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EVALUATION OF BIOMIMETIC REMINERALIZATION OF DEMINERALIZED ENAMEL USING DIFFERENT CONCENTRATIONS OF NANOCOMPLEXES OF PHOSPHORYLATED CHITOSAN AND AMORPHOUS CALCIUM PHOSPHATE

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ABSTRACT

Objective: This study was conducted to evaluate the remineralizing efficacy of biomimetic remineralization of demineralized enamel using different concentrations and different time of application of nanocomplexes phosphorylated chitosan- amorphous calcium phosphate on artificially induced enamel lesions using mineral content assessment (EDX) at different storage time. **Material and methods:** A total number of 40 bovine incisors from the second dentition were selected for this in vitro study. Teeth with cracks, caries were excluded. They were divided into two main groups (n = 20 each) according to the concentration of remineralizing agent. Each group was divided into two subgroups (n=10 each) according to time of application (2 minutes and 3 minutes). **Results:** The present study has shown that a statistically significant difference was found between demineralized specimens and all experimental groups. Specimens stored for three months showed the highest mean value of remineralization (the best result), while specimens stored for 1month showed the lowest mean value of remineralization, regardless to different concentration of remineralizing agents and their time of application. **Conclusion:** Nanocomplexes phosphorylated chitosan amorphous calcium phosphate is considered a precious biomimetic remineralizing agent. Storage time plays an important role during remineralizing process as mineral precipitation to tooth structure is a continuous dynamic process.

KEYWORDS: Biomimetic remineralization, Subclinical carious lesions, Phosphorylated chitosan, Amorphous calcium phosphate, SEM/EDX.

INTRODUCTION

Dental caries is the major cause of the hard-dental tissue disease in the human population. Early enamel caries can be described as a subsurface carious lesion of the enamel due to unharmed enamel surface with a subsurface demineralized zone. With the new understanding of the dental caries process, dental societies are directed towards finding non-invasive, and cost-efficient techniques to prevent lesion progress. New approaches have been devised to manage dental caries, such as biomimetic remineralization[1,2].

Recently, a further innovative biomimetic strategy for remineralizing enamel subsurface lesions has been introduced using nanocomplexes-phosphorylated chitosan and amorphous calcium

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Phosphate (Pchi–ACP)[3,4]. Chitosan and its derivatives have emerged as a new class of novel biomaterials due to their versatile biological activity, excellent biocompatibility and complete biodegradability[5]. Among these derivatives of chitosan, Pchi exhibits bactericidal, biocompatible, bio absorbable, and metal chelating properties[6]. Phosphorylated chitosan could be used to stabilize ACP to form the nano-complexes of Pchi–ACP for remineralizing enamel subsurface lesion based on the biomimetic strategy [7,8].

The hypothesis tested is that nano-complexes phosphorylated chitosan-amorphous calcium phosphate have effective remineralization potential regarding to remineralizing early enamel caries lesion. The aim of this in vitro study was to evaluate the remineralizing efficacy of biomimetic remineralization of demineralized enamel using different concentrations (2.5%, and 5% nano complex Pchi–ACP), and different time of application (2 and 3 minutes) of nano-complexes phosphorylated chitosan- amorphous calcium phosphate (Pchi-ACP) on artificially induced enamel lesions using mineral content assessment (EDX) at different storage time (1 and 3 months) at baseline, after demineralization, after remineralization.

MATERIAL AND METHODS

- Materials used in this study were remineralizing agents (2.5%, and 5% nano complex Pchi–ACP), demineralizing solution and artificial saliva.
- A total number of 40 bovine incisors from the second dentition were selected for this in vitro study. Teeth with cracks, caries were excluded. They were divided into two main groups (n=20 each) according to the concentration of remineralizing agent. Each group was divided into two subgroups (n=10 each) according to time of application (2 minutes and 3 minutes). Then each subgroup was subdivided into two divisions according to time of storage in artificial saliva till evaluation time.

