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Evaluation of Remineralization Potential of Two Agents and pH Cycling on Early Enamel Lesions

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ABSTRACT

Purpose: The purpose of this study was to evaluate & compare the remineralization potential of two remineralizing agents on artificially induced enamel carious lesions via pH cycling. This assessment has been conducted via Surface microhardness analysis and energy dispersive X-ray(EDX) analysis. **Materials and Methods:** In this in vitro study, 20 human teeth were sectioned mesio-distally to obtain 40 enamel samples where artificial carious lesions were induced using an acidic buffer solution. Then samples were randomly divided into two groups (n=20) according to the material used. The first group was for Casein phosphopeptide–amorphous calcium phosphate(CPP-ACP) &the second was for Nano-hydroxyapatite paste(nHAP). Each group was further subdivided into two subgroups(n=10) according to whether subjected to pH cycling or not. The surface microhardness was measured for enamel by Vickers hardness tester at baseline, demineralization and after the application of remineralizing agents in a 15-days pH-cycling model and without it. Similarly, the mineral content of the samples was estimated by EDX analysis. The data were analyzed using independent t-test, ANOVA test and Tukey's post-hoc test. **Results:** Results of the microhardness showed that without pH cycling; CPP-ACP recorded a higher statistically significant difference than nHAP. Additionally, after pH cycling, CPP-ACP recorded a non-significant difference than nHAP. Moreover, EDX results revealed that the Ca/P ratio for the nHAP group recorded the highest statistically significant difference than all tested groups followed by CPP-ACP without pH cycling, baseline and CPP-ACP after pH cycling, respectively. **Conclusion:** CPP-ACP and nHAP both had a positive effect on causing remineralization of enamel. Nano-hydroxyapatite was more effective as compared to CPP-ACP, in increasing the calcium and phosphorus content of enamel in both presence and absence of pH cycling.

KEYWORDS

CPP-ACP, nano-hydroxyapatite, remineralization.

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INTRODUCTION

Dental caries is described as a cyclic process, the metabolism of a fermentable substrate by plaque flora results in periods of demineralization, followed by periods of remineralization [1]. The ratio between cyclic demineralization and remineralization process is the key that determines the progression or prevention of tooth caries[2()]. Demineralization process of enamel is the dissolution and finally loss of carbonated hydroxyapatite minerals from tooth structure due to pH decline of the oral environment [3]. High consumption of acidic beverages as well as acids produced by food fermentation by oral cariogenic bacteria leads to an oral environment with a lower level of mineral ion content in comparing to the enamel mineral and demineralization phase initiated [3,4].

Furthermore, the existence of the demineralization phase for a long period may lead to excessive mineral loss and finally enamel cavitation[3-5]. Fortunately, dental caries is a slow process [6] allowing the early stage of enamel demineralization to be reversible [3,7], when oral pH rise, mineral deposition back to the enamel takes place in the process of remineralization [3,4,5].

Remineralizing agents are part of a new era of dentistry aimed at controlling the demineralization/remineralization cycle, depending upon the microenvironment around the tooth. The rationale of these agents is the remineralization of early carious and non-carious white lesions, advocating a biological or therapeutic approach rather than the traditional surgical approach. With a clearer understanding of these remineralizing agents and new technologies accessible to dentists, we can provide quality dental care using minimally invasive methods[8]. Non-invasive treatment protocol of demineralized enamel lesions as a more conservative procedure by different remineralizing agents would bring a major approach in the clinical management of these enamel defects [9].

Numerous types of remineralizing agents and remineralizing techniques have been researched and many of them are being used clinically, with significantly predictable positive results. The recent researches on remineralization are based on biomimetic remineralization materials, having the capability to create apatite crystals within the completely demineralized collagen fibers [10]. Therefore, focus of dental research has shifted to the development of methodologies for the early detection and use of non-invasive techniques by using remineralizing agents for the effective management of carious lesions [11].

Casein phosphopeptides have recently been used extensively in the field of preventive dentistry. They are utilized alone or as (casein phosphopeptides with amorphous calcium phosphate) CPP-ACP or (casein phosphopeptides with amorphous calcium fluoride phosphate) CPP-ACFP. CPP-ACP has proved to promote the remineralization of the enamel subsurface carious lesions and decrease the demineralization [12]. One of the major properties of CPP is to improve the bioavailability of calcium and phosphate ions level via supersaturation with these ions to enhance remineralization [13]. The CPP-ACP also acts as a reservoir of bioavailable calcium and phosphate and maintains the solution supersaturated, thus facilitating remineralization [14].

