EGYPTIAN DENTAL JOURNAL
www.eda-egypt.org
VOL. 68, 1025:1041, JANUARY, 2022
PRINT ISSN 0070-9484 • ONLINE ISSN 2090-2360
CONSERVATIVE DENTISTRY AND ENDODONTICS
Submit Date : 25-10-2021 • Accept Date : 08-01-2022 • Available online: 30-01-2022 • DOI : 10.21608/edj.2022.102806.1840

A COMPARATIVE EVALUATION OF REMINERALIZATION POTENTIAL OF NANO-SEASHELL, NANO-PEARL, AND NANO-HYDROXYAPATITE PASTES VERSUS FLUORIDE-BASED TOOTHPASTE ON NON-CAVITATED INITIAL ENAMEL LESION: AN IN VITRO STUDY

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ABSTRACT

Abstract Aim: This in vitro study evaluated and compared the remineralization potential of nano-seashell paste, nano-pearl paste, and nano-hydroxyapatite (nHA) paste versus fluoride-based toothpaste by assessing enamel surface microhardness and laser fluorescence analysis (DIAGNOdent).

Materials and methods: Eighty premolars were used in this in vitro study. Premolars were distributed into 4 groups of 20 teeth each, according to the remineralizing agent used. Group A (n=20): teeth were treated with nano-seashell paste; Group B (n=20): teeth were treated with nano pearl paste; Group C (n=20): teeth were treated with nHA paste; Group D (n=20): teeth were treated with fluoride-based toothpaste as a positive control group. Ten teeth of each group were subjected to microhardness assessment at baseline, after demineralization for 72 hours and after 28 days of remineralization with different remineralizing materials to compare its effect on enamel surface microhardness. The rest 10 teeth of each group were assessed using the DIAGNOdent pen® pen at baseline, after demineralization, after 14 days, and after 28 days of remineralization with different materials. The data were statistically analyzed.

Results: After 28 days of remineralization, all groups showed increase in enamel surface microhardness. The highest mean percent increase of enamel microhardness was recorded in group D (458.59), followed by group B (405.97), then group A (286), with the least value recorded in group C (207.8). After 14 days remineralization, intergroup comparison of DIAGNOdent readings showed a percent decrease of laser fluorescence with no significant difference between groups. After 28 days remineralization, the greatest mean percent decrease was recorded in group A, followed by group D, then group C, with the least percent decrease recorded in group B.

Conclusion: Nano-seashell, nano-pearl, nHA pastes were demonstrated to own remineralization potential comparable with fluoride-based toothpaste on initial non-cavited enamel lesions in an effective noninvasive manner.

KEYWORDS: Nano seashell, Nano pearl, nHA DIAGNOdent, remineralization, initial enamel lesions.

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INTRODUCTION

The most recent trend in the management of caries is detecting the initial non cavitated enamel lesions as early as possible and managing these lesions in a non-invasive way using novel remineralization agents. Initial caries can be reversed; thus, the incompletely demineralized hydroxyapatite (HA) crystals in enamel could grow to their original size if they are exposed to oral environments that are rich with remineralizing elements. Dental caries can be stopped or restored by promoting teeth remineralization at initial phases [(1)].

Fluoride is considered the cornerstone of non-invasive caries management and proved to be an efficient remineralizing agent. Due to the improvement in dental studies, the trend has become to use natural ingredients as remineralizing agents. For the proper guidance of the researchers to properly select and apply the various technologies, these natural products need to be validated and compared with already accepted commercially used products.

The main component of seashell and pearl powders is calcium carbonate (which is around 95%), in addition to the phosphor, manganese, zinc, iron, potassium, magnesium, and other minerals. The importance of these powders as natural sources of calcium, both had been demonstrated to have a bacteriostatic function [(2)]. Therefore, the application of seashell and pearl powders in the medical field had attracted the attention of researchers.

Nano-hydroxyapatite (nHA) is characterized being able to protect the teeth by surrounding it with a new synthetic layer of enamel, instead of increasing the hardness of the existing layer with fluoride [(3)]. Nano-hydroxyapatite is similar to the morphology of the enamel of the tooth as well as the structure of its crystals. Therefore, hydroxyapatite is capable of substituting the natural enamel mineral constituent for repair biomimetically [(4)].

Nanoparticles are characterized by having a higher ion release profile than microparticles. Since it is far hard to directly remineralize the teeth in the oral cavity by using the nanomaterials, these materials are often added to restorative materials as inorganic fillers, such as resin composites to release calcium, phosphate, and fluoride ions for remineralization of dental hard tissues [(4).] The incorporation of nano-particles in remineralizing agents is one of the recent directions of researches.

Therefore, this study was carried out to compare the effectiveness of the three natural remineralizing agents nano-seashell, nano-pearl, and nHA particles with a fluoride-based toothpaste.

MATERIALS AND METHODS

Materials

Remineralizing pastes used:

a. Nano-seashell paste (10%) (Cockle seashells were collected from Red-sea beaches in Egypt)

b. Nano-pearl paste (10%) (NA'VI wild harvest, supreme pearl wisdom, 100% pure water levigated, micro ground powder, Navi Organics Ltd, UK)

c. Hydroxyapatite nanoparticles paste (10%).

d. Signal toothpaste with sodium fluoride (850-1150ppm)

Methods

Study Design

Eighty premolars were used in study. Premolars had been equally distributed into four groups, each of 20 teeth, according to the remineralizing agent used. Group A (n=20): teeth treated by nano-seashell paste; Group B (n=20): teeth treated by nano-pearl paste; Group C (n=20): teeth treated by nHA paste; Group D (n=20): teeth treated by fluoride-based toothpaste as a positive control group. Each group

was further subdivided into 2 equal subgroups of 10 teeth each. One subgroup group (n=10) was subjected to microhardness assessment at baseline, after demineralization for 72 hours, and after 28 days of remineralization. The other subgroup (n=10) was assessed using laser fluorescence (DIAGNOdent pen®) at baseline, after demineralization, after 14 and 28 days of remineralization. The data were statistically analyzed.

Nano-Seashell Powder Preparation

Cockle seashells were collected from Red-sea beaches in Egypt. The seashells were washed to remove all dirt. 100 grams seashells were boiled at 100° C for 30 min and dried for 2 days at 110° C in the oven. An agate mortar was used for hand-grounding the shells after being dried into powder. The grounded powder was milled (Nanogate Company-Egypt) using a ball mill machine (planetary-ball-mill-pm-400) for 10 h, speed 350 rpm, and 3min intervals. The obtained nano-seashell powder (of particle size ranged from 7.72 to 17.54 nm) was then dispersed while stirring in distilled water (10% w/v) to obtain 10% nano-seashell solution; then carboxymethyl cellulose (4%w/v) was gradually added to the mixture with continuous stirring until the suspension turned to a gel form of nano-seashell[5]. A percent weight / volume (%w/v) was calculated using the formula: %w/v = g of solute /100ml of solution.