TABLE (1) Variables of the study

Variable	Symbol	Refer to
Remineralizing agents (A,B)	A1	2.5% Nano-complexes Pchi–ACP (2 minutes)
	A2	2.5% Nano-complexes Pchi–ACP (3 minutes)
	B1	5% Nano-complexes Pchi–ACP (2 minutes)
	B2	5% Nano-complexes Pchi–ACP (3 minutes)
Time (T)	T1	1 month
	T2	3 month

- Freshly extracted bovine teeth were stored in distilled water. Crowns were cut off at the cemento-enamel junction to be separated from roots by diamond discs using a low-speed handpiece under copious water irrigation. A specially fabricated cylindrical polyvinyl chloride (PVC) ring of internal diameter 10 mm and 15mm height was fabricated. The mould was filled with self-curing acrylic resin. Each crown was embedded horizontally in middle of the mould with the labial surfaces facing upwards leaving about two mm from the labial surface projecting above the surface of the mould using caliber [9].
- The enamel surface of each specimen was covered with two coats of an acid-resistant, purple nail varnish. Digital caliber was used to standardize a window of sound enamel of about 6mm×6mm in the middle of the labial surface.
- White spot lesions were created with standardized lesion depth of 300 μm representative to ICDAS II score 1[10,11] by immersing the specimens in a demineralizing solution for four days without changing the solution to keep the lesion depth constant, then washed under running water for 30second [4]. The specimens were

divided into two main groups according to the remineralizing agent. It was applied by brushing the surfaces of specimens by a soft brush with minimum pressure. Then immersed in a daily renewed artificial saliva for the specified storage period till testing (T1, T2).

- Assessment of mineral content of the enamel surface was done by using Energy Dispersive X-Ray (EDX). Assessment was done prior to demineralization, then after demineralization, one and three months from remineralization.

Statistical analysis

The mean and standard deviation values were calculated for each group in each test. Data were explored for normality using Kolmogorov-Smirnov & Shapiro-Wilk tests, data showed parametric (normal) distribution. Paired sample t-test was used to compare between two groups in related samples. Independent sample t-test was used to compare between two groups in non-related samples. Three-way ANOVA tests were used to test the interactions between different variables. The significance level was set at P≤0.05. Statistical analysis was performed with IBM® SPSS® Statistics Version 20 for Windows.

RESULTS

Mineral content assessment (EDX)

Ca/P ratio results:

1. ***Effect of concentration of remineralizing agents on Ca/P mineral wt%:***

Regarding both concentration (Group A: 2,5%, Group B:5 %) of remineralizing agents, there was no statistically significant difference between mean Ca/P mineral wt% values of subgroups 1T1, 1T2, 2T1, and 2T2. While the group B revealed the highest mean value of Ca/P mineral wt%. **Table (2), Figure (1)**

TABLE (2) The mean and standard deviation (SD) values of Ca/P with different concentrations of remineralizing agents in relation to different application time and storage time

Variables	Ca/P							
	Group 1 (2 minutes)				Group 2 (3 minutes)			
	T1 (1m)		T2 (3m)		T1 (1m)		T2 (3m)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Group A	1.48	0.06	1.79	0.04	1.50	0.11	1.81	0.04
Group B	1.56	0.07	1.84	0.06	1.57	0.12	1.87	0.08
p-value	**0.090ns**		**0.405ns**		**0.406ns**		**0.058ns**	

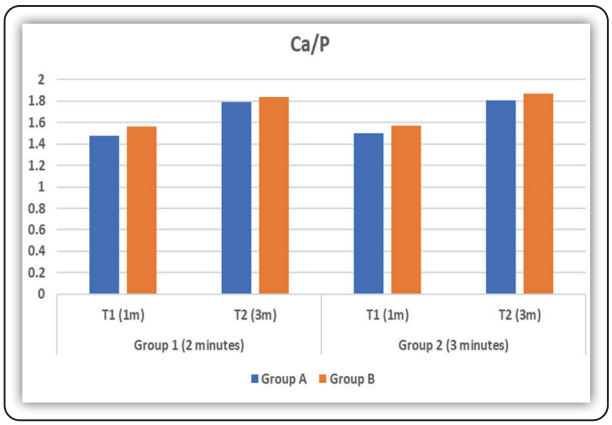

FIG (1) Bar chart representing effect of different concentrations on Ca/P mineral wt%

For Subgroup 1T1 (2 minutes application and 1 month storage time):

There was no statistically significant difference between A1T1 (1.48 ± 0.06), and B1T1 (1.56 ±0.07), where p=0.090. While the B1T1 showed the highest mean value of Ca/P mineral followed by A1T1.