Hydroxyapatite is a calcium phosphate compound with the molecular formula $Ca_{10} (PO_4) OH_2$ and a calcium-to phosphorus ratio of 1:67[15]. Nano-hydroxyapatite (n-HAP) is one of the most biocompatible and bioactive materials and has gained immense popularity in dentistry nowadays[10]. Recently, numerous in vitro attempts have been made to prepare enamel-like materials using biomimetic systems that contain nano-apatites or different organic materials used as analogues to organic matrix mainly in the form of slurries, solutions or pastes [16]. Due to the similarity between Nano- HAP (n-HAP) and the apatite crystal of tooth enamel in terms of morphology and crystal structure, it can substitute for the natural mineral constituent of enamel for repair biomimetically[10]. During the nano-HA remineralization, the circular crystals of nano-HA get sedimented onto the tooth enamel and directly fill up defects and micropores on demineralized surfaces. As a result, there is decrease in defects and cavities of the enamel surface and the increase in hardness of the enamel surface[8]. Also, the nanoscale ranges from 1 to 100nm. From these dimensions derives a distinct activity of the particles. Their large reaction surface and small size enhance the hydration of the material, thus gaining better physical and chemical characteristics [17]. Moreover, nanohydroxyapatite is hydrophilic and has a greater surface area than conventional hydroxyapatite crystals, so they have better wettability and form a thin layer on enamel surface that bonds to tooth structure[18].

Furthermore, remineralization of the teeth is directly related to changes in the mineral content, microhardness, and surface morphology. Therefore, in this study, Vickers microhardness and energy-dispersive X-ray spectroscopy (EDX) were used to evaluate the remineralizing potential of CPP-ACP and nHAP pastes on early enamel carious lesions in permanent teeth [19].

MATERIAL AND METHODS

A total of 20 freshly extracted human teeth were used in this study. Ethical approval for the use of extracted human teeth was obtained in accordance with guidelines from research ethics committee approval Faculty of Dental Medicine -Al-Azhar University for girls. Teeth with cracks, fractures, carious lesions of the crown or the root, restorations, and previous endodontic treatment were excluded. The teeth were stored at 4˚C in thymol solution until used. They were used not more than 1-month post-extraction [20]. The teeth were thoroughly washed, scrubbed, and scaled to remove surface debris and contaminants.

Enamel samples' preparation:

In this study, the teeth were sectioned horizontally at the level of the cement-enamel junction, separating the crown and root parts of the tooth. The root portion was discarded [20]. Separation was done by a diamond-coated band saw under continuous water spray. Each tooth was sectioned mesiodistally into two halves (n=40). Each half was trimmed to produce cubic shaped blocks thereafter. Each enamel block was (4mm x4mm) [21].

Polymethyl-methacrylate (PMMA) blocks were constructed on which the samples were individually mounted on them. The superficial surface of enamel was flattened with water-cooled carborundum discs (1200 grit; Water Proof Silicon Carbide Paper, Struers, Germany). Subsequently, any remnants of abrasive paper on the enamel blocks were cleaned with methanol. Then, a diamond paste (15 μm diamond paste, Struers) was used to polish the surfaces, removing about 100 μm of the superficial enamel layer resulting in a flat surface. Thereafter, these slabs were cleaned with distilled water and methanol [21].

Samples' grouping:

The samples were divided randomly into two groups (n=20) according to the type of remineralizing material applied; the first group was for CPP-ACP and the second was for nHAP.

Each of the two groups was further subdivided into two subgroups (n=10) according to whether subjected to pH cycling or not.

Preparation of nano-hydroxyapatite:

Hydroxyapatite nanorods were synthesized by a wet chemical reaction method and preparation of nano grade calcium Phosphate (Ca_2PO_4). Preparation was done by Nano-Gate company. Nasr city (Cairo, Egypt).

Solution preparation:

Demineralization solution:

The demineralization solution was composed of calcium chloride (CaCl2.2H2O) 2.2 mmol/l, potassium dihydrogen phosphate (KH2PO4.7H2O) 2.2 mmol/l, and lactic acid 0.05 mmol/l. The final pH was adjusted to 4.5 with 50% sodium hydroxide (NaOH). It was used thereafter during the test in pH cycling [22].

