Original Article

A comparative evaluation of human enamel remineralization ability of biomimetic nacre against casein phosphopeptide-amorphous calcium phosphate: An *in vitro* study

Vantari Swathi Reddy, Jayaprada Reddy Surakanti[1], Deepak Kumar Sharma[2]

Department of Conservative Dentistry and Endodontics, Government Dental College, Hyderabad, [1]Department of Conservative Dentistry and Endodontics, Kamineni Institute of Dental Sciences, Nalgonda, Telangana, [2]Department of Conservative Dentistry and Endodontics, Jaipur Dental College, Jaipur, Rajasthan, India

Abstract

Introduction: This study aimed to assess and compare the efficacy of Nacre and casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) on the remineralization of enamel using surface microhardness analysis, scanning electron microscopy (SEM), and energy dispersive X-ray (EDX) spectroscopy.

Materials and Methods: Twenty human maxillary premolars extracted for orthodontic reasons were collected. Under cool water spray, the crowns were sectioned mesiodistally into buccal and palatal halves using a diamond disc. The samples were subsequently mounted in self-cure acrylic resin. The samples were then subjected to Vickers hardness testing and SEM-EDX for baseline. To simulate carious lesions, all of the samples were acid-etched with 37% phosphoric acid for 30 s in a specific area on the enamel samples and subjected to surface microhardness testing and SEM-EDX. The enamel samples were randomly assigned to Group 1: Nacre water-soluble matrix (WSM), Group 2: Nacre varnish, and Group 3: CPP-ACP for remineralization. After 21 days, remineralization assessment of the test samples was done using SMH analysis and SEM-EDX analysis. Data obtained were statistically analyzed using the one-way analysis of variance to reveal the significant differences between the groups. Tukey's test was used for *post hoc* comparisons.

Results: All three groups showed a significant increase in surface microhardness. All three groups showed a significant calcium and phosphorous ratio increase after remineralization. Among the three groups, the highest Ca:P ratio was seen in the Nacre WSM group (0.58) followed by the Nacre Varnish (0.57) and CPP-ACP group (0.57). SEM images of the Nacre surface revealed the presence of extensive interlocking. A layer of packed hydroxyapatite particles was formed on the surface of the nacre through surface reactions.

Conclusion: All the groups in the present study showed some extent of remineralizing ability irrespective of the different materials and mechanisms of action. Nacre WSM showed a remarkable hardness spike close to natural enamel after demineralization.

Keywords: Biomimetic; casein phosphopeptide-amorphous calcium phosphate; enamel remineralization; nacre; varnish

Address for correspondence:
Dr. Jayaprada Reddy Surakanti,
Department of Conservative Dentistry and Endodontics,
Kamineni Institute of Dental Sciences, Sreepuram, Narketpally,
Nalgonda, Telangana, India.
E-mail: drsjayapradareddy@gmail.com

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INTRODUCTION

Tooth enamel is a highly mineralized tissue in the human body and its primary component is apatite.[1] The special features of enamel rely on its composite nature of organic materials

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and 95%–97% hydroxyapatite (HAP) by weight. Regulated by enamel matrix proteins, HAP nanorods in native enamel bundles are parallel and densely arranged into a highly ordered unit, forming the hardest tissue in the body. However, when the substantial mineral is lost, the mature enamel can hardly be recovered *in vivo*, leading to dental caries.

A pivotal target of contemporary dentistry is to manage noncavitated lesions noninvasively through remineralization in an attempt to prevent disease progression and improve esthetics, strength, and function.

The ideal remineralizing agent will provide adequate amounts of calcium and phosphate ions to the body of the carious lesion where they are needed and will not readily precipitate on the tooth surface or increase calculus formation.[2] A variety of compounds are currently available such as fluoride, novamin, tricalcium phosphate, sodium monofluorophosphate, casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), etc.[2] These agents are the part of a new era in dentistry that is aimed at improving the dental hard tissue with different modes of action, depending on the microenvironment around the tooth.

Methods for *in situ* tooth remineralization are becoming more diverse and follow biomimetic and bioinspired directions. Bioinspired materials have received considerable attention for the remineralization of enamel due to their excellent bioactivity, biocompatibility, and ability to remineralize the early enamel lesion by forming a new layer of synthetic enamel around the tooth, instead of hardening the existing layer with fluoride.

