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Original Article

Effect of Incorporating Nano-Pearl and Nano-Seashell particles into Fluoride-Based Pits and Fissure Sealants on Enamel Remineralization of Extracted Permanent Molars: An Invitro Study.

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Abstract:

Objective: This in-vitro study evaluated the remineralizing effect of nano-pearl and nano-seashell incorporated into fluoride-based pits-and-fissures sealants by assessing the enamel surface microhardness and the enamel elemental analysis.
Materials and Methods: 75 extracted third molars were randomly allocated to one of the following 5 groups of fluoride containing sealant: 10% nano-pearl; 15% nano-pearl; 10% nano-seashell; 15% nano-seashell; or plain fluoride containing sealant. After 2 weeks of dynamic pH-cycling using Pepsi as demineralizing solution; 10 molars of each group were subjected to microhardness assessment, while 5 molars of each group were subjected to Energy Dispersive X-ray Analysis.
Results: Teeth treated with 15% nano-pearl reported the highest percentage increase (20.5%) while teeth treated with plain fluoride containing sealant showed a percentage decrease (-11.62 %). The XRD pattern results confirmed the presence of aragonite and calcite polymorphs of $CaCO_3$ in nano-pearl powder and the presence of aragonite in nano-seashell powder. Calcium ions recorded a significantly higher value in the groups treated with 10% and 15% nano-pearl incorporated pits and fissure sealant.
Conclusion: The incorporation of 10 % and 15 % nano-pearl and nano-seashell particles into fluoride-based pits and fissure sealant significantly improved the enamel surface microhardness as well as the enamel mineral content.

Keywords: Seashell; pearl; remineralization; pits and fissure sealant; calcium carbonate; enamel microhardness.

1. Introduction

Recent dental practice modalities manage early lesions by non-surgical intervention through remineralization to stop ongoing caries progression (Khouroushi and Hachuie, 2017). Most dental caries occurs in the pits and fissures due to their morphological complexity (Khanna,Pandey and Singh, 2015). Pits and fissure sealant prevents effectively pits and fissure caries as it reduces total bacterial counts (Oong *et al.*, 2008). Remineralizing agents incorporated into pits and fissure sealant can improve its mechanical properties and prevent secondary caries (Harris, García-Godoy and Nathe, 2004). It has been shown that remineralizing agents have anti-erosive and anti-cariogenic properties. It interacts with hydrogen ions of the enamel forming calcium hydrogen phosphate that releases calcium and phosphate ions preventing acid dissolution, hence protecting the enamel (Khoroushin and Kachuie, 2017).

Fluoride can effectively promote remineralization when calcium and phosphate ions are present in saliva. Unfortunately, overexposure to fluoride can cause adverse effects as dental fluorosis. Calcium phosphate based technologies are designed to increase the fluoride's ability to restore tooth minerals (Arifa, Ephraim and Rajamani, 2019).

Different forms of hydroxyapatite (HAP) can be prepared from biologically derived natural materials as eggshells and seashells (Akram and Ahmed, 2014) . Hydroxyapatite synthesized from seashell could be used as bone substitute and many other biomedical applications (Brundavanam, Fawcett and Poinern, 2017).

Pearl is one of the medicines used in China for treating bone-related diseases due to its high protein and mineral contents and hence its high osteogenic ability (Yang *et al.*, 2014). Calcium carbonate ($CaCO_3$) is the most essential chemical component of pearl. Pearl powder contains high calcium content, amino acids, zinc, magnesium, iron, and other elements (Brundavanam, Fawcett and Poinern, 2017).

Nano-crystallization has recently been implemented as a new technique for increasing powder drug dissolubility and absorption. Nano-pearl powder is used in a range of medical and cosmetic applications. It was found that the amount of released proteins increased when the size of pearl powder decreased. The nano-pearl powder achieved a long term absorption of calcium in rat femurs when compared to other pearl powder of different particle sizes (Chen *et al.*, 2019).

Nano-pearl powder was demonstrated to have excellent in vitro bone-forming bioactivity (Chen *et al.*, 2013)**.** Few researchers evaluated the enamel remineralizing efficacy of nano-pearl powder. No studies till now evaluated the enamel remineralizing effects of nanoparticles of seashell and pearl powder. The hypothesis of the research work was: would the incorporation of nanoparticles of seashell and pearl powder into fluoride containing pits and fissures sealant add a value to the enamel remineralization? Therefore, this in vitro study aimed to assess and compare the remineralizing effect of both nano-pearl and nano-seashell incorporated into fluoride-based pits and fissure sealants through enamel microhardness assessment and energy dispersive x-ray (EDX) analysis.

2. Materials and Methods

2.1 Materials:

Three materials were used in this study:

a. Seashell powder (Cockle seashells were collected from Red-sea beaches in Egypt)

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

c. Fluoride containing pits and fissure sealant (Imicryl Fissured Nova, IMICRYL, Turkey), of high inorganic filler (55 wt%), self-adhesive to the enamel.