Nano-Pearl Powder Preparation

Pearl powder (NA'VI wild harvest, supreme pearl wisdom, 100% pure water levigated, micro-ground powder, Navi Organics Ltd, UK) was bought from the Bio store market in Braunschweig, Germany. The powder was milled (Nano-gate company-Egypt) by using a ball-mill machine (planetary-ball-mill-pm-400) for 10h, speed 350 rpm, and 3min intervals. The obtained nano-pearl powder (of particle size ranged from 11.79 to 17.10 nm) was then dispersed in distilled water (10%w/v) while stirring to obtain a 10% nano-pearl concentration. Carboxymethyl cellulose 4%w/v was gradually added to the mixture with continuous stirring until the suspension turned to the gel form.

Nano- Hydroxyapatite Powder Preparation

Nano-hydroxyapatite powder was obtained from Nanotech company for Photo-Electronics, Egypt. The powder was then dispersed in distilled water (10%w/v) while stirring to obtain 10% nano-hydroxyapatite. Carboxymethyl cellulose 4%w/v was gradually added to the mixture; then the suspension turned to nHA in gel form.

Characterization of the prepared powders using different analytical methods:

a) Transmission Electron Microscope (TEM)

Characterization of nano-seashell, nano-pearl and nHA powders was performed using Transmission Electron Microscope (TEM) on JEOL JEM-2100 high resolution transmission electron microscope at an accelerating voltage of 200 kV, respectively.

b) X-Ray Diffraction analysis (XRD)

An XRD pattern had been performed using XPERT-PRO Powder Diffractometer system, with 2 theta (20^{o} - 80^{o}), with Minimum step size 2 Theta: 0.001, and at wavelength (Kα) = 1.54614^{o}.

c) Energy Dispersive X-ray (EDX) analysis:

Energy Dispersive X-ray analysis was performed to determine weight % of different elements present in nano-seashell, nano-pearl and nano-hydroxyapatite powders.

Sample Preparation

Eighty sound extracted premolars for orthodontic reasons were used in this study. Teeth were collected from patients (ranged from 18 to 25 years old) who visited the Surgery Clinic, Faculty of Dental Medicine, Al Ahram Canadian University. Teeth

with restorations, enamel cracks, caries, erosion, developmental defects, or white spot lesions were excluded [6]. 5.25% sodium hypochlorite solution was used for the disinfection of the selected teeth for 60 min [7]. The crowns were scraped with a hand scaler and washed under running tap water to remove any residual tissues and debris; then polished with fluoride-free pumice paste [8]. Each group of samples was put in a separate glass container containing 10 ml of artificial saliva at 37°C in the CO_2 incubator.

a) Sample Preparation for Enamel Surface Microhardness Assessment:

Each tooth was sectioned into 2 halves mesiodistally using a water-cooled diamond saw (Isomet® 5000 Linear Precision Saw; Buehler Ltd., Lake Bluff, USA) [3]. Custom-made plastic molds were prepared with the dimension of 3 mm height and 20 mm diameter poured with cold cure acrylic resin. Both halves of each tooth were fixed using superglue on the custom-made acrylic resin block; so that the buccal surfaces were available for treatment to be treated. A transparent acid-resistant nail polish was used to coat the surface leaving a 4 × 4 mm window on it. An artificial caries lesion was developed on this part of the tooth [3]. For easy identification, each acrylic disc with the glued sample was numerically coded at its base using a waterproof permanent marker**.**

b) Sample Preparation for Laser Fluorescence Analysis:

The teeth were mounted from the root portion in self-cure acrylic resin blocks (Acrostone dental factory, Egypt) showing the crown only. All the crown surfaces were coated with a transparent acid-resistant nail polish leaving a 4 × 4 mm window on the buccal and lingual surfaces where the artificial non-cavitated initial enamel lesion was developed [3]. For easy identification, each acrylic block was numerically coded at its base using a waterproof permanent marker.

Artificial Saliva Preparation

Artificial saliva was prepared in the laboratory by dissolving [0.4gm sodium chloride (NaCl), 1.21 gm potassium chloride (KCl), 0.78 gm sodium dihydrogen dehydrate ($NaH_2PO_4.2H_2O$), 0.005 gm hydrated sodium sulfide ($Na_2S.9H_2O$), 1gm urea $CO(NH_2)_2$] in 1000 ml of deionized water. 10N sodium hydroxide was added to this mixture until the pH value was measured using a pH meter to be 6.75±0.15 [9].

Artificial Non-cavitated Initial Enamel Lesion Formation

Storage of each sample was done in freshly 15 mL demineralizing solution for 72 hours at 37°C in incubator [1]. The demineralizing solution used in this study was Pepsi® of pH 1.28 (Carbonated water, 42.5 g/35 cl sucrose, phosphoric acid) [10,11]. The demineralizing solutions were changed every day to avoid pH change [3]. Deionized water was used for thorough rinsing of the samples and the samples were then kept for wash out period of 24 hours.

Enamel Surface treatment with the remineralizing pastes

The samples in the experimental groups (A, B, C, D) were treated with the corresponding pastes: nano-seashell paste, nano pearl paste, nHA paste, and signal toothpaste once daily for 28 days. A plastic scoop was used to standardize the quantity applied on the enamel surface. One scoop from each paste was rubbed on the enamel surface in a circular motion using a micro brush for 2 minutes [12]. Then the paste was kept on the surface for 30 seconds. Finally, the samples were rinsed carefully under running tap water to remove any excess paste. After drying the samples with clean absorbent, each group was returned to its container containing artificial saliva (10ml) in the incubator.

Enamel Surface Microhardness Assessment (SMH)

Enamel surface microhardness was measured at baseline of sound untreated enamel, after 72 h of the

demineralization, and after 28 days of remineralization. Digital Display Vickers Microhardness Tester with a Vickers diamond indenter and a 20X objective lens was used to measure the surface microhardness. A load of 100g was applied on the surface of the specimens for 10 s. Three indentations were equally placed on the surface of each specimen and not closer than 0.5 mm to the adjacent indentations. The length of the of the indentations were measured by a built-in scaled microscope and Vickers values were converted into microhardness values. Microhardness value was obtained using the following equation: $HV=1.854\ P/d^2$; Where HV is Vickers hardness in Kgf /mm^2, P is the load in Kgf and d is the length of the diagonals in mm.