Biomineralization is an integral process governed by genetic predisposition through which living organisms form mineralized tissues such as shells, bone, pearl, eggshells, and exoskeletons, usually taking place at ambient temperature and pressure. The formed biominerals and nanocomposites made of inorganic and organic substances are renowned for their mechanical properties.[3]

Nacre or mother-of-pearl present in mollusc shells is composed of calcium carbonate crystals organized in the multiple layers of thin tablets of aragonite, surrounded by an organic matrix. A Nacre-based tooth implant was found in the skull of the Mayan tribe, which fused well with the surrounding bone. Various studies have been conducted that show the potential content of Nacre as a new ingredient in regeneration.[4-6]

The organic matter from Nacre could be separated into water soluble and insoluble matrices. The water-soluble matrix (WSM) is responsible for inducing the crystallization of highly-ordered calcium carbonate in Nacre.[7] Moreover, Lamghari *et al*. suggested that WSM, like the bone morphogenetic protein, could induce the growth of HAP crystals at 37°C *in vitro* on rat bone.[8] Subsequently, Ni and Ratner reported the successful formation of HAP on the Nacre surface with the stimulation of WSM.[9] Since the extraction of WSM is facile and inexpensive, Nacre WSM may be a promising mineral template for dental remineralization. Nacre varnish is the processed form of Nacre WSM which is prepared for direct application on the enamel surface to assess remineralization.

Modern prospective caries studies require the measurement of small changes in a tooth's mineral content, especially in a single caries lesion. One such technique is scanning electron microscopy (SEM) with an energy dispersive X-ray (EDX) analysis attachment. It is a microanalytical technique that is employed to quantitatively estimate the amounts of minerals in a given tooth sample.[10] As enamel surface microhardness is related to its mineral content, this index is measured as a criterion to determine enamel demineralization and remineralization.[11]

Hence, this study was conducted to compare the remineralizing ability of the Nacre in WSM and varnish form with the CPP-ACP in an *in vitro* condition, assessed by SMH, SEM-EDX analysis.

MATERIALS AND METHODS

Twenty human maxillary premolars extracted for orthodontic reasons were collected. The teeth were rinsed under running deionized water for 5 min and then refrigerated in 0.1% thymol at 4°C before the experiment.[12] Teeth with caries, deformities, cracks, fractures, restorations, abrasions, developmental anomalies, white spots, and stains were excluded from the study. The flow chart summarizes the protocol of the study.

Preparation of enamel samples

The crowns were sectioned mesiodistally into buccal and palatal halves using a diamond disc under cool water spray. Teeth were sectioned 1 mm below the cemento-enamel junction with a slow-speed diamond disc. Roots were discarded and the crowns were used for the study. The enamel sections were flattened and polished using a series of silicon carbide grit papers (200, 400, 600, 800, 1000, and 1200 grits). Following the polishing of the samples, a 3 mm × 3 mm working window was marked on the sectioned enamel surfaces of all the samples using the adhesive tape. The area of the crown other than the working window was covered with nail varnish making it resistant to acid attack [Figure 1].[13]

The samples were subsequently mounted in self-cure acrylic resin with a buccal or palatal surface facing upward and exposed. The acrylic resin blocks were made using silicone molds of diameter 10 mm to standardize the study.[14] Out of 40 samples, properly mounted 36 samples were used for the study. The samples were then subjected to Vickers

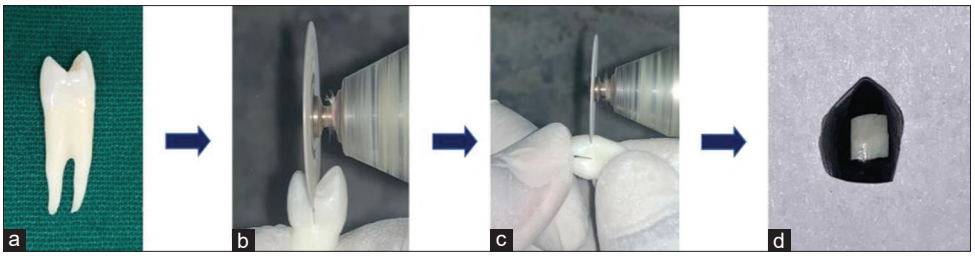

Figure 1: Preparation of enamel samples. (a) extracted premolar, (b) vertical section, (c) horizontal section 1mm below CEJ, (d) working window

hardness testing and SEM-EDX for the baseline analysis of microhardness, surface morphology, and chemical composition, respectively.