2.2. Methods:

2.2.1. Research Ethical Approval:

Ethical approval was obtained from Institutional Review Board Organization IORG0010866, Faculty of Oral & Dental Medicine, Ahram Canadian University. Research Number: IRB 00012891 #8

2.2.2. Study Design:

The study was a randomized controlled in vitro study, with five parallel groups design with 1:1 allocation ratio. Seventy-five extracted third molars were randomly allocated into five groups (n=15) according to the type of pits and fissure sealant used, where (group A)was treated with 10% nano-pearl incorporated pits and fissure sealant , (group B) was treated with 15 % nano-pearl incorporated pits and fissure sealant, (group C) was treated with 10% nano-seashell incorporated pits and fissure sealant , (group D) was treated with 15% nano-seashell incorporated pits and fissure sealant and positive control group was treated with the plain fluoride containing pits and fissure sealant without any addition. After 2 wk of the dynamic pH-cycling; 10 teeth of each group were subjected to microhardness assessment, while 5 teeth of each group were subjected to energy dispersive x-ray analysis.

2.2.3. Blinding:

The study was a double blinded study as the data analyst and the 2 independent laboratory technicians (who assessed the study outcomes) were blinded.

2.2.4. Sequence Random Allocation:

The specimen allocation to the 5 groups (group A, group B, group C, group D, Control group) was done using block randomization method. The randomization list was generated using www.sealedenvelope.com/simple-randomiser/v1/lists.

2.2.5. Sample Preparation:

Seventy-five sound extracted third molars for orthodontic reasons were used in this study. Consent was obtained from patients (ranged from 18 to 25 years old) before extraction with the approval of using their extracted teeth for research work. Teeth with restorations, enamel cracks, caries, erosion were excluded (Kumar *et al.*, 2018). The teeth were disinfected in 5.25% sodium hypochlorite solution for 60 min (Ceci *et al.*, 2016). All teeth were decoronated by sectioning the roots 2 mm cervical to the cementoenamel junction using a water-cooled diamond saw (Isomet® 5000 Linear Precision Saw: Buehler Ltd., Lake Bluff, USA). All the crowns were embedded vertically in an auto polymerizing acrylic resin block (3.0 cm × 3.0 cm × 3.0 cm). The crowns were scraped with hand scaler; any debris were removed by washing the crowns under running water; then polished with fluoride-free pumice paste (AHL generic, regular medium grain paste, Advanced Health care, Tonbridge, Kent) (Abdelaziz, Mohamed and Talaat, 2019).

Two coats of acid-resistant nail varnish were applied to the crown surfaces except a window of 1 mm width surrounding the occlusal margin (Zawaideh, Owais and Kawaja, 2016). For easy identification, the blocks were numerically coded using a waterproof permanent marker. Each group of teeth was put in a separate glass container containing 30 ml of artificial saliva in the incubator at 37 °C.

2.2.6. Preparation of Artificial Saliva:

Artificial saliva was prepared by adding [0.4 g sodium chloride (NaCl), 1.21 g potassium chloride (KCl), 0.78 g sodium dihydrogen dehydrate ($NaH_2PO_4.2H_2O$), 0.005 g hydrated sodium sulphide ($Na_2S.9H_2O$), and 1 g urea $CO(NH_2)_2$] in 1000 ml deionized water. The pH of this mixture was modified with 10N sodium hydroxide until it reached 6.750.15 on a pH meter (Bali, Prabhakar and Basappa, 2015).

2.2.7. 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 Biostore market in Braunshweig, Germany. The powder was milled (in 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-pearl powder (of particle size ranged from 11.79 to 17.10 nm) was then dispersed in distilled water (10%w/v and 15% w/v) while stirring to obtain 10% and 15 % nano-pearl concentration. A percent weight / volume (%w/v) was calculated using the formula: %w/v = g of solute /100ml of solution. Carboxymethyl cellulose 4%w/v was gradually added to the mixture with continuous stirring until the suspension turned to the gel form.

2.2.8. 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 d at 110° C in the oven. The dried shells were then hand-grounded into powder using agate mortar. The grounded powder was milled (in 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 and 15% w/v); then carboxymethyl cellulose (4%w/v) was gradually added to the suspension with continuous stirring until the suspension turned to a gel form of nano-seashell (Tram, 2020).

2.2.9. Morphological Examination of Nano-pearl and Nano-seashell Powders:

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

2.2.10. Chemical Analysis of Nano-pearl and Nano-seashell Powders:

An X-Ray Diffraction analysis (XRD) had been performed using the XPERT-PRO Powder Diffractometer system, with 2 theta (20^o - 80^o), with Minimum step size 2 Theta: 0.001, and at a wavelength (Kα) = 1.54614^o.