Laser Fluorescence Analysis

Laser fluorescence analysis was done using DIAGNOdent pen® (2190, Kaltenbach & Voigt GmbH, Germany) to measure the lesions objectively. The instrument was calibrated against its own ceramic disk provided by the manufacturer before every measurement session. The instrument probe B was used in this study as it is indicated for smooth surface caries' detection. The specimens were placed horizontally with the lesion facing upward, which allowed the standardization of the measurement by holding the tip 90° to the lesion surface. Measurements were interpreted according to the manufacturer of the DIAGNOdent pen® (0–7 Healthy tooth structure, 8–15 initial demineralization, >16 strong demineralization). Teeth were assessed at baseline, after 72 h demineralization, and post remineralization at 14 days, and 28 days intervals.

Statistical Analysis

Values were presented as mean, standard deviation (SD) values. Data were explored for normality using Kolmogorov-Smirnov test of normality. The results of Kolmogorov-Smirnov test indicated that microhardness and DIAGNOdent pen data were normally distributed (parametric data), therefore, independent one-way analysis of variance, followed by Tukey's post hoc test were used to compare between groups. Most values of percent change were non-parametric and were compared using Kruskall Wallis test. The percent change was calculated by the formula:

$$\frac{\text{Value after-value before}}{\text{Value before}} \text{X100}$$

The significance level was set at $p \leq 0.05$. Statistical analysis was performed with SPSS 18.0 (Statistical Package for Scientific Studies, SPSS, Inc., Chicago, IL, USA) for Windows.

RESULTS

Characterization of the prepared powders using different analytical methods:

a) TEM Results of Nano-Seashell, Nano-Pearl and Nano-Hydroxyapatite Powders:

TEM analysis of nano-seashell powder confirmed the presence of the orthorhombic crystal system of the prepared powder with the particle size range from 7.72 to 17.54 nm (Figure 1). While the morphological examination of nano-pearl powder confirmed the presence of the rhombohedral crystal system of the prepared powder with a particle size range from 11.79 to 17.10 nm (Figure 2). The TEM analysis of the prepared nHA powder showed rod-like shape particles of particle size 100 ± 30 nm (Length), 20 ± 5 nm (Diameter) (Figure 3).

b) X-ray diffraction (XRD) analysis of Nano-Seashell, Nano-Pearl and Nano-Hydroxyapatite powders:

The XRD pattern of nano-seashell powder suggested the presence of aragonite crystals (Figure 4); while the XRD pattern of nano-pearl powder suggested the presence of aragonite and calcite crystals (Figure 5). Aragonite has its greatest peak 221 at relatively small °2Θ, whereas calcite has a booming 104 peak a bit to the right of the aragonite large peak, and few and comparatively small other

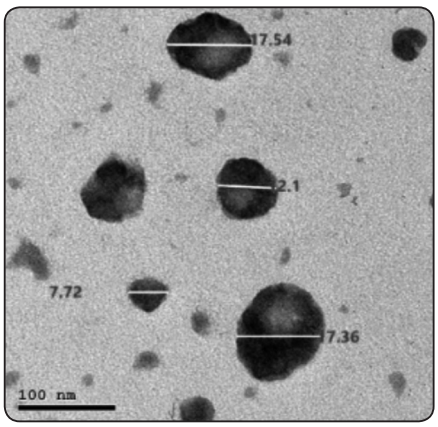

Fig. (1): TEM nanographs for sea shell powder

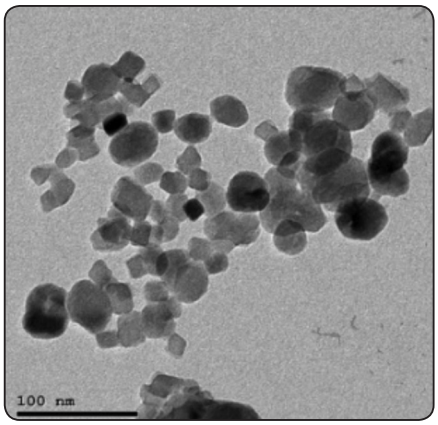

Fig. (2): TEM nanographs for pearl powder

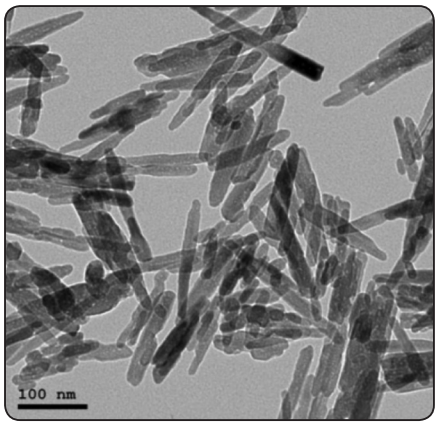

Fig. (3): TEM nanographs for HA powder

peaks. The XRD pattern of nano-hydroxyapatite powder suggested the presence of amorphous HA particles (Figure 6). The sharp diffraction characteristic peaks that appeared at around 26˚, 33˚ and 40˚ are the HA particles. The XRD patterns showed diffraction peaks with high intensities which conform the nano size with crystalline nature.

c. EDX analysis for nano-seashell, nano-pearl and nano-hydroxyapatite powders:

The standard EDX spectra were recorded on the examined powders. Calcium, carbon, oxygen, and sodium were detected in the EDX spectrum of nano-seashell powder. Calcium, carbon, oxygen, sodium, aluminium, silica, and sulfur were detected in the EDX spectrum of nano-pearl powder. Carbon, oxygen, sodium, magnesium, phosphorus,