Demineralization protocol

To simulate carious lesions, all of the samples were acid-etched with 37% phosphoric acid for 30 s in a specific area on the enamel samples. Following the demineralization procedure, all demineralized samples were washed with copious quantities of deionized water, damped dry, and subjected to surface microhardness testing and SEM-EDX.[15]

Remineralization protocol

Extraction of the nacre water-soluble matrix

Nacre powder (particle size 50–100 mm) was obtained from the inner shell layer of the giant oyster *Pinctada maxima*. Powdered Nacre (100 g) was suspended in 200 ml of ultra-pure water (Milli-Q) for 20 h at the room temperature, with continuous stirring using a magnetic stirrer. The suspension was then centrifuged for 20 min at 3500 revs/min. The supernatant is known as the WSM.[16]

Preparation of SBF

The SBF solution was prepared by dissolving the appropriate amounts of reagent grade chemicals; NaCl, $NaHC0_3$, KCl, $K_2HP0_4.3H_20$, $MgC1_2.6H_20$, Na_2S0_4, and $[(CH_20H)_3CNH_2]$ in distilled water and buffered with HCI to pH 7.4 at 37°C.[17] The remineralizing solution was a mixed solution of SBF and 0.6 mg/mL WSM.

Preparation of nacre varnish

The obtained WSM was subjected to a Rota evaporator to remove water and obtain a fine powder. The resultant powder is mixed with plain varnish (a mixture of fully hydrogenated rosin, hydrophilic fumed silica, and absolute ethanol) to obtain Nacre varnish which was later stored in a sealed vial and used for the study.[18]

The enamel samples were randomly assigned to three groups: GROUP 1: NACRE Water soluble matrix, Group 2: NACRE Varnish, and Group 3: CPP-ACP.

Group-1 - NACRE WSM - (n = 12). The demineralized samples were immersed in freshly prepared remineralizing solution at 37°C, and then, they were sealed in a 20 mL scintillation vial and remineralized for 72 h. The solution was refreshed every 24 h. After remineralization, the samples were removed from the mineralization solution, gently rinsed with double distilled water, and dried at the room temperature followed by incubation in artificial saliva at 37°C.[15] Group-2 - NACRE VARNISH- (n = 12) - Nacre varnish was applied with cotton applicator tips on a demineralized surface once daily and rinsed with deionized water thoroughly for 30 s after application followed by incubation in artificial saliva at 37°C. Group-3 - CPP-ACP- (n = 12) CCP-ACP was applied with cotton applicator tips on a demineralized surface twice daily for 3 min for 7 days, followed by incubation in artificial saliva at 37°C.[19]

The samples from three groups were incubated in a beaker of 30 ml of artificial saliva at 37 degrees for 21 days. The storage medium was changed daily.[20]

After 21 days, remineralization assessment of the test samples was done using SMH analysis and SEM-EDX analysis.

Microhardness analysis

Ten specimens from each experimental group were rinsed thoroughly with deionized water and air-dried at the room temperature. The microhardness of the enamel surface was measured using a Vickers microhardness tester at 300 g for 5 s. The VHN was obtained using the following equation: VHN = 1854.4 P/d^2, where P is the applied load in grams and d is the average length of the indentation measured in millimeters.[21] The indentation formed was viewed and measured on the display monitor with a ×10 objective lens. The average microhardness of the specimen was determined from two indentations to avoid any discrepancy since the enamel surface has a curvature.[22]

Structural analysis and elemental analysis

The surface characteristics of the demineralized and remineralized enamel specimens were analyzed by SEM. The specimens were placed on a metal mounting block and then kept inside the gold sputter coater. After sputtering, the specimens were observed under SEM. The same specimens were prepared for the detection of calcium, phosphorus, fluorine, and silicon using EDX analysis. EDX

has been used for elemental analysis at the ultra-structural level. It is a microanalytical technique that was used in conjunction with the SEM wherein SEM do the structural analysis and elemental analysis was done by EDX.[23]

RESULTS

The results of the microhardness tests of all experimental groups were analyzed using the one-way analysis of variance to reveal significant differences between the groups. Tukey's test was used for *post hoc* comparisons. Data were entered in a Microsoft Excel spreadsheet and analyzed using the SPSS software. For a test, a $P < 0.05$ is considered statistically significant.