For Subgroup 1T2 (2 minutes application and 3 month storage time):

There was no statistically significant difference between A1T2 (1.79±0.04), and B1T2 (1.84 ±0.06), where p=0.405. While the B1T2 showed highest mean value of Ca/P mineral followed by A1T2.

For Subgroup 2T1 (3 minutes application and 1 month storage time):

There was no statistically significant difference between A2T1 (1.50 ± 0.11), and B2T1(1.57 ±0.12), where p=0.406. While the B2T1 showed highest mean value of Ca/P mineral followed by A2T1.

For Subgroup 2T2 (3 minutes application and 3 month storage time):

There was no statistically significant difference between A2T2 (1.81 ± 0.04), and B2T2 (1.87 ±0.08), where p=0.058. While the B2T2 showed highest mean value of Ca/P mineral followed by A2T2

2. Effect of application time of remineralizing agents on Ca/P mineral wt%:

Regarding both application time of remineralizing agent subgroup 1 (2 minutes) and subgroup 2 (3 minutes) of application, there was no statistically significant difference between mean Ca/P mineral wt% values of subgroups AT1, AT2, BT1, BT2. While subgroup 2 (3 minutes application) revealed the highest mean value of Ca/P mineral wt%. ***Table (3), Figure (2)***

TABLE(3) The mean and standard deviation (SD) values of Ca/P with different application time [1 (2 minutes) and 2 (3 minutes)] in relation to different concentration and storage time

Variables	Ca/P							
	Group A (2.5%)				Group B (5%)			
	T1 (1m)		T2 (3m)		T1 (1m)		T2 (3m)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Group 1 (2 minutes)	1.48	0.06	1.79	0.04	1.56	0.07	1.84	0.06
Group 2 (3 minutes)	1.50	0.11	1.81	0.04	1.57	0.12	1.87	0.08
p-value	**0.569ns**		**0.486ns**		**0.861ns**		**0.346ns**	

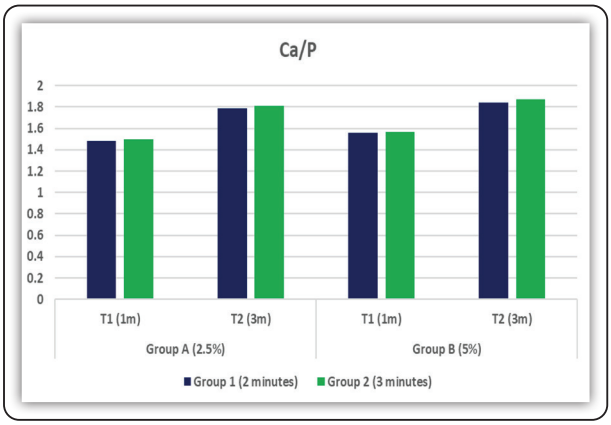

FIG (2) Bar chart representing effect of different application time on Ca/P mineral wt%

For subgroup AT1 (2.5% concentration and 1 month storage time):

There was no statistically significant difference between A1T1 (1.48 ± 0.06), and A2T1 (1.50 ± 0.11), where p=0.569. While the A2T1 revealed the highest mean value of Ca/P mineral followed by A1T1.

For subgroup BT1 (5% concentration and 1 month storage time):

There was no statistically significant difference between B1T1 (1.56±0.07), and B2T1 (1.57±0.12), where p=0.861. While the B2T1 revealed the highest mean value of Ca/P mineral followed by B1T1.

For subgroup AT2 (2.5% concentration and 3 month storage time):

There was no statistically significant difference between A1T2 (1.79 ± 0.04), and A2T2 (1.81±0.04), where p=0.486. While the A2T2 revealed the highest mean value of Ca/P mineral followed by A1T2.