Remineralization solution:

The remineralization solution was composed of calcium chloride (CaCl2) 1.5 mmol/l, potassium chloride (KCl) 50 mmol/l, potassium dihydrogen phosphate (KH2PO4) 0.9 mmol/l, and Tris buffer 20 mmol/l [22].

Treatment solution:

The pastes of the remineralization materials (CPP-ACP and nHAP) and the remineralizing solution were diluted together in the ratio 1(9 g): 3 (27 mL) to prepare the treatment solution. The mixture was rendered homogenous by using a magnetic stirrer at 350 rpm for 1 min. From this mixture 4.0 mL was recommended for each tooth. Just before treatment, fresh slurry should be prepared for each group. The treatment solutions of nHAP were ultrasonicated immediately after preparation [23].

Preparation of early artificial caries lesions:

All enamel specimens were immersed in 8 mL of demineralization solution for 72 h to induce artificial caries formation and then rinsed 3-consecutive times by 20 mL distilled water for 20 seconds [21].

pH-cycling model:

After demineralization of all the tested samples, half of the samples (n=10) were subjected to pH cycling model and the other half (n=10) were treated directly without pH cycling. The samples subjected to pH cycling were first immersed in treatment solution for 3min followed by remineralizing solution for 1hr then reimmersed in treatment solution for 3 min. The samples were then exposed to remineralizing solution for 2hrs then to demineralizing solution for 2hrs followed by remineralizing solution for 2 more hours. After that, the samples were immersed in treatment solution for 3 min followed by remineralization for 1 hr then reimmersed in treatment solution for 3min. Lastly, the samples were left in the remineralizing solution over night for 16 hrs. After each treatment, the specimens were washed under running distilled water. The treatment regime was repeated daily for 15 days. [21].

Samples' testing:

For both microhardness and EDX analysis, all samples were first tested at baseline and then after demineralization. After that, all the samples were retested after treatment with remineralizing agents with pH cycling and with no pH cycling.

Microhardness testing:

Microhardness values of all samples were measured using digital Vickers microhardness tester and Vickers Microhardness Software (Wilson® Instruments 402 MVD Microhardness Tester). A load of 100 g was applied to the surface for 15 sec. Three indentations were placed on the surface, and the average value of the three readings was recorded. Distances between the indentations were set as minimum 120μm. Vickers microhardness number (VHN) was then computed automatically by the software program and displayed on the screen of the microhardness tester [22].

Quantitative analysis by EDX:

The samples were evaluated quantitatively using the EDX. The energy-dispersive X-ray spectroscopy (EDX) (Jeol JSM- IT200 InTouchScope™ was used to measure the lesion quantitatively; it is a method of microanalysis integrated into a scanning electron microscope. An electron is expelled from an inner shell of an atom, and when an outer shell electron takes the place of the missing electron, energy is released in the form of "X-ray radiation". The X-rays are then analyzed to provide details on the surface's elemental distribution "Calcium and phosphorous" ions concentrations. Then, they were converted into Ca/P ratio for each tested group [24].

Statistical analysis:

All data were statistically analyzed by computer using statistical program SPSS ver. 18.0 (Statistical Package for Scientific Studies, SPSS, Inc., Chicago, IL, USA) for Windows. Numerical data was presented as mean and standard deviation values. The mean and the standard deviation were estimated for quantitative data. The data analysis was performed using Paired t-test, independent t-test, ANOVA test and Tukey's post-hoc test.

RESULTS

Microhardness of enamel:

The results of Vickers microhardness (VHN) of the enamel at baseline, after demineralization and after treatment with and without pH cycling were illustrated in table (1).

Results showed that the highest mean value was recorded at baseline (334.02± 49.65) and the lowest value was recorded after demineralization (232.8±50.69). Moreover, all tested groups recorded statistically significant lower mean values than the baseline except for CPP-ACP group with no pH cycling which showed a non-significant difference with baseline as revealed by Tukey's post hoc test.

Effect of pH cycling:

Within each treatment group CPP-ACP and nHAP, the pH cycling had no statistically significant effect on the microhardness mean values. Additionally, comparing the two treatment groups, results showed that after pH cycling, non-significant difference was recorded between CPP-ACP (274.62±42.93) and nHAP (263.03±54.81). Furthermore, without pH cycling; CPP-ACP (290.30±24.53) recorded a higher statistically significant difference than nHAP (242.25±21.79).