Vickers microhardness test

At baseline, there was no statistically significant difference in microhardness values of enamel specimens observed between the interventional groups which allowed for valid comparisons between groups postintervention. *P* value changes of microhardness from the baseline, when compared to post demineralization, show a significant result ($P < 0.05$) for three groups, which shows that significant demineralization of enamel has taken place when 37% phosphoric acid was used [Figure 2]. The specimens treated with WSM showed the highest microhardness value after remineralization followed by CPP-ACP and Varnish but were statistically not significant ($P > 0.05$) [Table 1]. Multiple comparison results indicated a significant difference in the mean microhardness at baseline, after demineralization, and after the remineralization process ($P < 0.05$) [Table 2].

Scanning electron microscopy-energy dispersive X-ray analysis

The calcium and phosphorus content was then converted into Ca/P ratios in each group. This clearly shows a fall in the Ca:P ratio after demineralization in all three groups [Figure 2]. It also shows a significant rise in the amount the remineralization compared to demineralization in all the three samples. Baseline enamel composed of HAP has a smooth, regular surface. The high-resolution images illustrate that HAP nanorods are densely arranged [Figure 2].

Demineralized enamel shows an irregular surface with variable sizes of porosities and the surface texture appears to be disorganized and coarse. The high-resolution images illustrate that HAP nanorods are irregularly arranged with porosities. It shows clear destruction of the enamel surface, destruction of enamel rods, and the dissolution of enamel crystals resulting in significant depressions and irregularities.