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

2.2.11. Pits and Fissure Sealant Application:

Equal amounts by volume from both nano-pearl gel (10% and 15%) and nano-seashell gel (10% and 15%) were mixed on a glass slab with the plain fluoride containing pits and fissure sealant used (1:1). In all groups, the polished occlusal surfaces were etched with 37% phosphoric acid for 20 s, rinsed for 20 s, and then air-dried as per the manufacturer's instructions. The etched pits and fissures were treated with the corresponding pits and fissure sealants that were previously mixed: 10% nano-pearl incorporated pits and fissure sealant (Group A), 15% nano-pearl incorporated pits and fissure sealant (Group B), 10% nano-seashell incorporated pits and fissure sealant (Group C) and 15% nano-seashell incorporated pits and fissure sealant (Group D) as well as the positive control group that was treated with the plain Fluoride containing pits and fissure sealant without any addition. All treated teeth were light-cured as per the manufacturer's instructions. Then each group of teeth had been stored in a separate glass container containing artificial saliva (30ml) in the CO_2 incubator (ST-45, Benchmark Scientific Company, USA) at 37˚ C. The artificial saliva was changed daily.

2.2.12. Dynamic pH-cycling:

All teeth were subjected to the pH-cycling regimen for 2 wk to simulate the long-term high humidity and acid challenge present in the oral cavity. Two complete cycles were done in 24 h, in other words one cycle every 11.5 h. Each group of teeth was put in artificial saliva (30ml) for 11.5 h , then removed and immersed in the demineralizing solution (10 ml)for 30 min (Choudhary *et al.*, 2012). The demineralizing solution used in this study was Pepsi® (Sikri *et al.*, 2016). After 30 min, the teeth were removed from Pepsi®; then each group was washed separately in 10 ml distilled water for 5 min. Finally, the teeth were returned to the glass containers containing the artificial saliva (30ml) in the CO_2 incubator at 37˚C till next pH-cycle.

2.2.13. Demineralizing Solution:

Pepsi® Soft drink was used as demineralizing solution with pH 2.49 (For each 100 ml: sodium 30 mg 1%, potassium 10 mg, phosphorus 53 mg 4%, carbohydrates 41 mg 14%, citric acid 5 %) (Bakri *et al.*, 2017).

2.2.14. Enamel Surface Microhardness Assessment (SMH):

After the 2 wk of the pH-cycling, the teeth were sectioned buccolingually using a diamond cutoff disk mounted on a cutting machine (ISOMET 1000 Precision Saw, Buehler, Lake Bluff, USA). The teeth halves were embedded horizontally in epoxy resin so that the outer enamel surface was inside the resin while the inner treated surface was outside visible and ready for SMH assessment.

Microhardness measurements were done using a Vickers test apparatus under a 200-gm load for 10 s (Leitz Miniload Model LL, Wilson Mechanical Instrument Hardness Tester, Connecticut, USA). For each half tooth specimen, 3 measurements were performed on different regions of the fissure enamel: closer to the sealant on the fissure side wall, far from the sealant on the fissure side wall; closer to the sealant on the fissure base (Haznedaroglu, Sozkes and Mentes, 2014). Microhardness value was obtained using the equation: $HV=1.854\ P/d^2$. HV is Vickers hardness in Kgf/mm^2, P is the load in Kgf and d is the length of the diagonals in mm (Taher and Bayoumi, 2018).

2.2.15. Chemical Analysis of Enamel Surface:

At baseline and after 2 wk of pH-cycling, the enamel specimens were examined for elements such as carbon, fluoride, calcium, and phosphorus. The enamel specimens were sputter-coated with a thin gold foil film. Then, the specimens were subjected to Energy Dispersive X-ray (EDX) analysis to determine its mineral content (mass/atomic percentage). The digital outputs of the EDX values were interpreted numerically at baseline and after 2 wk of pH-cycling (Taher and Bayoumi, 2018).

2.2.16. Statistical Analysis:

The data was provided as a mean and standard deviation (SD). The Kolmogorov-Smirnov test of normality was used to analyze the data for normality. The Kolmogorov-Smirnov test suggested that data were normally distributed (parametric data), so an independent one-way study of variance was used to compare groups, followed by Tukey's post hoc test. The percent change of enamel surface microhardness as well as enamel surface chemical analysis was calculated by the formula:

Value after sealant application - value before sealant application X100

Value before sealant application

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.

3. Results:

3.1. Morphological Examination of Nano-pearl and Nano-seashell Powders:

TEM analysis 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. While 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.

3.2. Chemical Analysis of Nano-pearl and Nano-seashell Powder:

The XRD pattern of nano-pearl powder suggested the presence of aragonite and calcite crystals, while the XRD pattern of nano-seashell powder suggested the presence of aragonite crystals. The two minerals have their highest-intensity peaks at different positions. 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 peaks.

The standard EDX spectra were recorded on the examined nano-pearl and nano-seashell powders. Calcium, carbon, oxygen, sodium, aluminium, silica, and sulfur were detected in the EDX spectrum of nano-pearl powder. Calcium, carbon, oxygen, and sodium were detected in the EDX spectrum of nano-seashell powder. Table 1, and table 2 are showing the presented spectrum of the elements' values of the prepared powders.

