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REMINERALIZATION OF INITIAL ENAMEL LIKE LESIONS WITH CHICKEN EGG SHELL POWDER SOLUTION VERSUS AMORPHOUS CALCIUM PHOSPHATE

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ABSTRACT

Aim: To compare the efficacy of chicken egg shell solution powder (CESP) and amorphous calcium phosphate (ACP) on the remineralization of enamel subsurface lesions.

Materials and Methods: Twenty bovine central incisor teeth were used in this study. The microhardness and the minerals content of all specimens were initially assessed using Vickers hardness tester and EDXA respectively (positive control group).The specimens were artificially demineralized and then reassessed directly after demineralization (negative control group). All demineralized specimens were randomly classified into 2 equal groups,group I :treated with Chicken eggshell powder solution (10 specimens), while, groupII: treated with ACP (10 specimens). The remineralizing agent were applied twice daily for 15 min each for seven successive days. Finally all specimens were reassessed for minerals content (Ca and PO4 weight %) and surface microhardness. The data were statistically analyzed using repeated measures analysis of variance (ANOVA), the significance level was set at $P \leq 0.05$.

Results: Microhardness was significantly decreased in all specimens after demineralization and then it was significantly increased after exposure to therapeutic solutions. There was a significant increase ($P<0.001$) in both Calcium and Phosphorus levels after remineralization using both agents, as detected by EDAX. However, there was no significant difference between the two remineralized groups.

Conclusions: Within the limitations of this in vitro study, it can be concluded that both remineralizing agents were similarly able to increase the microhardness and tooth remineralization. However, being natural products, CESP can be considered as an optimal alternative to the commercial ones.

KEY WORDS: Demineralization, EDAX, Eggshell, Enamel, remineralization, surface microhardness

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INTRODUCTION

Enamel demineralization usually begins with dissolution of the subsurface carbonated hydroxyapatite crystals under the effect of low pH of surrounding acidic media. That's creating fine micro pores and gaps in between the enamel rods [1]. At pH 5.5 H+ ions, produced by the bacterial byproducts or any acidic activity, react preferentially with the phosphate group of the enamel crystals, altering the $(PO4)^{-2}$ ions to $(HPO_4)^{-2}$ ions which, once formed, no more crystal lattice can be formed and at the same time H+ ions are buffered. This leads to enamel dissolution which indicates the beginning of early enamel loss. [2, 3]

Management of initial carious lesions should be of a preventive rather than curative approach. Enamel repair occurs through the deposition of depleted minerals in the demineralized defects at a molecular level. This process needs neutral pH at which sufficient calcium and phosphate ions are available in the surrounding environment. So, remineralizing the exhausted enamel surface with the needed calcium and phosphate ions,increased its resistance to further acid challenge, particularly with the use of enhanced remineralization treatments. [4]

Fluoride containing products have long been recognized as they promote caries lesion remineralization and inhibits demineralization of tooth surfaces subjected to acids related to the caries process [5]. However, utilizing fluoride alone seems questionable as the formation of each fluorapatite molecule requires calcium and phosphate in addition to fluoride ions.[6] Thus, it is necessary to find an efficient, safe alternative to fluoride to completely prevent caries and remineralize the incipient enamel lesions.

Amorphous calcium phosphate(ACP) has been widely applied in biomedical field due to its excellent bioactivity, high cell adhesion, adjustable biodegradation rate and good osteoconduction[7-9] .The first quantitative studies on synthetic ACP were done in the mid-1960s[10]. Afterward, more and more attention has been attracted in the development and the application of ACP-containing products, especially in the dental fields. It is also used as filler in ionomer cements to fill carious lesions or as a colloidal suspension in toothpastes, chewing gums or mouthwashes to promote remineralization of carious lesions and/or to prevent tooth demineralization[11]. As the ACP is not a stabilized structure, it requires a two-phase delivery system to keep the calcium and phosphorus components from reacting with each other before use. [12,13]

Throughout the ages humans have relied on nature to benefit for their basic needs, as medicines for the treatment of a wide spectrum of diseases. [14] Chicken egg shell solution powder (CESP) has been tested in various fields regarding its potential medical use. It is known as Ca rich source which contains about 93% (w/w) elemental Ca with a high degree of bioavailability due to its high $CaCO_3$content. [15]A previous study found that the high pH of the chicken egg shell solution along with its rich bioavailable calcium content provided it with great potentiality to favor the remineralization process.[16] Therefore, further research is necessaryon the efficacy of CESP for remineralization of early enamel lesions.