For subgroup BT2 (5% concentration and 3 month storage time):

There was no statistically significant difference between B1T2 (1.84±0.06), and B2T2 (1.87±0.08), where p=0.346. While the B2T2 revealed the highest mean value of Ca/P mineral followed by B1T2.

3. *Effect of storage time of remineralizing agents on Ca/P mineral wt%:*

Regarding both storage time (T1: 1 month and T2: 3 months) of remineralizing agents, there was a statistically significant difference between mean Ca/P mineral wt% values of subgroups A1, A2, B1, and B2. While the group T2 revealed the highest mean value of Ca/P mineral wt%. ***Table (4), Figure (3).***

TABLE (4) The mean and standard deviation (SD) values of Ca/P with different storage time (T1 (1 month) and T2 (3 months)) in relation to different concentration and application time

Variables	Ca/P							
	Group A (2.5%)				**Group B (5%)**			
	A1 (2 minutes)		**A2 (3 minutes)**		**B1 (2 minutes)**		**B2 (3 minutes)**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
T1 (1m)	1.48	0.06	1.50	0.11	1.56	0.07	1.57	0.12
T2 (3m)	1.79	0.04	1.81	0.04	1.84	0.06	1.87	0.08
p-value	**<0.001***		**0.003***		**0.006***		**0.006***	

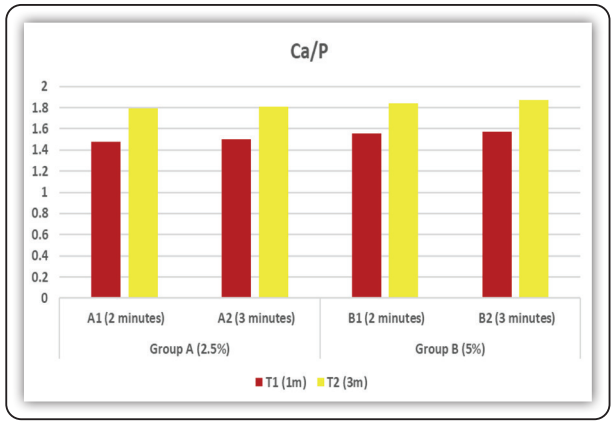

FIG (3) Bar chart representing effect of storage time on Ca/P mineral wt%

For subgroup A1 (2.5% concentration and 2 minutes application):

There was a statistically significant difference between A1T1 (1.48±0.06), and A1T2 (1.79± 0.04), where p<0.001. While the A1T2 revealed the highest mean value of Ca/P mineral followed by A1T1.

For subgroup A2 (2.5% concentration and 3 minutes application):

There was a statistically significant difference between A2T1 (1.50±0.11), and A2T2 (1.81±0.04), where p=0.003. While the A2T2 revealed the highest mean value of Ca/P mineral followed by A2T1.

For subgroup B1 (5% concentration and 2 minutes application):

There was a statistically significant difference between B1T1 (1.56±0.07), and B1T2 (1.84±0.06), where p=0.006. While the B1T2 revealed the highest mean value of Ca/P mineral followed by B1T1.

For subgroup B2 (5% concentration and 3 minutes application):

There was a statistically significant difference between B2T1 (1.57±0.12), and B2T2 (1.87±0.08), where p=0.006. While the B2T2 revealed the highest mean value of Ca/P mineral followed by B2T1.

1. Three-way ANOVA:

Data in ***table (5)*** shows the results of Three-way ANOVA analysis. The results showed that different concentrations had no statistically significant effect. Also, application time had no statistically significant effect. Storage time had a statistically significant effect.

TABLE (5) Results of Three-way ANOVA for the effect of different variables on Ca/P.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	0.934	7	0.133	22.078	0.000
Intercept	112.536	1	112.536	18629.281	0.000
Concentration	0.042	1	0.042	6.886	0.130
Application time	0.001	1	0.001	0.170	0.683
Storage time	0.886	1	0.886	146.688	0.000
Concentration* Application time	0.001	1	0.001	0.220	0.642
Conc.* Storage time	0.000	1	0.000	0.061	0.807
Application time * Storage time	8.362E-05	1	8.362E-05	0.014	0.907
Conc.* Application time* Storage time	0.003	1	0.003	0.509	0.481
Error	0.193	32	0.006		
Total	113.663	40			
Corrected Total	1.127	39			