3.3. Enamel Surface Microhardness:

Before pits and fissure sealants application (Baseline), there was no significant difference between groups (p=0.273). After pits and fissure sealants application (After 2 wk of pH-cycling), the highest mean value was recorded in group B (327.94 Kgf/mm^2); followed by group D (279.62 Kgf/mm^2), then group A (276.8 Kgf/mm^2), and group C (260.99 Kgf/mm^2), with the least value recorded in the positive control group (205.95 Kgf/mm^2) (as shown in table 3 and figure1). ANOVA test revealed that the difference between groups was statistically significant (p=0.00). Tukey’s post hoc test revealed no significant difference between A, C, D groups.

Regarding the mean percentage of change of enamel microhardness after 2 wk of pH-cycling, all experimental groups (A, B, C, and D) showed an increase of the percentage of change of enamel microhardness. Group B reported the highest percentage of change of enamel microhardness (20.5%) while the positive control group showed a decrease of the percentage of change (-11.62 %)

(as shown in table 3 and figure 2). ANOVA test revealed that the difference between groups was statistically significant (p=0.00). Tukey's post hoc test revealed no significant difference between A, C, D groups.

Based on the results of ANOVA test for the enamel surface microhardness, a large effect size (0.676) was detected yielding 99% power for the study.

3.4. Chemical Analysis of Enamel Surface:

Elemental analysis results for all teeth samples before and after application of different pits and fissure sealants are presented in table (4) and figures (3-4). Regarding the percentage of change of each element in each group, experimental groups (A, B, C, and D) showed a decrease of the percentage of change of carbon (C) ions (-74.52%, -69.98%, -61.91%, -66.55%) respectively which was significantly different from the increase of percentage change observed in the control group (24.39%) (p=0.00). Phosphorous (P) ions recorded a significantly lower value in the control group (-54.04%) (p=0.000). The greatest percentage increase of (P) ions was observed in group A (163.45%). All experimental groups showed a significant percentage increase of calcium (Ca) ions. Group B recorded a significantly higher value (1729%) followed by group A (821%); while a significantly lower value was recorded in the control group (-37.11%) (p=0.000).

4. Discussion

Fluoride remineralization is considered the backbone for caries prevention. However, fluoride remineralization was seen to be restricted to the superficial layer of the tooth, not the subsurface lesion (Philip, 2019). Additionally, fluoride has been considered as a chemical neurotoxicant when used in high concentrations (Grandjean and Landrigan, 2014). Moreover, it was found that fluoride failed to form well-organized apatite crystals (Ruan *et al.*, 2016).

Natural remineralizing agents could be more effective than fluoride to remineralize the dental tissues without the adverse effects of fluoride. Marine biomaterials are considered good candidates for biomedical applications. To reduce the pollution and give good value to waste shells, nanohydroxyapatite (nHAP) had been extracted from the seashell waste and pearl (Dhanaraj and Suresh, 2018). Nano HAP similarity to the mineral tooth structure and its biocompatibility encouraged the researchers to use it as remineralizing agent (Thimmaiah *et al.*, 2019).

This study used biologically derived natural materials such as pearl and seashells (Anadara granosa) as remineralizing agents and incorporated it into pits and fissure sealants. Both nano- pearl and nano-seashell particles had been reported by many previous studies to be biocompatible and toxic-free (Brundavanam, Fawcett and Poinern, 2017). Cockle seashells presented high percentage viability (>80%) even when used at high concentrations (Fu *et al.*, 2017). As nanotechnology has been introduced as a strategy that increases the dissolubility and absorption of powder drugs (Takebe *et al.*, 2017), the pearl powder and seashell powder used in this study were obtained in nanoparticle size.

Pits and fissure sealing reduces occlusal early caries lesions (Deery, 2017). Successful caries prevention by sealing deep pits and fissures depends mainly on the stability of the sealant in the oral environment, its resistance to acid dissolution, as well as its remineralizing efficiency. Many researchers had investigated the effect of pits and fissure sealants containing fluoride, amorphous calcium phosphate, or nanoparticles. However, the present study is the first study that investigated the effect of incorporating nano-pearl, nano-seashell of different concentrations into fluoride containing pits and fissure sealants. Plain fluoride containing pits and fissure sealant was used in the current study as the positive control group to compare the remineralizing effects of the tested sealants. Acidic soft drink (Pepsi®) was used as a demineralizing solution in the dynamic pH-cycle to simulate the effect of soft drinks on the pits and fissure sealants used in this study. The dynamic pH-cycling model was performed to mimic the active process of demineralization and remineralization that normally occurs in the oral environment(Choudhary *et al.*, 2012).

