highly significant difference in calcium and phosphorus concentration between the four studied groups after demineralization with cola based beverage. Group IV has a highly significant difference in comparison to group I, group II, and group III.

The direction of calcium and phosphorous concentrations were inversely increased. So when the calcium concentration increased, the phosphorous concentration was decreased. This result is in agreement with Hegde et, al., [15].

From the results of this study mentioned above, it has been concluded that both the remineralizing agents (NHA and CPP-ACP) were able to provide protective effect against erosive enamel loss. NHA has better protective effect than CPP-ACP. So using NHA as a mouth wash or adding to tooth paste or sport drink or chewing gum can enhance remineralization and prevent loss of minerals from enamel surface.

On the other hand, both the remineralizing agents (NHA and CPP-ACP) were found to be effectivein inhibiting the demineralization caused by cola based beverage. Among the remineralizing agents used in the present study, NHA was found to be more effective than CPP-ACP and combination of NHA andCPP-ACP. The combination of NHA and CPP-ACP had no synergistic effect on remineralization.

REFERENCES

1- Neville BW, Damm D, Allen GM, Bouquot JE. Oral and Maxillo Facial Pathology. 2nd ed. New York: Sheefer John; 2002. p. 55-58.
2- Bello LL, Al-Hammed N. Pattern of fluid consumption in a sample of Saudi Arabian adolescents aged 12-13 years. Int J Pediatr Dent 2006; 16(3): 168-73.
3- Haghgoo R, Abbasi F, Rezvani MB. Evaluation of the effect of nano-hydroxyapatite on erosive lesions of the enamel of permanent teeth following exposure to soft beer in vitro. Scientific Research and Essays 2011; 6(26): 5933-6.
4- Freitas RA. Nanodentistry. J Am Dent Assoc 2000; 131:1559-65.
5- Jhaver HM. Nanotechnology: The future of dentistry.J Nano Sci Nano Technol 2005; 5: 15-7.
6- Huang S, Gao S, Yu H. Effect of nano- hydroxyapatite concentration on remineralization of initial enamel lesion in vitro. Biomed Mater 2009; 4(3): 034101.
7- Min JH, Kwon HK, Kim BI. The addition of nano-sized hydroxyapatite to a sports drink to inhibit dental erosion: in vitro study using bovine enamel. J Dent 2011; 39(9): 629-35.
8- Reynolds EC. Remineralization of enamel sub surface lesions by casein phosphopeptide stabilized calcium phosphate solutions. Dent Res J 1997; 76: 1587-95.

9- Reynolds EC, Cai F, Cochrane NJ, Shen P, Walker GD, Morgan MV. Fluoride and casein phosphopeptide - amorphous calcium phosphate. Res Dent J 2008; 87: 344-8.
10- Parry J, Shaw L, Arnand MJ, Smith AJ. Investigation of mineral waters and soft drinks in relation to dental erosion. J Oral Rehabil 2001; 28: 766-72.
11- Bartlett DW, Coward PY. Comparison of the erosive potential of gastric juice and a carbonated drink in vitro. J Oral Rehabil 2001; 28: 1045-7.
12- Huang S, Gao S, Cheng L, Yu H. Combined effects of nano-hydroxyapatite and gallachinensis on remineralization of initial enamel lesion in vitro. Journal of Dentistry 2010; 38: 811–9 (IVSL).
13- Tarautino LM. Opinion of an expert panel on the generally recognized as safe (GRAS) status of Calcium casien peptone-calcium phosphate, 1998.
14- Björklund M, Ouwehand A, Forssten S. Improved artificial saliva for studying the cariogenic effect of carbohydrates. Curr Microbiol 2011; 63(1): 46-9.
15- Hegde MN, Devadiga D, Jemsily PA. Comparative evaluation of effect of acidic beverage on enamel surface pre-treated with various remineralizing agents: An In vitro study: J Cons Dent 2012; 15(4): 351-6.
16- Xu HH, Weir MD, Sun L. Nanocomposites with Ca and PO4 release: effects of reinforcement, dicalcium phosphate particle size and silanization. Dental Materials 2007; 23:1482-91.
17- Weir E, Lawlor A, Whelan A, Regan F. The use of nanoparticles in antimicrobial materials and their characterization. Analyst 2008; 133(7): 835-45.
18- Sun L, Chow LC. Preparation and properties of nano-sized calcium fluoride for dental applications. Dental Materials 2008; 24:111–6.
19- Kaehler T. Nanotechnology: Basic concepts and definitions. Clin Chem 1994; 40: 1797-9.
20- Devlin H, Bassiouny MA, Boston D. Hardness of enamel exposed to Coca-Cola and artificial saliva. J Oral Rehabil 2006; 33: 26-30.
21- Tantbirojn D, Huang A, Ericson MD, Poolthong S. Change in surface hardness of enamel by a cola drink and a CPP-ACP paste. J Dent 2008; 36(1): 74-9.
22- Johansson AK, Lingstrom P, Birkhed D. Comparison of factors potentially related to the occurrence of dental erosion in high and low-erosion groups. Eur J Oral Sci 2002; 110: 204-122.