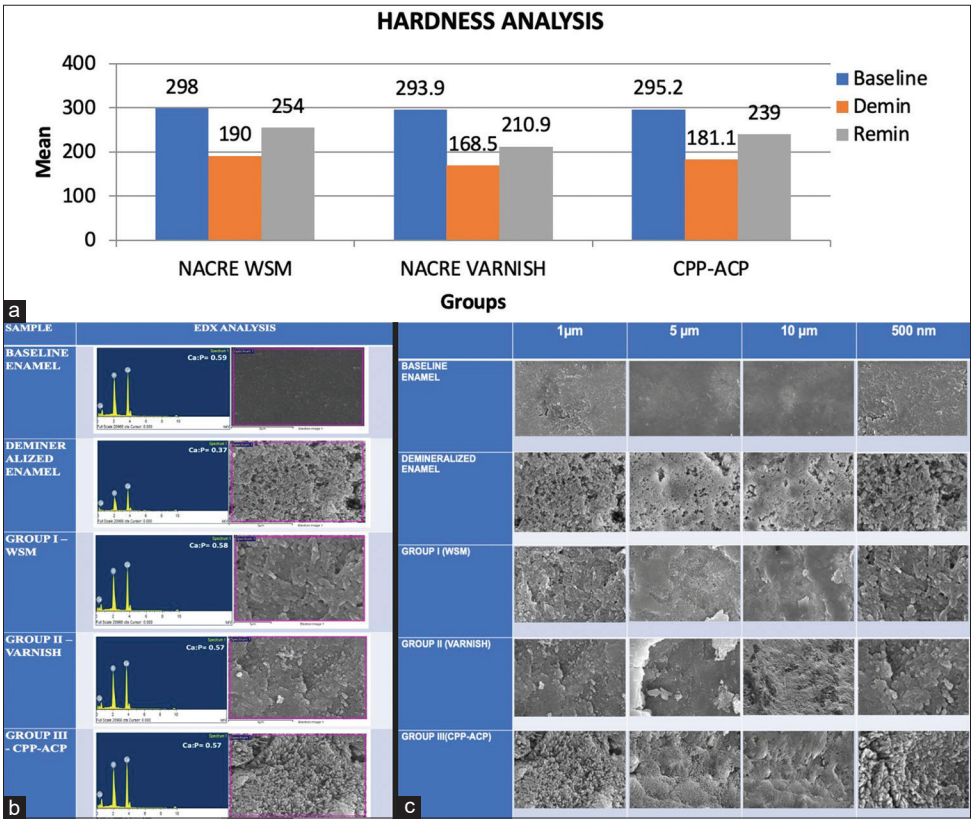

Figure 2: (a) Bar garph showing the comparison of remineralization values of three tested groups showing no statistically significant difference ($P > 0.05$). (b) Elemental analysis energy dispersive X-ray (c) Structural analysis by scanning electron microscopy

Table 1: The microhardness (mean, standard deviation, and 95% confidence interval) of the enamel samples in water-soluble matrix, varnish, and casein phosphopeptide-amorphous calcium phosphate groups using one-way ANOVA

	n	Mean	SD	SE	95% CI for mean lower bound	95% CI for mean upper bound	Minimum	Maximum
Baseline								
WSM	10	298.00	50.379	15.931	261.96	334.04	234	378
Varnish	10	293.90	29.734	9.403	272.63	315.17	248	354
CPP-ACP	10	295.20	47.646	15.067	261.12	329.28	234	378
Total	30	295.70	42.067	7.680	279.99	311.41	234	378
Demin								
WSM	10	190.00	39.004	12.334	162.10	217.90	148	261
Varnish	10	168.50	27.945	8.837	148.51	188.49	120	210
CPP-ACP	10	181.10	35.165	11.120	155.94	206.26	148	256
Total	30	179.87	34.333	6.268	167.05	192.69	120	261
Remin								
WSM	10	254.70	63.666	20.133	209.16	300.24	167	362
Varnish	10	210.90	38.960	12.320	183.03	238.77	137	268
CPP-ACP	10	239.00	39.084	12.359	211.04	266.96	187	300
Total	30	234.87	50.425	9.206	216.04	253.70	137	362

SD: Standard deviation, SE: Standard error, CI: Confidence interval, WSM: Water-soluble matrix, CPP-ACP: Casein phosphopeptide-amorphous calcium phosphate

Table 2: Results of Tukey *post hoc* test from multiple comparisons of enamel microhardness in the tested groups

Dependent variable	(I) groups - intra	(J) groups - intra	Mean difference (I−J)	SE	*P*	95% CI lower bound	95% CI upper bound
Baseline	WSM	Varnish	4.100	19.480	0.976	−44.20	52.40
		CPP-ACP	2.800	19.480	0.989	−45.50	51.10
	Varnish	WSM	−4.100	19.480	0.976	−52.40	44.20
		CPP-ACP	−1.300	19.480	0.998	−49.60	47.00
	CPP-ACP	WSM	−2.800	19.480	0.989	−51.10	45.50
		Varnish	1.300	19.480	0.998	−47.00	49.60
Demin	WSM	Varnish	21.500	15.360	0.355	−16.58	59.58
		CPP-ACP	8.900	15.360	0.832	−29.18	46.98
	Varnish	WSM	−21.500	15.360	0.355	−59.58	16.58
		CPP-ACP	−12.600	15.360	0.694	−50.68	25.48
	CPP-ACP	WSM	−8.900	15.360	0.832	−46.98	29.18
		Varnish	12.600	15.360	0.694	−25.48	50.68
Remin	WSM	Varnish	43.800	21.754	0.128	−10.14	97.74
		CPP-ACP	15.700	21.754	0.753	−38.24	69.64
	Varnish	WSM	−43.800	21.754	0.128	−97.74	10.14
		CPP-ACP	−28.100	21.754	0.412	−82.04	25.84
	CPP-ACP	WSM	−15.700	21.754	0.753	−69.64	38.24
		Varnish	28.100	21.754	0.412	−25.84	82.04

SE: Standard error, CI: Confidence interval, WSM: Water-soluble matrix, CPP-ACP: Casein phosphopeptide-amorphous calcium phosphate

After the remineralization process with Nacre WSM, the surface was fully coated with a crystalline structure. The remineralized surface showed dense HAP coating and the gaps appear to be filled with the remineralized layer. The crystal structure formed shows extensive interlocking between the matrix. The surface morphology of the Nacre Varnish appears to be regular, smooth, and densely packed with the remineralized layer. The high-resolution images illustrate that HAP nanorods were densely compacted with the least number of porosities. The remineralized surface of the CPP-ACP group was densely compacted with HAP rods which were arranged as parallel bundles. The remineralized structure also resembles the regular keyhole pattern of natural enamel.