Table 1: The weight % and quantitative results of EDX spectrum of nano-pearl powder

Element	Weight %	Atomic %	Error %
C K	6.93	11.69	7.82
O K	53.49	67.72	9.94
Na K	0.97	0.85	12.64
Al K	0.53	0.4	9.57
Si K	0.32	0.23	7.83
S K	0.16	0.1	22.23
Ca K	37.59	18.99	1.41

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

Table 2: The weight % and quantitative results of EDX spectrum of nano-seashell powder

Element	Weight %	Atomic %	Error %
C K	4.81	8.29	19.52
O K	54.01	69.92	11.51
Na K	1.32	1.19	30.62
Ca K	39.86	20.6	1.54

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

Table 3: Descriptive statistics and comparison between enamel surface microhardness (Kgf/mm^2) after treatment with different pits and fissure sealants after dynamic pH-cycling. (ANOVA test)

		Mean (Kgf/mm^2)	Std. Dev	Std. Error	95% CI for Mean Lower Bound	95% CI for Mean Upper Bound	Min	Max	F	P
Baseline	**Group A**	244.92	29.26	9.25	223.99	265.85	191.73	285.13		
	Group B	276.00	48.40	15.31	241.38	310.62	194.2	341.57	1.33	0.273 ns
	Group C	240.25	41.40	13.09	210.63	269.87	190.7	317.93		
	Group D	256.33	67.18	21.24	208.28	304.39	146.27	339.63		
	Control group	231.47	40.56	12.83	202.46	260.49	193.77	315.27		
After2 wk of pH-cycling	**Group A**	276.80^{a,b}	29.73	9.40	255.53	298.07	222.47	315.2	8.46	0.00*
	Group B	327.94^{a}	38.13	12.06	300.66	355.22	250.9	382.8		
	Group C	260.99^{b}	41.74	13.20	231.13	290.85	217.23	338.73		
	Group D	279.62^{a,b}	71.02	22.46	228.82	330.42	167.4	374.57		
	Control group	205.95^{c}	47.09	14.89	172.27	239.63	151.8	299.47		
Percent change	**Group A**	13.18^{b}	2.75	0.87	11.21	15.15	9.22	16.22		
	Group B	20.57^{a}	13.69	4.33	10.78	30.37	5.07	41.68	27.14	0.00*
	Group C	8.87^{b}	2.80	0.89	6.86	10.87	5.21	14.6		
	Group D	9.45^{b}	2.70	0.85	7.52	11.38	5.42	14.45		
	Control group	-11.62^{c}	7.32	2.32	-16.86	-6.38	-24.28	-1.77		

Significance level p≤0.05, *significant

Tukey's post hoc test: means sharing the same superscript letter are not significantly different

Table 4: Descriptive statistics and comparison between elemental analysis of enamel (wt%) before and after treatment with different remineralizing pits and fissure sealants after dynamic pH-cycling (ANOVA test)

		Group A	Group B	Group C	Group D	Control Group	F value	P value
C	**Before**	50.35^{a} ±6.12	$47.97^{a,b}$ ±5.1	48.41^{a} ±5.3	45.32^{b} ±7.9	39.98^{c} ±7.4	3.739	0.01*
	After	12.83^{b} ±2.7	14.40^{b} ±3.1	18.44^{b} ±4.9	15.16^{b} ±3.8	49.73^{a} ±12.1	61.47	0.00*
	% change	-74.52^{a} ±16.5	-69.98^{a} ±15.5	-61.91^{a} ±14.8	-66.55^{a} ±20.1	24.39^{b} ±8.6	72.79	0.00*
P	**Before**	$2.38^{b,c}$ ±0.9	3.14^{b} ±0.8	4.55^{b} ±1.1	8.85^{a} ±2.3	3.96^{b} ±1	38.32	0.00*
	After	6.27^{a} ±1.3	3.42^{b} ±0.6	5.68^{a} ±1.6	1.47^{c} ±0.3	1.82^{c} ±0.4	48.73	0.00*
	% change	163.45^{a} ±34	8.92^{c} ±1.6	24.84^{b} ±4.8	-83.4^{e} ±11.5	-54.04^{d} ±9.6	315.62	0.00*
Ca	**Before**	1.14^{c}±0.3	1.17^{c}±0.3	2.92^{b}±0.4	3.27^{b}±0.4	4.15^{a}±0.5	118.43	0.00*
	After	10.50^{c} ±2.1	21.40^{a} ±3.6	11.98^{c} ±2.1	15.75^{b} ±3.4	2.61^{d}±0.6	71.86	0.00*
	% change	821.05^{b} ±87.3	1729.06^{a} ±298.2	310.27^{c} ±60.3	381.7^{c} ±53.5	-37.11^{d} ±5.4	212.8	0.00*

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

Tukey's post hoc test: Within the same comparison (each row), means sharing the same superscript letter are not significantly different