Thus the aim of this in vitro study was to compare the efficacy of amorphous calcium phosphate (ACP) and Chicken eggshell powder (CESP) in the remineralization of initial enamel subsurface lesions using quantitative energy dispersive X-ray analysis (EDXA) and microhardness analysis. The null hypothesis was that CESP has the same potent remineralizing effect as ACP material.

MATERIALS AND METHODS

Materials:

Two different remineralizing agents were used in the current study; commercial relief ACP and laboratory prepared egg shell solution. Materials, compositions, manufacturers and batch number of the materials used in this study are shown in table I

TABLE (I) Materials, compositions, manufacturers and batch number of the used materials.

Materials	Commercial Product and Manufacturer/ Laboratory Prepared	Compositions	Batch Number
Relief ACP	Dash Philips USA	5%potassium nitrate, 0.22% sodium fluoride, 0.375% amorphous calcium phosphates,water, poloxamer 338, natural peppermint, calcium nitrate, sodium phosphate, sodium saccharin	16314001
Egg shell powder	Laboratory prepared	94% calcium carbonate,1% calcium phosphate, 1% magnesium carbonate, 4% organic matrix [17]	.....
Artificial saliva	Laboratory prepared	(Na-3PO4 (3.90mM), NaCl (4.29mM), KCI (17.98mM), CaCl2 (1.10mM), MgCl2 (0.08mM), H2SO4 (0.50mM), NaHCO3 (3.27 mM)[18]	.....
Demineralizing solution	Laboratory prepared	50 mM acetic acid derivation, 2.25 mM $CaCl_2$ $2H_2O$, 1.35 mMKH_2PO_4; 130 mm KCl[19,20]	.....

Methodology:

Specimen preparation:

Twenty sound bovine incisor teeth were selected for this study. The teeth were examined using magnifying loupes at 3X (Univet custom made lopes, Italy) and head light to ensure the absence of any deformity or pathological defects. The selected teeth were thoroughly cleaned to remove debris or any attached periodontal tissue using a hand scaler Scaler 10A, NOVA instruments Ltd, Berkshire, UK) then they were stored in deionized water containing 0.2% thymol solution. Radicular portions of all teeth were removed with a slow speed diamond saw (Hard tissue microtome,Bronwill,E.McGranthinc, MA,USA) under water irrigation. The coronal portion of each tooth was imbedded in self-cured acrylic resin with labial surface facing upward.The labial enamel surfaces of the specimens were ground using silicon carbide paper (grades 600-1200) under water irrigation to produce more consistent reproducible flat enamel surfaces.

Baseline micro-hardness test

The baseline microhardness was evaluated for all specimens (positive control group/ n=20 specimens) using Digital Display Vickers Microhardness Tester (Wilsonminiaturized scale hardness analyzer, display Tukon**1102** Germany) with a Vickers diamond indenter and a 20X objective lens. A load of 100g was applied to the surface of the specimens for 20 seconds. Three indentations, which were equally placed over a circle and not closer than 0.5 mm to the adjacent indentations, were made on the surface of each specimen. The diagonal lengths of indentations were measured by built-in scaled micrometer and values were changed over into Vicker's numbers. The values were averaged to produce one hardness value for each specimen Micro-hardness values were obtained utilizing the following equation: $HV=1.854\ P/d^2$ [21] Where, HV was Vickers hardness in Kg/mm^2, P was the load in Kg and d was the average length of the diagonals in mm.

Quantitative elemental analysis (weight %) by EDXA:

The mineral content of all positive control specimens was assessed quantitatively using Energy Dispersive X-ray analysis (EDXA) (Ametek, Materials Analysis Division, Netherlands), computer controlled software Genesis using an accelerating voltage of 20–25 kV. Elemental level including Calcium (Ca) and Phosphorus (P) were evaluated in weight percentage.