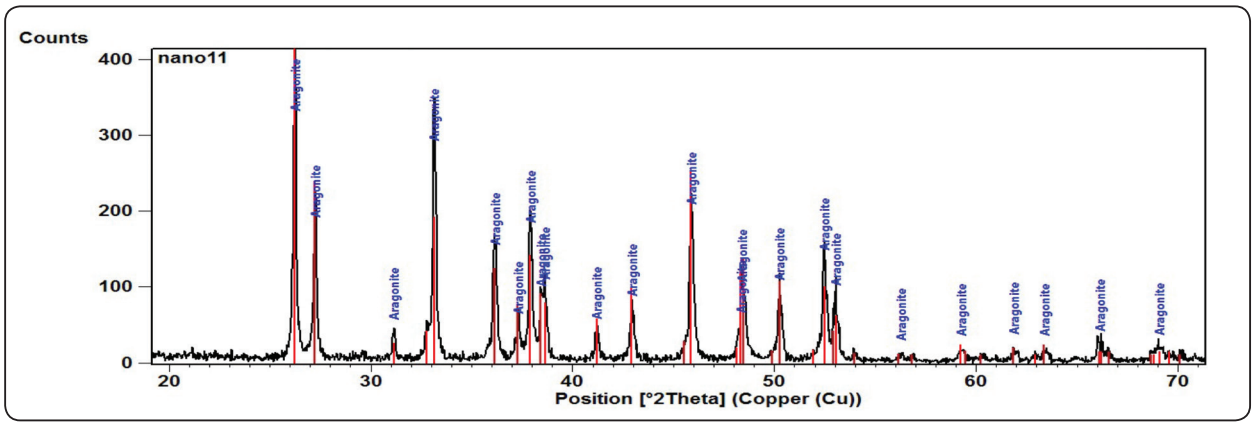

Fig. (4): XRD spectra of the aragonite present in nano-seashell powder

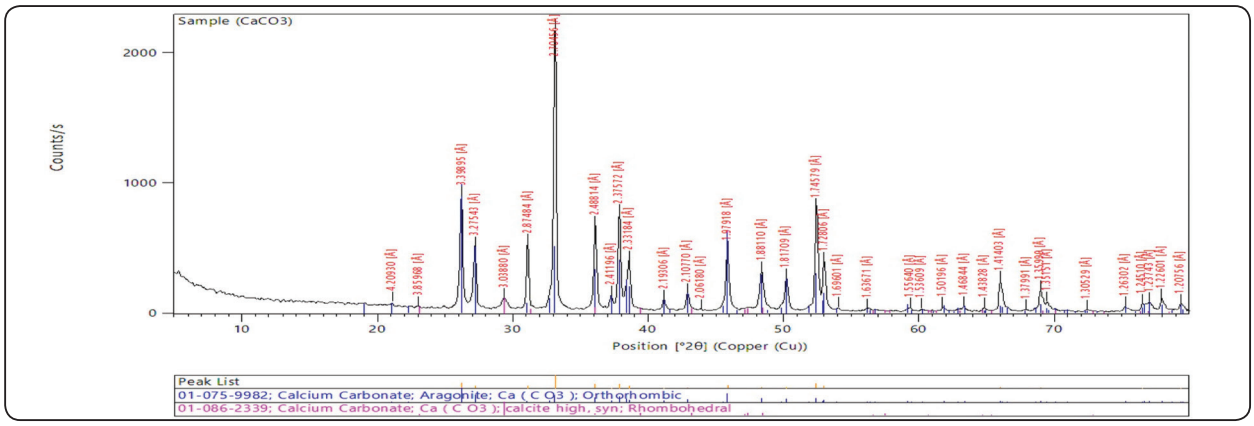

Fig. (5): XRD spectra of the calcite and aragonite present in nano-pearl powder

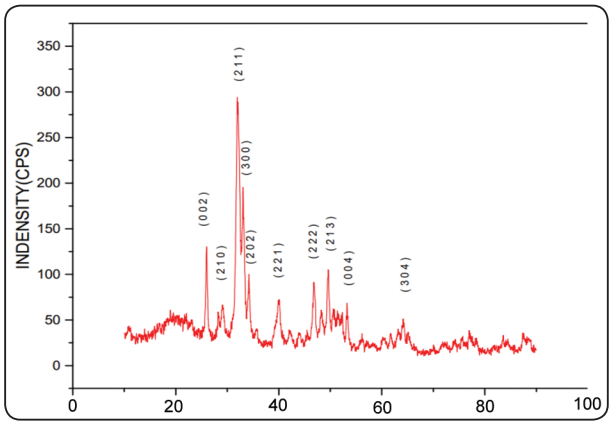

Fig. (6): XRD spectra of nHA powder

and calcium were detected in the EDX spectrum of nano-hydroxyapatite. Table 1, and diagram 1, table 2, and diagram 2, table 3, and diagram 3 are showing the presented spectrum of the elements' values of the prepared powders.

TABLE (1): The weight % and quantitative results of EDX spectrum of nano-seashell powder

Element	Weight %
C K	4.81
O K	54.01
Na K	1.32
Ca K	39.86

C: Carbon, O: Oxygen, Na: Sodium Ca: Calcium

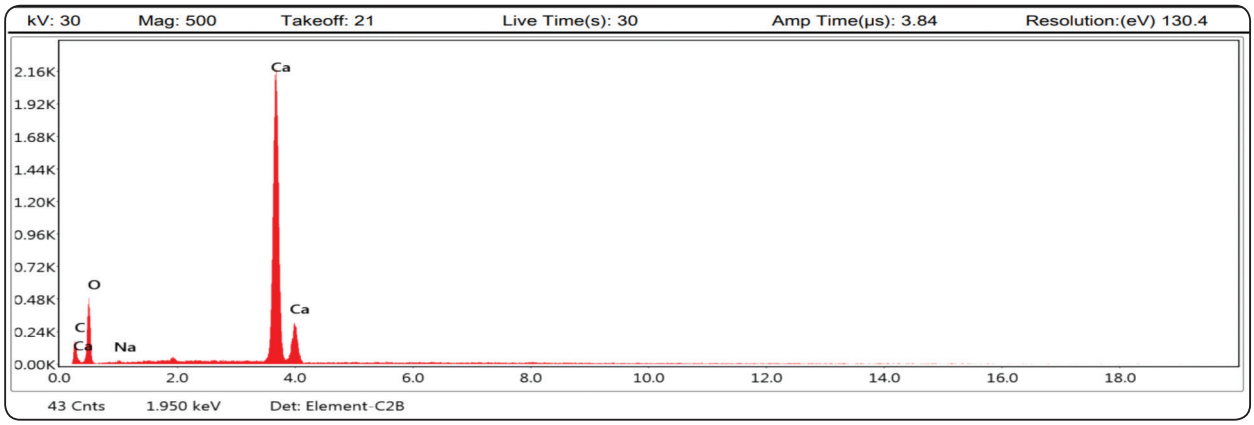

Diagram (1): Showing the weight % and quantitative results of EDX spectrum of nano-seashell powder

TABLE (2): The weight % and quantitative results of EDX spectrum of nano-pearl powder

Element	Weight %
C K	6.93
O k	53.49
Na K	0.97
Al K	0.53
Si K	0.32
S K	0.16
Ca K	37.59

C: Carbon, O: Oxygen, Na: Sodium, Al: Aluminum, Si: Silica, S: Sulfur, Ca: Calcium

Table 3: The weight % and quantitative results of EDX spectrum of nHA powder

Element	Weight %
C K	7.37
O k	49.08
Na K	0.65
Mg K	0.48
P K	15.8
S K	0.16
Ca K	26.5

C: Carbon, O: Oxygen, Na: Sodium, Mg: Magnesium, P: Phosphorus, S: Sulfur, Ca: Calcium