EDX:

The mineral content of the samples estimated by EDX analysis was represented in table (2). The mass % of both Ca and P ions were recorded by EDX at baseline, after demineralization and after treatment and without pH cycling, then the Ca/P ratio was obtained for each group.

The results revealed that Ca and P mass% after demineralization recorded statistically lowest significant difference compared to all other tested groups. The nHAP group recorded the highest statistically significant difference in both Ca mass % and P mass% compared to all other tested groups.

Moreover, the Ca/P ratio for the nHAP group recorded the highest statistically significant difference compared to all tested groups with Ca /P ratio (2.23±0.2), (2.10±0.11) after pH cycling and without pH cycling, respectively. Followed by CPP-ACP without pH cycling (1.95±0.08), baseline (1.90±0.08) and CPP-ACP after pH cycling (1.89±0.06), whereas, the least Ca/P ratio was recorded after demineralization (1.76±0.09).

Effect of pH cycling:

Within each treatment group, pH cycling had a statistically non-significant effect on the mineral content (Ca and P mass %) of the enamel and on the Ca/P ratio.

Table (1): *Comparison of enamel microhardness (VHN) according to treatment.*

	Baseline	After demineralization	CPP-ACP		n-HAP		P- value
			After pH cycling	Without pH cycling	After pH cycling	Without pH cycling	
Mean ±SD	334.02^{a} ±49.65	232.81^{c} ±50.69	274.62^{b} ±42.93	290.30^{a,b} ±24.53	263.03^{b,c}±54.81	242.25^{c} ±21.79	0.00*
P-value	0.00 *		0.3292 ns		0.2799 ns		

*Significance level p≤0.05, * significant, ns=non-significant*

Table (2): *EDX calcium and phosphorus analysis of enamel according to treatment.*

Element		Base-line	After demine-ralization	CPP-ACP		nHAP		P-value
				After pH cycling	Without pH cycling	After pH cycling	Without pH cycling	
Calcium mass%	Mean	29.55^{b}	26.23^{c}	31.14^{b}	31.53^{b}	39.9^{a}	36.40^{a}	0.00*
	SD	1.52	2.29	1.43	1.09	3.5	2.14	
	P (pairwise comparison)	0.0013*		0.64ns		0.09ns		
Phosphorus mass%	Mean	15.57^{c}	14.87^{d}	16.48^{b}	16.22^{b}	17.9^{a}	17.35^{a}	0.00*
	SD	0.56	0.68	0.37	0.92	0.4	0.45	
	P (pairwise comparison)	0.022*		0.574ns		0.074ns		
Ca/P ratio	Mean	1.90^{b}	1.76^{c}	1.89^{b}	1.95^{b}	2.23^{a}	2.10^{a}	0.00*
	SD	0.08	0.09	0.06	0.08	0.20	0.11	
	P (pairwise comparison)	0.0017*		0.217ns		0.239ns		

*Significance level p≤0.05, * significant Tukey's post hoc test: Within the same comparison, means sharing the same superscript letter are not significantly different.*

DISCUSSION

In the recent era of minimal intervention dentistry, various agents have been produced to enhance remineralization potential of the demineralized enamel [(25)]. Therefore, the aim of this study was to evaluate the remineralization ability of Casein phosphopeptide agent versus nano-hydroxyapatite agent. The effect of pH cycling was also investigated.

There is a high-level of evidence supporting the remineralizing efficacy of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) from studies around the world. Evidence is now emerging that CPP-ACP may also have a beneficial influence on the dental plaque, microbial ecology and homeostasis. The ecological cariostatic effects of CPP-ACP are believed to be mediated predominantly through its anti-adhesion, buffering and biofilm disrupting actions [(26)].

In this study, HAP nanorods was used as one of the most biocompatible and bioactive materials[(27)]. As, the coating effect of the micro-structured hydroxyapatite nanoparticles reintegrates the enamel with a biomimetic film reproducing the structure and the morphology of the biologic hydroxyapatite of the enamel. It was demonstrated that the coating is due to the deposit of a new layer of apatite, which presents fewer particles than the natural enamel, not based on the chemical-physical changes occurring in fluorinated toothpastes. Moreover, it shows resistance to brushing as a consequence of chemical bonds between the synthetic and natural crystals of the enamel[28()]. The remineralization process of early carious lesions has been proved to be influenced by the morphological structure and the surface chemical properties of nHAP. The concentration as well as the precipitation rate and amount of nHAP are in direct relation. As the concentration increases the other properties would also increase leading to the deposition of considerable amounts of Ca^{+2} and $PO3^{-4}$, hence extensively improving remineralization [(29)].