Demineralizing Protocol

The early artificial demineralization of enamel subsurface was achieved by immersing the specimens into glass container containing 20ml of demineralizing solution. The solution was adjusted with a 1.0 M of NaOH to a pH of 5.0 using pH meter (Deluxe deep vision, model no: 101, California, USA), at room temperature for 72 hours. [19,20] Microhardness and elemental analysis were revaluated for the demineralized specimens (negative control group/ n=20 specimens).

Grouping of the specimens

Specimens were divided into two groups(n=10 specimens/gp) according to the remineralizing material used, as follow :

Group (I): Remineralized using prepared egg shell solution.

Group (II): Remineralized using amorphous calcium phosphate ACP (Relief, Zoom Philips, USA)

Preparation of Chicken Eggshell Powder:

Powder was prepared by the calcination protocol given by World Property Intellectual organization [22]. Chicken Egg Shell has about 95 % of calcium carbonate which is converted into basic calcium oxide by calcination process. Twenty chicken eggs were obtained from a local hatchery, the contents were removed and the eggshells were cleaned in distilled water. The eggshells were then kept in hot water bath at 100°C for 10 minutes followed by removing the inner membrane. These eggshells were then crushed using a sterile mortar and pestle. The crushed particles then heated at 1200°C in a furnace (Lava Furnace 200,3M,USA)™ to go through the calcination process. This process was done to increase the mixture alkalinity and to make sure that it was pathogen free.[22,23]

Preparation of Eggshell Powder Solution:

One gram of CEP was dissolved in 20 ml of 4% acetic acid (India Chemicals, Pvt ltd. Mumbai, India) in a test tube. The clear fluid which was collected at the top was then transferred to a beaker .The pH of the solution was measured by using a pH meter(Deluxe deep vision, model no: 101, California, USA) and it was adjusted to 11.7 [16]

Application of the remineralizing agents

The demineralized specimens were immersed in the egg shell powder solution for 15 minutes/twice daily for seven consecutive days. During this seven days, the specimens were stored in artificial saliva with pH = 7.2. A fresh egg shell powder solution was used daily to enhance the process of remineralization. While, in the second group, specimens were painted using disposable brushes with the ACP using the same testing period. Finally, Microhardness and elemental analysis were conducted again after the remineralization procedure.

Statistical analysis

Numerical data were explored for normality by checking the distribution of data and using tests of normality (Kolmogorov-Smirnov and Shapiro-Wilk tests). All data showed normal (parametric) distribution. Data were presented as mean and standard deviation (SD) values. One-way ANOVA was used to compare between the groups. Tukey's post-hoc test was used for pair-wise comparisons. The significance level was set at $P \leq 0.05$. Statistical analysis was performed with IBM® SPSS® Statistics Version 20 for Windows.

® IBM Corporation, NY, USA.
® SPSS, Inc., an IBM Company.

RESULTS

Microhardness

One-way ANOVA results showed a statistically significant difference between the groups at P<0.001. Result revealed that the (+ve) control group had the statistically significantly highest mean vicker's hardness number (295.1 ±25.6 VHN) while, the demineralization group (-ve control) showed the statistically significantly lowest mean hardness (204.1 ±41.2.1 VHN). Moreover, there was no statistically significant difference between Egg Shell groups (gp I) and ACP (gp II) (235.9 ±30.5 VHN and 244.8 ± 51.1. VHN respectively); both showed a statistically significantly higher mean hardness than demineralization group and lower hardness than (+ve) control group as summarized in table (1) and graphically represented in figure (1).

Results of quantitative elemental analysis

The changes in concentrations of the total mineral components of bovine enamel in the different groups are summarized in table (2) and graphically represented in figure(2)

One-way ANOVA results showed that the (+ve) control group had the statistically significantly highest mean minerals content (35.9 ±6.4 Ca wt % and 15.2 ±3.1 P wt%). While demineralized group (-ve control gp) showed the statistically significantly lowest mean minerals content (24.1 ±4.5 Ca wt % and 9.7 ± 2.2 P wt %). Irrespective of the treatment group there was no statistically significant difference between GpI and Gp II; both showed statistically significantly higher mean minerals content than -ve control group and lower mean mineral content than +ve control group.