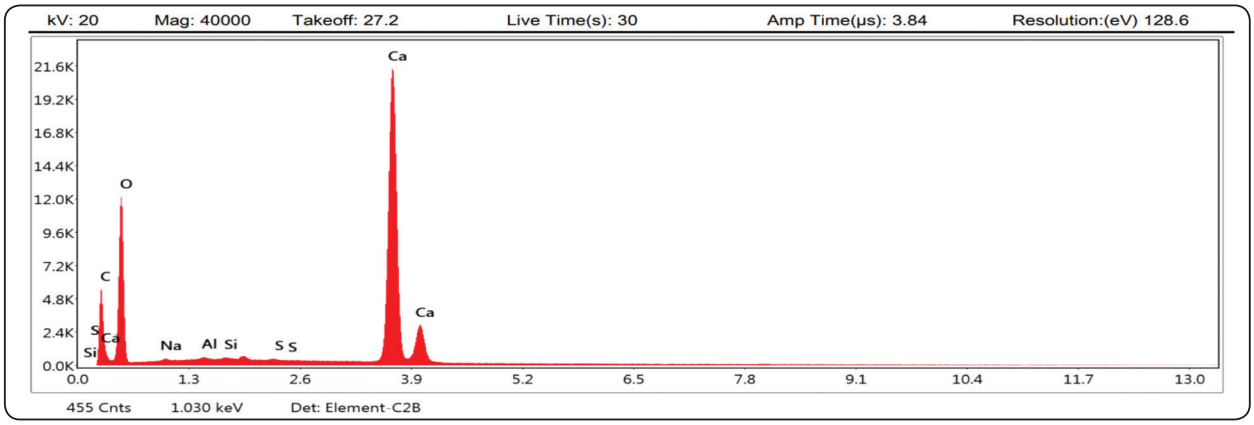

Diagram (2): Showing the weight % and quantitative results of EDX spectrum of nano-pearl powder

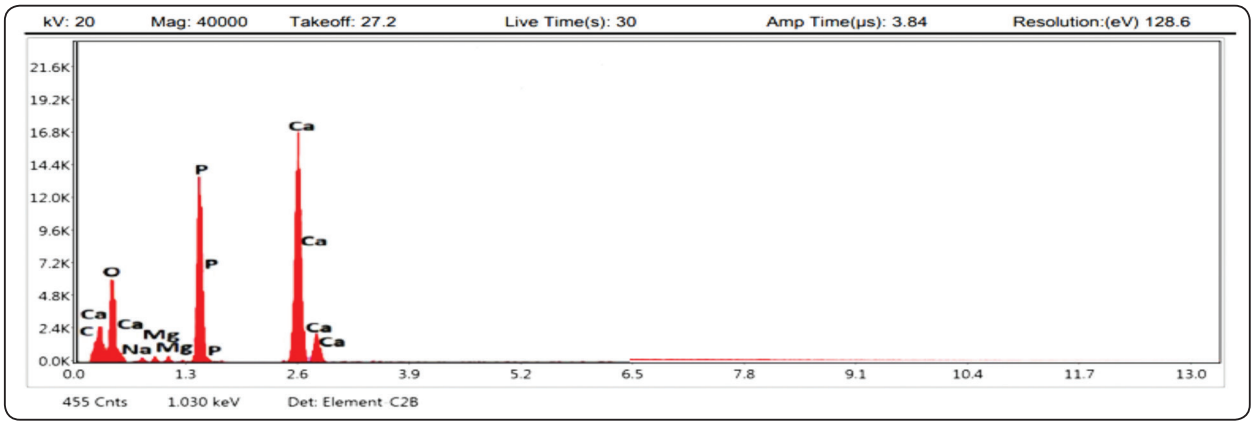

Diagram (3): Showing the weight % and quantitative results of EDX spectrum of nHA powder

Enamel Surface Microhardness Results

At baseline and after demineralization, all groups showed decrease in enamel surface microhardness with no statistically significant difference between groups (p=0.245, p=0.634 respectively). After remineralization, all groups showed increase in enamel surface microhardness. ANOVA test revealed that the difference between groups was statistically significant (p=0.00) (Table 4, Figure 7). The highest mean percent increase was recorded in group D (458.59±17.88), followed by group B (405.97±54.45), then group A (286.26±3.87), with the least value recorded in group C (207.8±2.51). Kruskall Wallis test revealed that the difference between groups was statistically significant (p=0.00). Post hoc test revealed a significant difference between each 2 groups (Table 5, Figure 8).

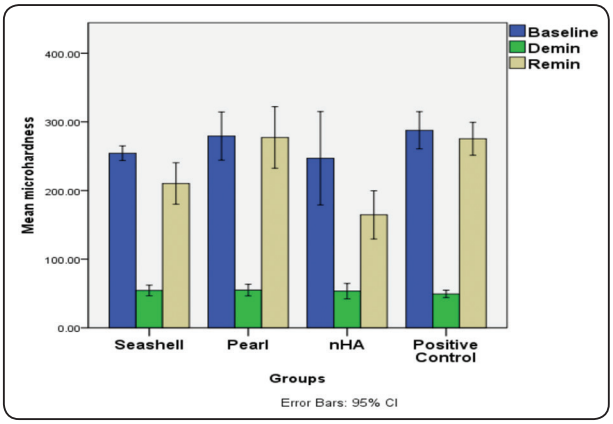

Fig. (7): Bar chart illustrating mean microhardness value in different groups.

Laser fluorescence (DIAGNOdent pen) analysis:

At baseline and after demineralization, there was no statistically significant difference between groups (p=0.119, p=0.146 respectively). After 14 days remineralization, the highest mean value was

TABLE (4): Descriptive statistics of microhardness at baseline, demineralization and remineralization (ANOVA test)

		Mean	Std. Dev	Std. Error	95% Confidence Interval for Mean		Min	Max	F	P
					Lower Bound	Upper Bound				
Baseline	**Group A**	254.33	10.15	4.14	243.68	264.97	240.33	266.00	1.49	.245ns
	Group B	279.31	33.53	13.69	244.12	314.50	235.67	324.00		
	Group C	247.01	64.84	26.47	178.96	315.05	184.33	367.37		
	Group D	287.76	25.87	10.56	260.60	314.91	253.33	311.67		
After Demineralization	**Group A**	54.44	7.47	3.05	46.60	62.28	43.33	65.30	.58	.634ns
	Group B	54.96	8.04	3.28	46.52	63.39	44.07	65.00		
	Group C	53.45	10.76	4.39	42.15	64.75	38.67	69.03		
	Group D	49.40	5.08	2.07	44.07	54.73	43.67	54.97		
After Remineralization	**Group A**	210.27[b]	28.82	11.76	180.03	240.51	167.67	251.50	16.63	.000*
	Group B	277.26[a]	42.74	17.45	232.41	322.11	213.67	317.33		
	Group C	164.58[b]	33.52	13.69	129.40	199.76	118.43	213.47		
	Group D	275.39[a]	22.90	9.35	251.36	299.42	249.33	302.30		