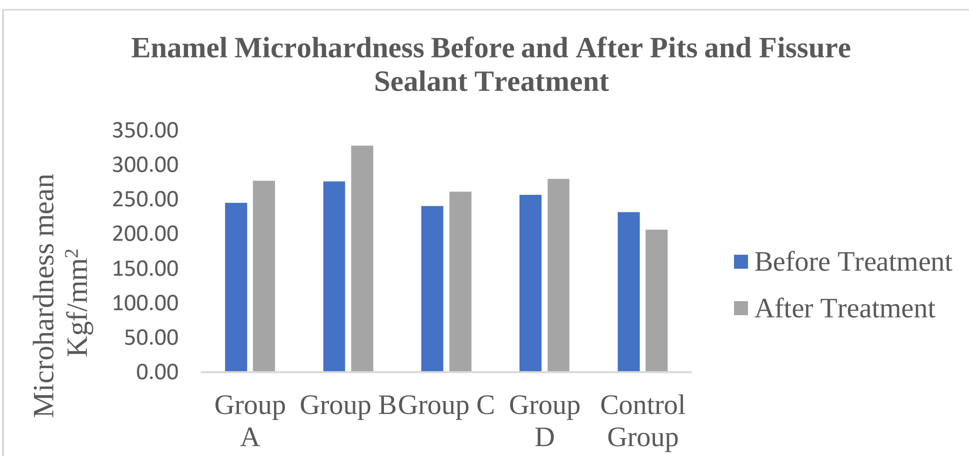

Figure 1: Bar chart illustrating mean enamel microhardness before and after treatment with different pits and fissure sealants (Group A: 10 % nano-pearl incorporated pits and fissure sealants, Group B: 15 % nano-pearl incorporated pits and fissure sealants, Group C: 10 % nano-seashell incorporated pits and fissure sealants, Group D: 15 % nano-seashell incorporated pits and fissure sealants, Control Group: Plain Fluoride-based pits and fissure sealant).

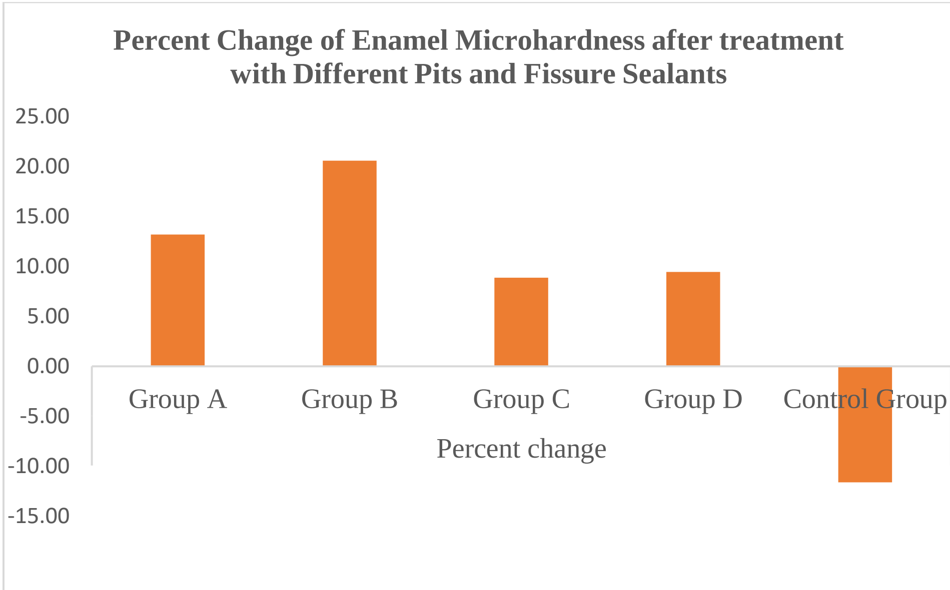

Figure 2: Bar chart illustrating percent change of enamel microhardness after treatment with different pits and fissure sealants (Group A: 10 % nano- pearl incorporated pits and fissure sealants, Group B: 15 % nano-pearl incorporated pits and fissure sealants, Group C: 10 % nano-seashell incorporated pits and fissure sealants, Group D: 15 % nano-seashell incorporated pits and fissure sealants, Control Group: Plain Fluoride-based pits and fissure sealant).