TABLE (1) The mean, standard deviation (SD) values of the microhardness(VHN) of the different groups

+ve control (n = 20)		-ve control (n = 20)		Gp I (n = 10)		Gp II (n = 10)		*P*-value
Mean	SD	Mean	SD	Mean	SD	Mean	SD	
295.1 ^{A}	25.6	204.1 ^{C}	41.2	235.9 ^{B}	30.5	244.8 ^{B}	51.1	<0.001*

: Significant at P ≤ 0.05, different superscripts are statistically significantly different.

TABLE (2) The mean and standard deviation (SD) values of minerals content (Ca and P weight %) of the different groups

	+ve control (n = 20)		-ve control (n = 20)		GpI (n = 10)		GpII (n = 10)		*P*-value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Ca	35.9 ^{A}	6.4	24.1 ^{C}	4.5	31.2 ^{B}	7.4	30.1 ^{B}	6.5	<0.001*
P	15.2 ^{A}	3.1	9.7 ^{C}	2.2	11.5 ^{B}	3.0	10.9 ^{B}	2.5	<0.001*

: Significant at P ≤ 0.05, Different superscripts in the same row are statistically significantly different

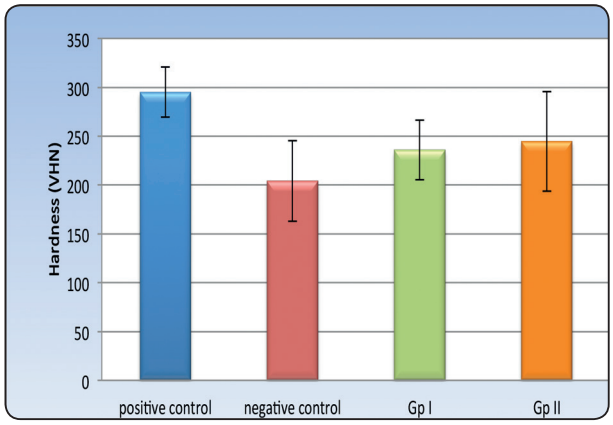


Fig. (1): Column chart representing mean and standard deviation values for microhardness (VHN) in the different groups

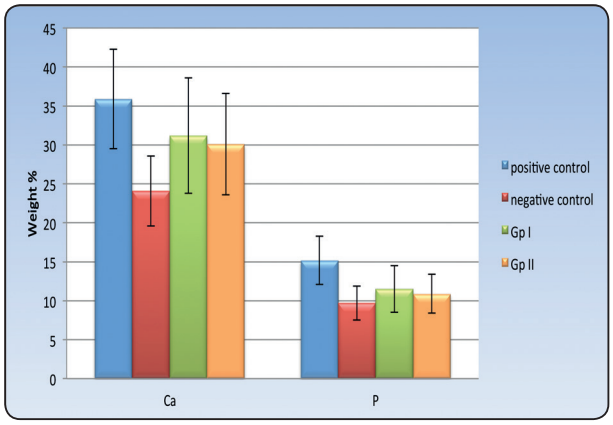
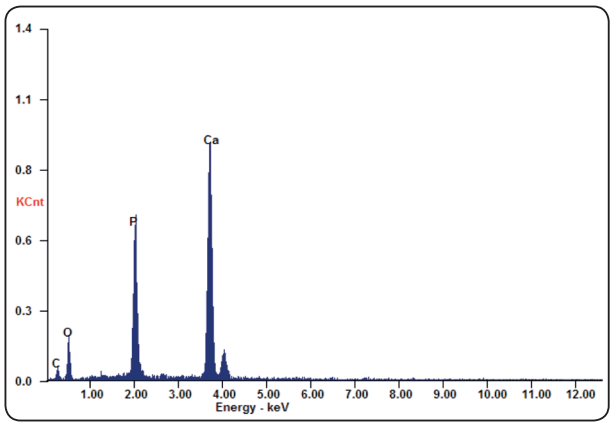
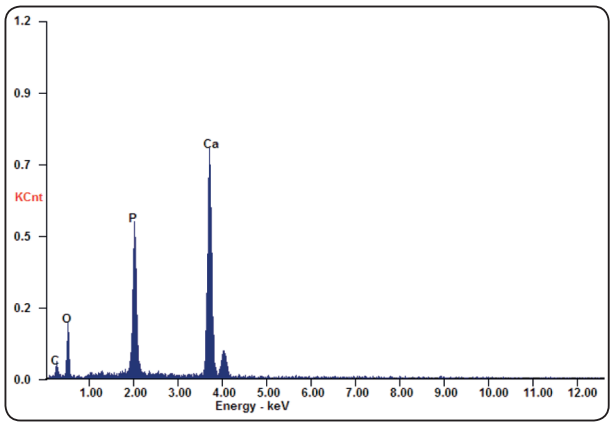