DISCUSSION

Modern prospective caries research studies focus on bringing out small changes in the mineral content of the teeth enabling them to withstand strong demineralizing challenges. A goal of modern dentistry is to manage noncavitated caries lesions noninvasively through remineralization in an attempt to prevent disease progression and improve esthetics, strength, and function.

As the "Minimal Intervention" concept becomes widely spread, various products with tooth surface protection and anticariogenic or remineralizing effects have gained increasing attention.[24] Concentrating mainly on prevention and early intervention of caries, minimal intervention dentistry's first basic principle is the remineralization of early carious lesions, advocating a biological or therapeutic approach for early surface lesions.[25] Ideally, a remineralization system should supply stabilized bioavailable calcium, phosphate, and fluoride ions that favor subsurface mineral gain rather than deposition only in the surface layer.

Nacre, because of its highly organized internal structure, chemical complexity, mechanical properties, and optical

effects stands as the best-studied example of calcium carbonate biomineralization. Nacre is composed of aragonite crystal tablets covered by an organic matrix. The bulk of the water-soluble fraction is thought to consist of a complex mixture of proteins and peptides. Nacre WSM acts as both a template and binder to form the tightly oriented array of HAP nanorods, leading to the remarkable hardness of the surface.

Giles *et al*.[26] have stated that WSM is the natural template for calcium carbonate in nacre layers and is thought to comprise a complex mixture of proteins and organic macromolecules. It has been revealed that WSM specifically controls aragonite crystal morphology of the nacre layers either through selective adsorption or spatial constraint on the growing crystals. Ni and Ratner[9] have stated that the organic matrix of Nacre serves as a template for calcium carbonate crystallization. This organic matrix may also serve as a template for HAP crystallization. Cariolou and Morse[27] have stated that the water-soluble organic matrix dictates which crystal structure is formed and when it is deposited.

Nacre WSM can further facilitate well-arranged nanorods and accelerate the growth of HAP crystals. The two carboxyl oxygen atoms of WSM proteins behave like a "claw" to coordinate with calcium cations and assemble them on its surface in order, which would induce a large amount of HAP nuclei to form onto the HAP surface.[28] The assembled calcium cations help HAP crystals to preferably grow along the c-axis which is parallel to the direction of WSM.[29]

As a commercially available agent, CPP-ACP has been proven effective for remineralizing enamel *in vitro* and *in vivo*. CPP is a saliva biomimetic that can stabilize calcium and phosphate ions, and thereby enhance mineral solubility and bioavailability.[30] Therefore, CPP-ACP nanocomplexes can maintain a state of supersaturation of ACP nanoparticles in the oral environment and facilitate remineralization through their release during acidic attacks or changes in ion concentration. According to Philip,[31] CPP-ACP can induce subsurface remineralization and significantly improve the strength, esthetics, and acid resistance of WSLs.

CPP-ACP acts as a potent remineralizing agent according to Reynolds.[32] who conducted a study in which the mechanism of action of CPP-ACP is stated as the formation of HAP in the lesion would lead to the generation of acid and phosphate, which would diffuse out of the lesion down a concentration gradient. The CPP, by stabilizing calcium phosphate in a metastable solution, facilitate high concentrations of calcium and phosphate ions, which can diffuse into the enamel subsurface lesion. The CPP will also maintain the high activities of the free calcium and phosphate ions during remineralization through the reservoir of bound ACP. The bound ACP, by being in dynamic equilibrium with free calcium and phosphate ions, will maintain the concentrations of the species involved in diffusion into the lesion. Furthermore, dissociation of the CPP-bound ACP will be facilitated by the acid generated during enamel remineralization.