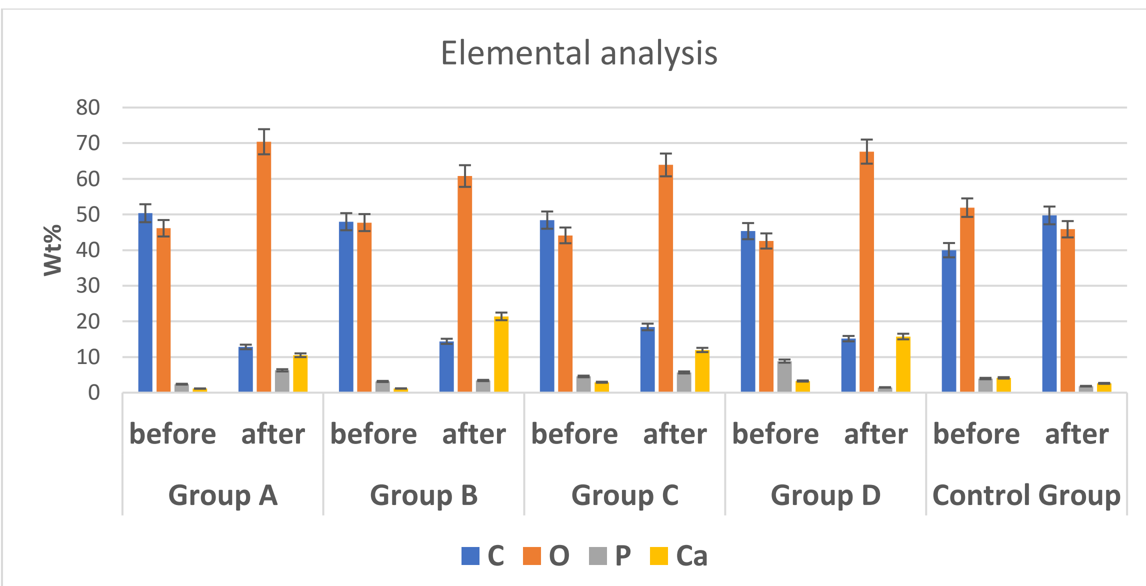

Figure 3: Bar chart illustrating the mean value of enamel elemental analysis obtained by EDX analysis (Carbon, Oxygen, Phosphorous and Calcium) before and after pits and fissure sealant application and pH-cycling (Group A: 10 % pearl incorporated pits and fissure sealants, Group B: 15 % pearl incorporated pits and fissure sealants, Group C: 10 % seashell incorporated pits and fissure sealants, Group D: 15 % seashell incorporated pits and fissure sealants, Control Group: Plain Fluoride-based pits and fissure sealant).

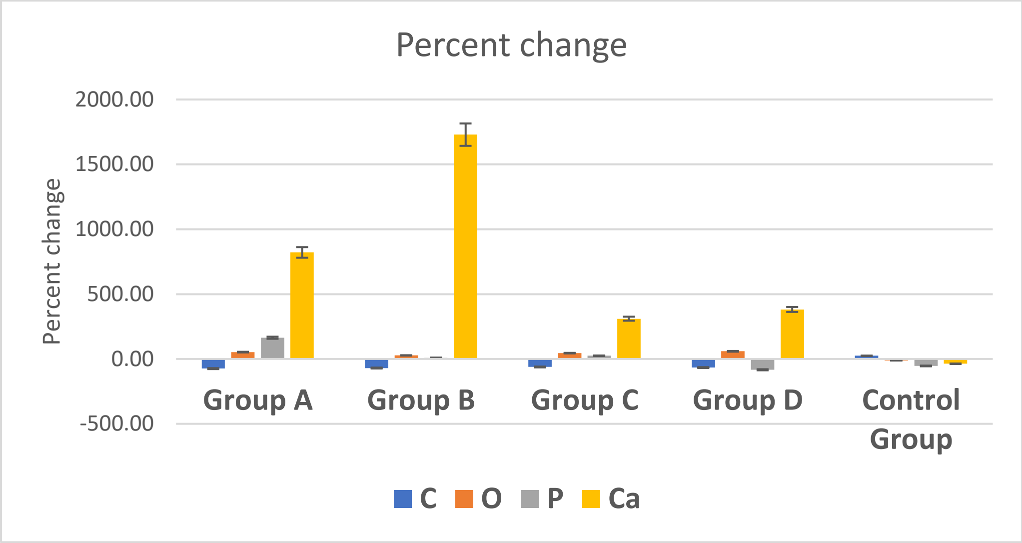

Figure 4: Bar chart illustrating percent change of elemental analysis obtained by EDX analysis (Carbon, Oxygen, Phosphorous and Calcium)before and after pits and fissure sealant application and pH-cycling (Group A: 10 % pearl incorporated pits and fissure sealants, Group B: 15 % pearl incorporated pits and fissure sealants, Group C: 10 % seashell incorporated pits and fissure sealants, Group D:15 % seashell incorporated pits and fissure sealants, Control Group: Plain Fluoride-based pits and fissure sealant).

All teeth were subjected to the pH-cycling regimen for 2 weeks to simulate the long-term high humidity and acid challenge present in the oral cavity that may cause dissolution of the sealant. Artificial saliva was used as a storage medium for all experimental and positive control teeth receiving different sealants, to simulate the remineralizing capacity of human saliva (Ionta *et al.*, 2013).

Vickers surface microhardness (SMH) test, as well as elemental analysis (EDX analysis), were used in the present study to evaluate the enamel remineralization effect of different tested sealants. The objective of baseline SMH assessment was to compare and calculate the changes that occur after applying the pits and fissure sealants during the pH-cycling (Bandekar *et al.*, 2019). The microhardness test results showed that the highest mean value was observed in group B treated with 15% nano-pearl powder incorporated pits and fissure sealant (327.94 Kgf/mm^2). The presence of silica in nano-pearl composition observed by EDX analysis (shown in table 1) might be one of the causes of the remarkable increase of enamel surface microhardness observed in group B. The high surface microhardness of enamel as well as its significant high value of Ca ions (1729%) confirmed the high remineralizing effect of 15% nano-pearl incorporated pits and fissure sealant. This could be attributed to the biomimetic ability of pearl powder to form HAP crystals (Yang *et al.*, 2014). Nano-pearl powder had previously been shown to stimulate the formation of an apatite layer on bone surfaces (Chen *et al.*, 2013). It was previously proven that pearl nano-crystallization improved its calcium bioavailability as a result of its enlarged specific surface area per unit volume with subsequently increased solubility (Chen, Chang and Wu, 2008) ; this could be the cause of promoting apatite layer formation on enamel surfaces. Nacre is the material of which pearls are composed. Nacre also called 'mother of pearl', is composed of a crystalline $CaCO_3$ mineral phase suspended in an organic matrix. The individual crystals are enclosed by a thin layer of an organic matrix composed mainly of protein and polysaccharide. Perlucin, lustrin A, perlwapin, perlinhibin, and perlustrin are some of the proteins that form the organic matrix of nacre. Perlucin had been shown to facilitate biomineralization and promote $CaCO_3$ nucleation and crystal orientation (Mailafiya *et al.*, 2019) (Raj and Patil, 2020). The collected evidence from the previous studies confirmed the biomimetic remineralizing effect of nano-pearl to form well-organized apatite crystals (Yang *et al.*, 2014). This explains the lower enamel surface microhardness treated with plain fluoride containing pits and fissure sealant (205.95 Kgf/mm^2) when compared to that treated with nano-pearl incorporated pits and fissure sealant (327.94 Kgf/mm^2). A possible explanation for our findings is that fluoride alone could not ensure biomimetic remineralization. Fluoride (in the plain sealant with no pearl or seashell addition) failed to regulate the growth, shape, and arrangement of apatite crystals during enamel remineralization (Ruan *et al.*, 2016).