Fig. (2). Column chart representing mean and standard deviation values for Calcium and Phosphorus weight % in the different group



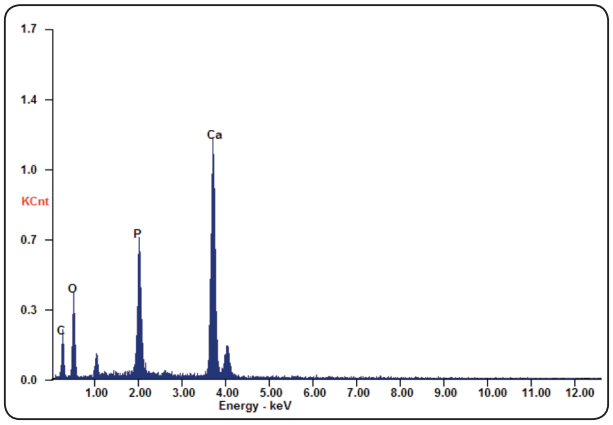
Element	Wt %	At %
C K	16.82	29.36
O K	31.28	41.00
P K	16.20	10.97
CaK	35.70	18.67

Fig. (3) Representive figure for the elemental analysis by EDX of enamel surface of specimen befor any treatment (+ve control group)



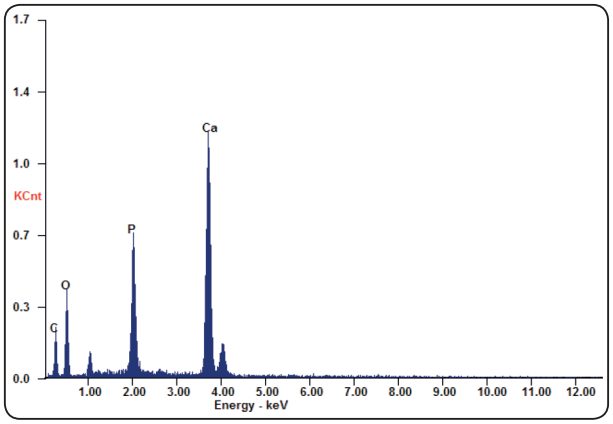
Element	Wt %	At %
C K	28.60	42.31
O K	37.00	41.11
P K	10.17	05.83
CaK	24.23	10.75

Fig. (4): Representive figure for the elemental analysis by EDX of enamel surface of specimen after demineralization (-ve control group)



Element	Wt %	At %
C K	17.57	29.80
O K	33.95	43.22
P K	15.75	10.36
CaK	32.72	16.63

Fig. (5) Representive figure for the elemental analysis by EDX of enamel surface of specimen after remineralization using egg shell powder solution (Gp I).



Element	Wt %	At %
C K	20.55	34.00
O K	32.81	40.76
P K	14.26	09.15
CaK	31.75	15.74

Fig. (6) Representive figure for the elemental analysis by EDX of enamel surface of specimen after remineralization using ACP varnish (Gp II)

DISCUSSION

The modern dental practice is now continuously shifting towards the concept of minimal invasion dentistry (MID) which is a conservative concept that mainly emphasizes upon early detection of carious lesions, remineralization of tooth surfaces and preservation of surrounding tooth structure[24,25]. Studies based on this concept revealed that the incipient enamel caries are reversible provided that a super saturated state with calcium and phosphorous ions are kept in the adjacent enamel.[26,27] Super saturation can be achieved by using different remineralizing agents that are able to release sufficient amount of Ca and P ions. These agents include amorphous calcium phosphate, bioactive glass, casein phosphopeptide, fluoride and some natural agents.[4,28,29] In the current study, two different remineralizing agents were used; the first is derived from natural which was egg shell powder solution (CESP) and the second one is a commercial available ACP