In the present study, artificial saliva was changed every 24 h during the remineralization regimen to ensure ionic balance and maintenance of PH. This is in accordance with Patil *et al*.[33] Due to the limitations of using natural saliva in *in vitro* studies, substitutive formulations are used to simulate the oral environment in remineralization studies. A different mechanism was adopted in this study whereby artificial saliva was used to mimic the physiological conditions. This was challenging because of the limited number of ions available in saliva for remineralization.

Considering the importance of the surface layer in caries progression, the evaluation of changes in this region was relevant. Surface microhardness measurement is a suitable technique for this purpose. Surface microhardness indentation provides a relatively simple, nondestructive, and rapid method in demineralization and remineralization studies.

The average hardness value for enamel is in the range of 270–350 KHN or 250–360 VHN.[34] Surface microhardness tests have been widely used to evaluate the degree of enamel mineralization, for which an increase suggests mineral regain and improvement in enamel crystalline structure. In the present study, the Vickers hardness test was employed as it is suitable for determining the hardness of very brittle materials. Vickers indenter has proven to be more effective because of the conservative square shape and its ease in the detection of errors.[35]

The scanning electron microscope determines and compares the morphological variations between the demineralized and remineralized samples. SEM is one of the most sensitive and least time-consuming techniques for assessing and comparing the changes before and after the application of remineralizing agents *in vitro*.

In the present study, the Nacre WSM group showed a significant increase in the mean microhardness value (254.70 ± 63.666) after remineralization when compared to the Nacre varnish group (210 ± 38.960) and the CPP-ACP group (239 ± 39.084), whereas no statistically significant difference was observed between the three groups ($P > 0.05$). This was to the results of Li *et al*. who found the *in vitro* HAP remineralization ability using the Nacre WSM as a template.[29]

All three groups showed a significant calcium and phosphorous ratio increase after remineralization with respective remineralizing agents. This indicates the

deposition of crystal structure on the eroded enamel. These results are following the studies conducted by Li *et al*.,[29] Hegde *et al*.,[10] and Hegde and Moany[19] Among the three groups, the highest Ca:P ratio was seen in the Nacre WSM group (0.58) followed by the Nacre Varnish (0.57) and CPP-ACP group (0.57).

SEM image of Nacre surface revealed the presence of extensive interlocking which is following Katti *et al*.[36] Layer of packed HAP particles was formed on the surface of Nacre through surface reactions as seen in the study conducted by Ni and Ratner.[9] This shows the ability of NACRE to restore the uniform, thick, compact, and homogenous layer of calcific deposits that well-sealed interprismatic cavities.

These surface reactions comprise a dissolution–precipitation mechanism in which calcium ions released from the bulk Nacre surface enter the phosphate buffer solution and then precipitate as HA on the Nacre surface after interacting with free phosphate. According to the study conducted by Li *et al*.,[29] Nacre WSM has a potent remineralizing ability which is also proven in the present study with a clinically acceptable form as Nacre Varnish.

The limitations of this *in vitro* study include the difficulty of precisely simulating the biological aspects of caries and the multitude of intraoral conditions that contribute to dental caries. The factors such as the complexity of the tooth-pellicle-plaque-saliva interface were not simulated. The lack of bacteria in the demineralizing protocol, adds to one of the limitations. Other confounding factors involve the possibility of experimental errors and dissimilarities in the micro-structure of the enamel between specimens.

CONCLUSION

Within the limitations of this study, it can be concluded that Nacre and CPP-ACP act as potent enamel remineralizing agents. Nacre, a facile biomaterial, has remineralizing ability closer to the natural enamel structure in terms of hardness, morphological structure, and chemical composition. Having remarkable hardness and strength the ability of Nacre can be explored in *in vivo* studies to treat the teeth that lost enamel.

Nacre's more important structural characteristics and mechanical properties are exposed as a base that has inspired scientists and engineers to develop biomimetic strategies that could be useful in further research. A strong emphasis can be given to the synthetic design and production of nacre-inspired materials and coatings, in particular, to be used in biomedical applications and the field of dentistry.

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Conflicts of interest
There are no conflicts of interest.

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