The XRD pattern results in the present study confirmed the presence of aragonite and calcite polymorphs of $CaCO_3$ in nano-pearl powder and the presence of aragonite in nano-seashell powder. Both calcite and aragonite are two different forms of $CaCO_3$. The key difference between calcite and aragonite is that the crystal system of calcite is trigonal, whereas the crystal system of aragonite is orthorhombic. Since they are different structure 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 $CaCO_3$ (Yang *et al.*, 2014). This explains the high release of calcium ions (1729 %) from calcite present in nano-pearl and the subsequent increase of enamel surface microhardness treated with 15% nano-pearl incorporated pits and fissure sealant (327.94 Kgf/mm^2). Enamel surface microhardness results suggested that increasing nanoparticles percentage of nano-pearl and nano-seashell incorporated in sealants was positively affecting the remineralizing effect of the tested sealants. Moreover, the results confirmed the synergistic effect of adding nano-pearl and nano-seashell particles to fluoride. Our results were in agreement with other previous studies that reported the synergistic effect of bioavailable calcium ion and fluoride (Shen *et al.*, 2018).

The low pH of the used demineralizing solution in the dynamic pH-cycling could trigger the Ca ion release and leach out from aragonite and calcite polymorphs of $CaCO_3$. Both nano-pearl and nano-seashell incorporated pits and fissure sealants were "smart" by increasing the Ca and P ions release considerably more than the plain fluoride containing pits and fissure group when the pH was reduced from neutral to a cariogenic pH of 2.4. Adding the nano-pearl or nano-seashell particles into fluoride containing pits and fissure sealants might increase its remineralizing capacity through the massive Ca and P ions release as shown in table (4).

The enamel elemental analysis results confirmed the surface microhardness results. A significantly higher value of Ca ions in nano-pearl pits and fissure sealants groups (1729 %, 821%) than the nano-seashell groups (310%, 381%) was observed. The reason for this finding could be due to that $CaCO_3$ is pH-sensitive material (Mailafiya *et al.*, 2019). In the acidic solution of pH values below 2.9, the dissolution of calcite occurred by the transportation of the reactant to the surface (surface reaction) (Ryu *et al.*, 2010). This could be the reason for leaching out of Ca ions from calcite crystals much more than aragonite. $CaCO_3$ acts as a calcium reservoir, facilitating the release of Ca ions as they break down during acidic challenges and thus increasing Ca ions levels in artificial saliva. Consequently, saturated artificial saliva with Ca ions helped enamel surface mineralization. Also, released CO_3 ions act as a buffering element in the artificial saliva (Lynch and Ten Cate, 2005). On the other hand, nano-seashell powder which consists mainly of aragonite crystals; is characterized by its high mechanical strength and slow biodegradation (Mailafiya *et al.*, 2019) which could be the cause of its slower dissolution and its lower release of Ca ions when compared to nano-pearl groups.

After application of the plain fluoride containing pits and fissure sealant (Positive control group), a significant increase of the percentage of carbon ions was recorded (24.39%) ($p=0.00$). This great increase could be the main cause of HAP crystals dissolution. The carbon ions could replace phosphate ions or hydroxyl groups of enamel HAP leading to increase apatite solubility (Alkattan *et al.*, 2018). On the other hand, all experimental pits and fissure sealants (A, B, C, D groups) showed a decrease of the percentage of carbon ions, which was significantly different from the increase of carbon ions percentage recorded in the control group. These results were consistent with a previous study that reported significantly lower amounts of calcium and phosphorus with greater amounts of carbon and nitrogen in carious lesions when compared to sound enamel (Meyer-Lueckel, Hendrik Paris, 2008).

Conclusion

Under the limitations of the current in vitro study, it could be concluded that the incorporation of 10 % and 15 % nano-pearl and nano-seashell particles into fluoride containing pits and fissure sealant significantly improved the enamel surface microhardness as well as the enamel mineral content (Ca and P ions); although all groups were subjected to acid challenge.

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