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

TABLE (5): Descriptive statistics of percent change of enamel surface microhardness (Kruskall Wallis test)

Percent change		Mean	Std. Dev	Std. Error	95% Confidence Interval for Mean		Min	Max	P
					Lower Bound	Upper Bound			
From baseline to demin.	**Group A**	-78.64	2.43	.99	-81.19	-76.09	-82.22	-74.72	.103ns
	Group B	-80.20	2.83	1.16	-83.17	-77.23	-85.27	-77.02	
	Group C	-77.74	4.66	1.90	-82.63	-72.85	-84.94	-73.26	
	Group D	-82.60	3.25	1.33	-86.00	-79.19	-85.97	-79.25	
From demin. to remin.	**Group A**	286.26^{c}	3.87	1.58	282.20	290.33	282.76	293.45	.000*
	Group B	405.97^{b}	54.45	22.23	348.83	463.11	377.47	516.88	
	Group C	207.80^{d}	2.51	1.02	205.17	210.44	203.60	210.63	
	Group D	458.59^{a}	17.88	7.30	439.82	477.35	433.12	485.22	
From baseline to remin.	**Group A**	-17.50^{m,n}	9.23	3.77	-27.19	-7.82	-31.19	-2.63	.012*
	Group B	.59^{m}	20.73	8.46	-21.17	22.34	-28.59	34.37	
	Group C	-31.50^{n}	14.19	5.79	-46.39	-16.60	-53.86	-18.04	
	Group D	-3.17^{m}	15.74	6.43	-19.69	13.35	-19.87	14.12	

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

Post hoc test: within the same comparison (time interval), means sharing the same superscript letter are not significantly different

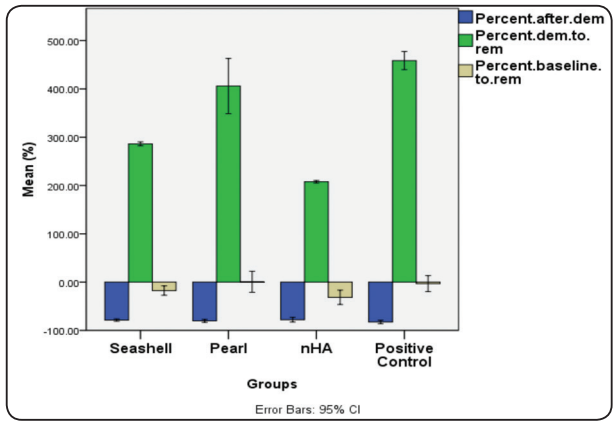

Fig. (8): Bar chart illustrating mean percent change of microhardness value in different groups.

recorded in group D (3.40±0.55), followed by group B (2.9±0.65), then group C (2.80±0.45), with the least value recorded in group A (2.50±0.71). ANOVA test revealed that the difference between groups was not statistically significant (p=0.160). After 28 days remineralization, the highest mean value was recorded in group D (1.9±0.42) followed by group B (1.9±0.96), then group C (1.80±0.27), with the least value recorded in group A (1±0.5). ANOVA test revealed that the difference between groups was not statistically significant (p=0.083) (Table 6, Figure 9).

In the interval from baseline to demineralization, all groups recorded a percent increase ranging from 522.62 % to 814.67 % with no statistically significant difference between groups (p=0.327). From demineralization to 14 days remineralization, all groups showed a percent decrease ranging from -87% to -89%, with no significant difference between groups (p=0.875). From 14 to 28 days remineralization, the greatest mean percent decrease was recorded in group A (-60 % ±14.91), followed by group D (-42.21% ±18.13), then group C (-35.24% ±9.21), with the least percent decrease recorded in group B (-34.33% ±37.12). Kruskall Wallis test revealed that the difference between groups was not statistically significant (p=0.140) (Table 7, Figure 10).

TABLE (6): Descriptive statistics of DIAGNOdent pen at baseline, demineralization and 14 & 28-days remineralization (ANOVA test)

		Mean	Std. Dev	Std. Error	95% Confidence Interval for Mean		Min	Max	F	P
					Lower Bound	Upper Bound				
Baseline	**Group A**	2.60	.42	.19	2.08	3.12	2.00	3.00	2.27	.119ns
	Group B	3.20	.84	.37	2.16	4.24	2.00	4.00		
	Group C	4.30	1.35	.60	2.62	5.98	3.00	6.00		
	Group D	4.30	1.89	.85	1.95	6.65	2.50	7.00		
After Demineralization	**Group A**	23.20	2.84	1.27	19.67	26.73	18.50	25.00	2.05	.146ns
	Group B	25.30	3.91	1.75	20.44	30.16	21.50	31.00		
	Group C	24.50	3.52	1.57	20.13	28.87	18.50	27.50		
	Group D	28.40	3.42	1.53	24.16	32.64	22.50	31.00		
After14 days remineralization	**Group A**	2.50	.71	.32	1.62	3.38	1.50	3.00	1.96	.160ns
	Group B	2.90	.65	.29	2.09	3.71	2.00	3.50		
	Group C	2.80	.45	.20	2.24	3.36	2.50	3.50		
	Group D	3.40	.55	.24	2.72	4.08	2.50	4.00		
After 28 days remineralization	**Group A**	1.00	.50	.22	.38	1.62	.50	1.50	2.66	.083ns
	Group B	1.90	.96	.43	.71	3.09	.50	3.00		
	Group C	1.80	.27	.12	1.46	2.14	1.50	2.00		
	Group D	1.90	.42	.19	1.38	2.42	1.50	2.50		

Significance level ≤0.05, ns=non-significant

TABLE (7): Descriptive statistics of percent change of DIAGNOdent pen results (Kruskall Wallis test)