Scanning Electron Microscopic (SEM)

A) Sound teeth

SEM analysis showed the smooth and homogeneous morphological pattern of the normal enamel surface ***Figure (4)***

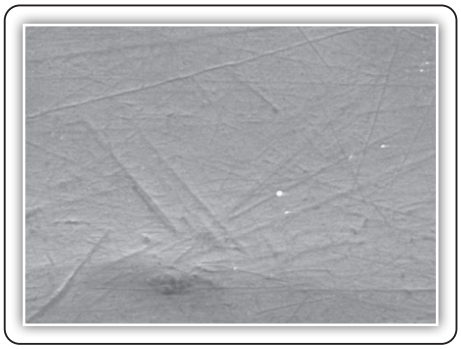

FIG (4) SEM showed sound enamel

B) After demineralization:

Enamel surfaces revealed loss of surface integrity with a number of porous defects suggestive of demineralization ***Figure (5)***

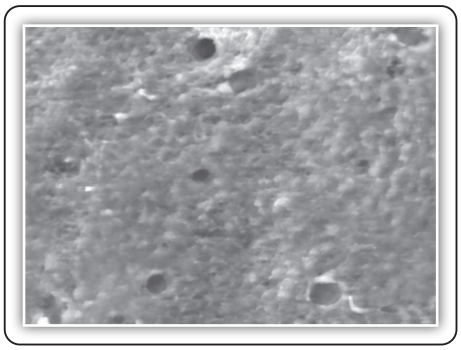

FIG (5) SEM showed demineralized enamel

C) After 1 month remineralization

Decrease in the surface irregularities and filling of some porosities were evident. ***Figure (6)***

D)After 3 months remineralization

This group showed a superior and uniform re-establishment of surface integrity in contrast to other groups. Enamel surface formed a homogeneous and dense layer of mineralized tissue were evident compared to the demineralized group. ***Figure (7)***

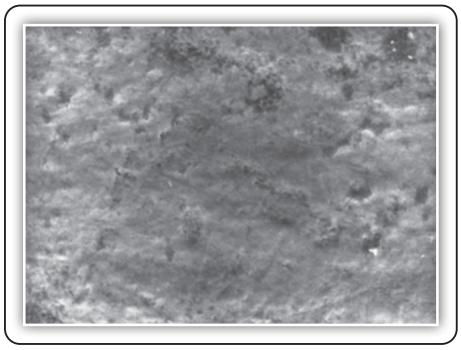

FIG (6) SEM showed enamel remineralization after 1 month

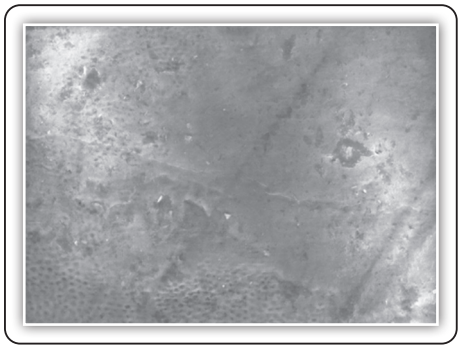

FIG (7) SEM showed enamel remineralization after 3 months

DISCUSSION

Remineralization and demineralization of dental hard tissues depends on the dynamic chemistry within the oral environment[12]. Biomimetic remineralization is a novel approach that mimics the natural process of mineralization. Biomimetic enamel reconstruction is a considerable issue in dentistry and material science as a unique method for the management of dental caries [13,14].

The results of this in vitro study have shown that a statistically significant difference was found between demineralized specimens and all experimental groups. There was no significant difference between all experimental groups regarding to different concentration of remineralizing agents and their time of application. There was a statistically significant difference between intervention groups according to time of storage, where specimens stored for three months showed the highest remineralizing efficacy, while specimens stored for 1month showed the lowest remineralizing efficacy. Hence, the hypothesis was accepted, as nano-complexes of phosphorylated chitosan-amorphous calcium phosphate have effective remineralization potential regarding to remineralization of early enamel caries lesion.