This study sought to assess and compare the efficacy of the CESP solution and ACP gel for remineralization of enamel initial like lesions in bovine teeth. Concerning the CESP, previous X-Ray fluorescence spectroscopic analysis of the egg shell have shown that it contains around 98% Calcium, 0.46% of phosphate, 0.53% of Magnesium, 0.18% of Strontium, 0.03% of Potassium and 0.03% Potassium. This high concentration of bio available

Ca plays a vital role in enamel remineralization when CESP applied topically.[16,25] Calcination process was done to obtain pure powder free of pathogens and to increase the alkalinity of powder. This high alkalinity (pH = 11.7) was approved by previous studies as it is the main reason for increased ion activity and availability of anions such as hydroxyl ions and phosphate ions for remineralization. If the pH of remineralizing solution is low (acidic) there will be more concentration of H^+ ions which combines with the available anions and thus less ions will be available for remineralization.[23,25]

The commercial Relief ÒACP was selected as it has a triple action, rebuilds enamel by depositing Ca and P on the enamel forming hydroxyapatite which lead to remineralization as proved by *Job et al* in 2018. Also it reduces sensitivity by occluding the dentin tubules as it contain potassium nitrate, in addition to the restoration of the enamel luster to create a smoother and glossier appearance.[30]

In order to simulate the subsurface demineralization lesions while maintaining the superficial enamel layer intact, weak organic acetic acid was included in the composition of the demineralizing agent[1,31]. Furthermore, the presence of calcium and phosphate, in the demineralizing solution helped preserve the superficial enamel layer while stimulating mineral loss from the subsurface layer. [32] This study was conducted in vitro as it would be difficult to control all the confounding factors in clinical studies such as diet, differences in the flow and composition of saliva, patient cooperation, and interpretation of results [1]

Microhardness measurement was done as it is appropriate for a material having fine microstructure, non homogenous and prone to cracking like enamel. Surface microhardness indentation provides relatively a simple, non-destructive, rapid method.[16] Results of this study showed that the microhardness was significantly decreased following exposure to demineralizing solution while it was significantly increased following exposure to remineralizing agents (Table 2). This indicates the efficacy of the agents used for the remineralization of incipient enamel caries-like lesions. The result shown in the current study was consistent with the results of previous study done by *Haghgooetal in 2016*[33] who found that the egg shell solution greatly increased the microhardness of the enamel by improving the remineralization of enamel like lesions.

In the current study, it was expected that CESP would induce more remineralization than ACP due to its high bioavailability of Ca content, neverthless, no significant difference was noted between the results of the specimens remineralized with the CESP and those remineralized with ACP as shown in table (2) and figure (1). This could be explained by the NaF contents of ACP which had a synergistic effect on the remineralization process, combined with deposition of extensive amounts of Ca^2+ and $PO4^{-3}$ significantly promoting the remineralization and making it equivalent to CESP.

Moreover, the results of the present study showed that the calcium and phosphorus levels have been significantly increased after remineralization process both in Gp I and GpII. This might be explained by the diffusion of extensive amounts of Ca^{+2} and phosphorus ions, as clarified and detected in the elemental analysis (figures 2), into the superficial layer of enamel and their deposition leading to complete obstruction of all the surface porosities,this led to the increase in the microhardness values of all the demineralized specimens.[33] In addition, Gamal et al in 2017 [34] demonstrated that the combination of calcium and the phosphates acted like a scaffold, or they could fill the gaps in between the enamel crystals, yielding a uniform crystalline enamel structure with high mineral content which may enhance the remineralization.

The null hypothesis was accepted as both ACP and CESP produced the same remineralizing effect on the demineralized enamel. However additional studies should be conducted to know what are the optimal concentration, form and maximum time of application of CESP to produce efficient remineralization.

CONCLUSIONS

Within the limitations of this in vitro study, it can be concluded that both remineralizing agents were similarly able to increase the microhardness and tooth remineralization. However, being natural products, CESP can be considered as an optimal alternative to the commercial ones.

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