Percent		Mean	Std. Dev	Std. Error	95% Confidence Interval for Mean		Min	Max	P
					Lower Bound	Upper Bound			
After demin	**Group A**	814.67	214.46	95.91	548.38	1080.95	640.00	1150.00	.327ns
	Group B	734.17	240.70	107.65	435.30	1033.04	462.50	1100.00	
	Group C	522.62	214.89	96.10	255.80	789.44	208.33	733.33	
	Group D	659.95	320.34	143.26	262.19	1057.70	307.14	1140.00	
Demin to 14 remin	**Group A**	-89.02	3.77	1.69	-93.71	-84.34	-93.33	-83.78	.875ns
	Group B	-88.42	2.89	1.29	-92.00	-84.83	-91.67	-83.72	
	Group C	-88.13	4.02	1.80	-93.13	-83.14	-90.91	-81.08	
	Group D	-87.73	3.43	1.54	-91.99	-83.47	-91.67	-82.22	
14 to 28 days remin	**Group A**	-60.00	14.91	6.67	-78.51	-41.49	-83.33	-50.00	.140ns
	Group B	-34.33	37.12	16.60	-80.42	11.75	-75.00	20.00	
	Group C	-35.24	9.21	4.12	-46.67	-23.80	-42.86	-20.00	
	Group D	-42.21	18.13	8.11	-64.72	-19.71	-62.50	-20.00	

Significance level ≤0.05, ns=non-significant

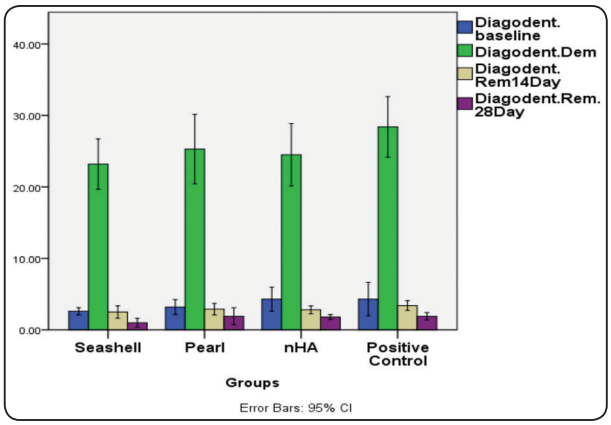

Fig. (9): Bar chart illustrating mean DIAGNOdent pen values in different groups

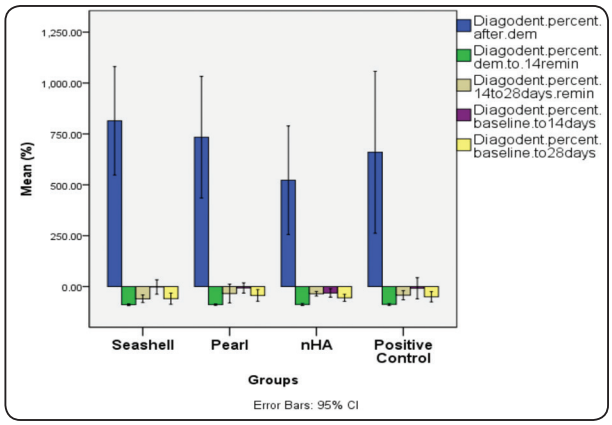

Fig. (10): Bar chart illustrating mean percent change of DIAGNOdent pen values in different groups

DISCUSSION

The rationale of minimally invasive dentistry is to gain the maximum preservation of healthy dental tissue either by caries prevention or by remineralizing the demineralized initial lesions[13]. Non-cavitated lesions can be arrested non-invasively by using remineralizing therapeutic agents which represents a promising effect over traditional invasive dentistry [14]. Although fluoride treatment has been the primary approach for non-invasive caries management and remineralizing initial lesions, caries still develop in high-risk individuals, irrespective of the dose of fluoride used [15]. In addition, dental fluorosis is considered an undesirable side effect of fluoride's preventive regimen that has been objected as an esthetic issue. Hence, the demand for using safer natural materials like pearl and seashell powder with higher remineralizing efficiency or at least the same efficiency of fluoride is rising each day.

Nano-seashell, nano-pearl, and nano-hydroxyapatite particles were used in the current in vitro study as remineralizing agents. Aragonite, the main structure of seashell and nacre pearl shell, is considered an important source of calcium carbonate ($CaCO_3$) that aid in teeth remineralization [16]. It was previously found that seashell nanoparticles as a remineralization agent could increase enamel surface microhardness which was nearly as effective as CPP-ACP paste [17]. Nacre pearl shell was found to share some similarities with bone. It was proved that nacre pearl shell could be used as bone graft in periodontal bone remodeling owing to its osteoconductive, osteoinductive, and osteogenetic properties [18]. Nano-hydroxyapatite is considered a biomaterial for teeth remineralization due to its chemical and structural nature that mimic the natural tooth mineral [19]. It had been used in many researches as a golden biomaterial for teeth remineralization [20]. After the improvement of nanotechnologies that have favored our understanding of dental tissues treatment at the nanoscale [21], many products showed benefits over the fluoride in the process of caries arresting [22]. Nanoparticles proved to have better ion release than microparticles [4]. Thus, the seashell, pearl and hydroxyapatite powders used in this study were obtained in nanoparticle size.

In this study, specimens were placed in the demineralizing solution at 37°C in the incubator for 72 h to simulate the duration of creating a subsurface demineralization with an intact surface simulating an early enamel lesion that is naturally occurring in the oral cavity of high caries risk individuals [1,23].

The remineralizing paste was rubbed on the enamel surface in a circular motion using a microbrush for 2 min to simulate the daily oral prophylaxis performed by the individual. Many studies evaluated the remineralization potential of various remineralizing agents over a period of one month and concluded that the extent of remineralization achieved was dose-dependent and increased with increasing the time of exposure and duration of the study [24]. For this reason, the remineralization process of the current study was carried out for 28 days. Owing to the remineralization potential of saliva, artificial saliva had been chosen as storage media of the samples throughout the experimental period to simulate the oral environment and to support the remineralization process by the inorganic ion needed for cluster formation [25].

Vickers microhardness test was utilized to evaluate demineralized and remineralized enamel surface microhardness, as it is considered a relatively simple, rapid, and non-destructive method [26]. Enamel surface microhardness was assessed at baseline and after 72 h of the demineralization to confirm the creation of non-cavitated initial enamel lesion after using Pepsi ®soft drink as a demineralizing solution. After demineralization, all groups showed a decrease in enamel surface microhardness with no statistically significant difference between groups ($p=0.634$). Initial enamel lesions have less mineral content at the surface layer when compared to sound enamel; thus, showing a lower microhardness value than that of baseline. Furthermore, enamel surface microhardness was measured after 28 days of

remineralization to compare the enamel microhardness at baseline (before remineralization) and after using the tested remineralizing pastes (28 days post-remineralization).