In the present research, Vickers hardness method was used to check microhardness because it was non-destructive, very reliable, rapid, and economical as compared to other hardness tests [(30)]. Moreover, EDX was used as it is a microanalytical technique that undergoes elemental analysis at the ultrastructural level [31].

In the current study, both nHAP and CPP-ACP pastes were able to remineralize early enamel lesions. The results displayed that the microhardness of demineralized enamel surfaces in all groups significantly decreased, implying loss of minerals. This could be attributed to the fact that all the samples were subjected to the same demineralizing solution and immersion time for the aim of standardization.

Comparing the results of the two treatment groups without pH cycling, CPP-ACP showed significantly higher mean microhardness value than nHAP. Additionally, CPP-ACP with no pH cycling recorded a non-significant difference than baseline. This could be clarified by the ability of Casien phosphopeptide to form nano clusters with amorphous calcium phosphate ensuring a reservoir of calcium and phosphate ions which can preserve the supersaturation of saliva. Meanwhile, CPP-ACP has a buffering effect by stabilizing these ions in the solution [32].

The pH-cycling model presented a good mimic of the oral environmental conditions of dental caries in comparison to individual demineralization and remineralization studies [33]. For this reason, this study assessed the pH cycling effect on the remineralization potential of CPP–ACP and nHAP pastes on initial enamel caries lesions. When comparing the materials under different pH cycling conditions, results demonstrated that there was a statistically insignificant change of microhardness within the same treatment group. This was inconsistent with other studies [34,35] which explained that the surface microhardness increased significantly when pH value dropped below 7.0 and the highest microhardness value was recorded at pH 4.0, when compared with neutral group. It was stated that an acidic solution had a significant potential to improve the mineral ions deposition during mineralization when compared to a neutral solution. Those findings could be related to differences in pH cycling regimes, microhardness assessment methods, different ingredients of the pastes, and differences in the tooth structures in these studies.

On another level, EDX analysis results indicated that there was a decrease in the mass % of both calcium and phosphorus with the least Ca/P ratio following the demineralization of samples due to loss of minerals from the enamel after exposure to the demineralizing solution. Moreover, after the treatment for 15 days, nHAP after pH cycling recorded the highest Ca/P ratio in addition to increased mineral content (calcium and phosphorus) as compared to CPP-ACP group. This could be a reflection of more mineral's precipitation in the deep parts coinciding with pH reduction which might be directly associated with the increased solubility of nHAP in the acidic condition that was also supported by another study [36]. Furthermore, nHAP with no pH cycling recorded a significantly higher Ca/P ratio than CPP-ACP group. This might be accounted for the calcium nano-phosphate crystals that have penetrated and deposited deeper into the defects of the carious enamel, forming a "reservoir-like" precipitate of Ca^{+2} and P^{-4} ions. This precipitate ensures availability of these ions coinciding with the cariogenic process, preserving a supersaturation with enamel minerals [37]. These results were in accordance with another study [25].

Furthermore, results showed that CPP-ACP recorded higher microhardness mean value as compared to nHAP. On the other side, it recorded lower Ca/P ratio than nHAP. This contradicting result might be explained by the presence of high calcium content which rapidly precipitate ions on the superficial layer of the enamel caries [38], thus increasing the surface hardness. While it prevented the remineralization process from occurring in the depth of the lesion that would inherently prevent rapid precipitation of calcium and phosphate and therefore led to lower Ca/ P ratio. This finding was in agreement with a study which concluded that although CPP-ACP can remineralize surface lesion, it is not effective in remineralizing the early enamel caries at the subsurface level [39].

CONCLUSIONS

Within the limitations of the present in vitro study, the following could be concluded:

1. Both agents nHAP and CPP-ACP were effective in causing remineralization of demineralized enamel.
2. CPP-ACP showed improved hardness of enamel over nHAP with no pH cycling.
3. nHAP was observed to be more effective in increasing the calcium and phosphorus content of enamel compared with CPP-ACP.

CONFLICT OF INTEREST

None declared.

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