Effect of concentration of remineralizing agents

There was no significant difference between all experimental groups regarding different concentration (2.5% and 5%). Chitosan acts as a template in the biomineralization process and controls the mineral crystallites through the molecular interaction between the polymer and minerals[15]. Saturation of subsurface lesion was achieved in both concentrations as increasing concentration of remineralizing agents is accompanied with higher viscosity which may inversely reduce chitosan permeability. The results obtained in this study are in agreement with studies done by Hanafy et al [16] , and Zhang et al.[6].

Study conducted by Arnaud TM et al.[17] proved that enamel treated with chitosan nanoparticle showed significantly higher calcium phosphate mineral content and higher surface hardness. For chitosan concentrations, both 2.5g/mL and 5.0g/mL, the penetration can be seen up to the dentin–enamel junction which act as a mechanical barrier for the acid penetration in the enamel coinciding with our results ***Figure (1)***.

Effect of application time of remineralizing agents

There was no significant difference between all experimental groups regarding different application time (2 minutes and 3 minutes). Two minutes application revealed high penetration action of Pchi which responsible for ACP stabilization. Nano-complexes of Pchi-ACP could be a good method to provide stable ACP to be absorbed on enamel surface with subsequent transforming into HAP crystals within lesions, the results obtained in this study

are in agreement with study done by Arnaud TM et al.[17] whose time of application was 30, 60, and 90 seconds. 30 s may be a too short time for chitosan action, while the best result for the inhibition of mineral loss, due to chitosan action, irrespective of application time (60 and 90 seconds[17].

In addition, studies done by Alsamolly.[18], and Noaman et al.[19] coincide with the present study proving that 3 minutes time of application showed high remineraliztion potential of demineralized enamel.

Effect of storage time of remineralizing agents

In this study, the results revealed that the remineralization potential of all the tested materials increased significantly over time. While 3 months storage time revealed the highest mean value of calcium and phosphorus mineral wt%. The results obtained in this study are in agreement with study done by Zhang et al.[6] concluded that effectively used phosphorylated chitosan and amorphous calcium phosphate (Pchi–ACP) as biomimetic remineralizing agent. They found changes in mineral profile over 3-months period after biomimetic remineralization using Pchi, while there was no mineral profile changes in the control group samples. This emphasises the importance of prolonged used remineralizing agents in order to increase remineralization potential.

The results obtained in this study are in agreement with study done by Alsamolly [18] and Noaman et al.[19]. Where their results revealed that remineralization potential increased significantly over time. Higher values were obtained after 6 months with a significant difference between 3 months and 6months. They illustrated that chitosan based nanocomplexes are able to induce biomimetic regeneration of early caries lesions.

The results obtained in this study are in agreement with studies done by Ibrahim et al.[20] who demonstrated that there is an evident precipitation of an enamel-like layer in both treatment groups 5 and 10 days storage time using the chitosan biomimetic hydrogel, in contrast with the control group. They revealed that 10 days storage time of chitosan hydrogel revealed a superior and uniform re-establishment of surface integrity in contrast to other groups. They denoted that remineralization is a time-dependent process.

The result of present study was in disagreement with the study carried by Beltrame et al.[21] as they approved that AmF/NaF/SnCl2 presented the most significant reduction in dentin loss upon erosive challenge. On the other hands, the phosphorylated chitosansolutions showed limitations in minimizing dentin surface loss after erosive challenge when compared to the fluoride and metal ion solutions. This difference can be explained by the difference in the time that the specimens were exposed demineralization. Furthermore, differences in the solubilization of chitosan across studies are possible, depending on the pH of the solution which can influence final total concentration.

Regarding to scanning electron microscopic evaluation, repair of the enamel with nanocomplexes Pchi-ACP was evidence by forming enamel-like layer for both incubation periods ***Figure (6,7)***. These findings were in accordance with other studies done by Ibrahim et al.[20] , Cao et al.[22], Hanafy et al.[16]

CONCLUSION

Nanocomplexes Pchi-ACP proved to be an excellent biomimetic remineralizing agent as it enhances formation of nucleating sites to regrow enamel like layer with well-ordered hydroxyapatite structure mimicking natural enamel in physical and mechanical properties the same as biomineralization process.

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