After 28 days of remineralization, all groups showed an increase in enamel surface microhardness. The highest mean percent increase of enamel microhardness was recorded in group D (458.59%±17.88), followed by group B (405.97% ±54.45), then group A (286.26%±3.87), with the least value recorded in group C (207.8%±2.51). The difference between groups was statistically significant (p=0.00). Fluoride treated group recorded the highest enamel surface microhardness (458.59%±17.88) which could be related to the fluorapatite layer formed on the enamel surface. Saturation of saliva with fluoride ions leads to decrease solubility and increases the surface microhardness of FA layer formed on the enamel surface in comparison with HA layer [27]. On the other hand, in previous studies showed no significant differences in surface microhardness and similar remineralization effectiveness between 10% n-HA and fluoride varnish under dynamic pH cycling [28,29,30] this may be attributed to the difference in experimental design, treatment regimens, sample preparation, equipment used, and data interpretation.

The XRD pattern results of the present study confirmed the presence of aragonite and calcite polymorphs of CaCO3 in nano-pearl powder and the presence of aragonite in nano-seashell powder. Both calcite and aragonite are two different forms of CaCO3. The main difference between calcite and aragonite is that the crystal system of calcite is trigonal, while the crystal system of aragonite is orthorhombic. Since they are the different structures of the same chemical compound, they are called polymorphs. However, they have distinct physical properties. Calcite can dissolve in acid while aragonite is a stable polymorph of $CaCO_3$ [31]. The low pH of the demineralizing solution could trigger the high release of calcium ions from calcite present in nano-pearl increasing calcium ions levels in the artificial saliva; with subsequent increase of enamel surface microhardness (405.97%). Furthermore, the presence of silica in nano-pearl powder composition observed by EDX analysis (Table 2) might be one of the causes of the increase of enamel surface microhardness observed in group B.

DIAGNOdent pen® had been chosen as a non-invasive diagnostic tool for assessing the laser fluorescence of non-cavitated initial enamel lesion. It is a device used for identifying initial enamel lesions and observing the effect of preventive interventions, because of its excellent sensitivity [32]. Many literature reports had shown the value of DIAGNOdent pen® as a reliable non-invasive caries detecting device[33]. The surface of the tooth absorbs the laser light and emits fluorescence within the infrared spectrum field. The device is then allowed directly to quantify the reflected laser light energy [34]. Intact healthy tooth structure showed little or no fluorescence resulting in very low scale display readings (0-7) adjusted by the manufacturer. Demineralized areas, however, displayed fluorescence proportionate to the degree of demineralization leading to elevated scale readings (8–15 initial demineralization and >16 strong demineralization) [34]. In the current study, the remineralization degree was quantified by the change in the number of units of fluorescence measured with DIAGNOdent pen® at baseline, after 72 h of demineralization then after 14, and 28 days of remineralization. In the interval from baseline to demineralization, all groups recorded a percent increase of laser fluorescence ranging from 522.62 % to 814.67% with no statistically significant difference between groups (p=0.327).

Both laser fluorescence and enamel surface microhardness results confirmed the development of non-cavitated initial enamel lesion; denoting the strong demineralizing effect of Pepsi of low pH used as demineralizing solution in this study [16]. After 14 and 28 days of remineralization, all groups showed a percent decrease of laser fluorescence with no significant difference between groups indicating the high remineralizing capacity of

nano-seashell, nano-pearl, nHA pastes as well as fluoride-based toothpaste. After 14 and 28 days of remineralization, Group A treated with nano-seashell paste showed the highest percentage decrease of laser fluorescence (-89.02%, -60% respectively). The highest remineralization potential reported by group A could be due to the highest Ca percentage in nano-seashell powder (39.86%) proved by the EDX characterization results of the present study (Table 1) in comparison with other groups.

According to Nugroho et al., 96.9% hydroxyapatite could be extracted from cockle seashells (Anadara granosa) that can be effectively used as a remineralizing agent for initial enamel lesion [17]. Ali Abdelnabi, et al compared the remineralizing effect of different concentrations of nHA with different concentrations of $CaCO_3$ and he proved that 10% nHA had a higher remineralizing effect than 10% $CaCO_3$ [35]. This inconsistency with our study results could be due to the different sources of calcium carbonate. Nano calcium carbonate has good retention to enamel surface because of its tiny particle, therefore, increasing the concentration of calcium ions as well as the saliva pH, hence facilitating the enamel remineralization [36].

Group B treated with nano-pearl paste showed a high mineral gain of enamel according to both laser fluorescence and enamel surface microhardness results. Many studies revealed the high osteogenic activity of pearl powder that would be a promising candidate as a novel osteoconductive material for bone repair [36,37]. Pearl powder could stimulate osteoblast proliferation more rapidly than HA and could be used in bone tissue engineering [38]. The effect of coral calcium topical application on enamel and its remineralizing capability on teeth is not yet adequately studied [36].

In some clinical studies, toothpaste contained nHA showed promising results regarding enamel remineralization rate and its acid resistance. However, nHA under neutral conditions has a superficial enamel remineralization effect but the complete remineralization process of the body lesion has not been observed [19]. This could explain the inferior laser fluorescence and enamel surface microhardness results of group C treated with nHA paste when compared to fluoride and nano-pearl paste groups. On the other hand, these results are inconsistent with other studies that concluded that nHA paste had a similar remineralizing potential of initial enamel lesion as fluoride varnish under dynamic pH cycling conditions [3,15,39]. After 28 days of remineralization, group C showed a percent decrease in laser fluorescence (-35.24%). Owing to its high affinity and adsorption to tooth surfaces, nHA has been shown to induce remineralization of initial caries lesions biomimetically by forming crystal nucleus, by promoting crystal deposition and continuously attracting large amounts of calcium and phosphate ions from the surrounding remineralization solution [40]. It was also proved that remineralization with nHA continued over an extended period [12,41].

Fluoride treated group showed a percent decrease of laser fluorescence (-42.21%) but higher than nano-seashell-treated group (-60%) denoting a higher remineralizing effect of nano-seashell paste. Researchers proved that for every two fluoride ions, ten calcium ions and six phosphate ions are required to form one-unit cell of fluorapatite. Accordingly, the inadequate amount of available calcium and phosphate ions in fluoride-based toothpaste can limit complete enamel remineralization [24]. These results are conflicting with a study that showed that the mineral deposits in the dentinal tubules openings formed by NaF were higher than those formed by Nano-seashell [20].

The difference of laser fluorescence and surface microhardness results of group D (treated with fluoride-based toothpaste) could be explained by Tomaz et al. who claimed that since Vickers Microhardness Tester evaluates only enamel surface microhardness not subsurface microhardness, the enamel surface microhardness recovery does not necessarily reflect the ability of fluoride toothpaste to remineralize the enamel subsurface lesion completely [42].

CONCLUSION

Nano-seashell, nano-pearl, nano-hydroxyapatite pastes, and fluoride-based toothpaste have high remineralizing potential. The clinical application of nano-seashell and nano-pearl particles will be a novel noninvasive and effective method to remineralize non-cavitated initial enamel lesions.

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