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Research Article

Efficacy of Casein Phosphopeptide - Amorphous Calcium Phosphate (CPP-ACP) and Grape Seed Extract (GSE) on Enamel Remineralization. An Invitro Study

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ABSTRACT

Background and objectives: Aim of the present in vitro study is to compare the remineralizing effect of Casein phosphopeptide and Amorphous Calcium phosphate (CPP-ACP) and Grape seed extract (GSE) by evaluation of DIAGNOdent values and scanning electron microscope (SEM) images. **Materials and methods:** 45 therapeutic maxillary first premolars were cut into buccal and lingual halves using with help of a diamond disc under water coolant. Buccal halves were taken into the study. A 5x5 mm window was created using a stick-on paper on the buccal surface and the remaining surfaces were coated with black color nail varnish. The teeth were placed in the demineralizing solution to induce artificial carious lesion on the occlusal surface .They were divided into three groups, Group 1 with artificial saliva (control), Group 2 and 3 were treated with 2 different remineralizing agents (Casein phosphopeptide and Amorphous Calcium phosphate) & (Grape seed extract) for a specific time period and stored in artificial saliva. In Each group some samples were evaluated with SEM for surface topography and all the samples were evaluated with DIAGNOdent at every step. Statistical analysis was done by using ANOVA to know the significant difference among three study groups. Tukeys post hoc test, repeated measures of ANOVA were used to identify which among the three groups differ significantly with respect to DIAGNOdent values after remineralization. Bonferroni post hoc test was used for pair-wise comparison of scores over different time intervals in each study group. Statistically Significant difference was observed at $p<0.05$. **Result**: After remineralization, mean ±S.D. of Artificial saliva (12.40±2.06), Tooth mousse (5.67±1.35) and Grape seed extract (GSE) (8.27±0.88). ANOVA showed that there was a statistically significant difference was observed among three groups. Overall CPP-ACP showed significant amount of remineralization over remaining two groups. **Conclusion:** The SEM pictures of the three groups suggest remineralization in the order, with the CPP-ACP showing the greatest amount of mineral deposits, followed by GSE and then artificial saliva.

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INTRODUCTION:

The enamel is the hardest tissue in the human body. It is the only ectodermal derivative of tooth. Inorganic constituents account for 96% by weight and they are mainly calcium phosphate in the form of hydroxyapatite crystals in the form of rods[1], small amount of carbonate, magnesium, potassium, sodium and fluoride, while the organic component is composed of 2 groups of proteins named as amelogenins and non-amelogenins.[2]

For many years, dental caries was considered as a progressive demineralization of enamel apatite followed by degradation of dentin.[3] However, the present concept identifies caries as a dynamic process which can be conceptualized as an imbalance between mineral loss called "Demineralization" and mineral gain called "Remineralization". Ultimately net loss of mineral determines progressive nature of caries.[4,5,6]

A variety of remineralizing agents like, Casein PhosphoPeptide-Amorphous Calcium Phosphate (CPP-ACP- Tooth Mousse), Bioglass (Novamin), Ozone, Xylitol, Grape seed extract (proanthrocyanidins) etc, that aid in remineralization of tooth structure are available commercially.

In contrast to enamel, the dentin contains more carbonated hydroxyapatite with smaller and soluble crystals, leading to more rapid demineralization by acids. The dentin reacts to any stimulation or irritation either by forming reparative dentin or secondary dentin by the action of odontoblasts, which is not seen in enamel because ameloblasts disappear once enamel is formed.[3]

Study conducted by Mankameh et al[7] on the effect of Grape seed extract (GSE) on artificial enamel caries in primary human teeth. The study concluded that GSE enhanced the remineralization process of artificial enamel lesions of primary teeth, and thus, might be considered an effective natural agent in non-invasive dentistry

Study conducted by Pai D et al,[6] to evaluate the remineralization of incipient enamel lesions by the topical application of Casein PhosphoPeptide-Amorphous Calcium Phosphate (CPP-ACP) using laser fluorescence and scanning electron microscope. The study concluded by saying that there is significant number of test samples observed under SEM showed high scores of remineralization.

MATERIALS AND METHODS

After obtaining the clearance from institutional ethical committee, the present study was carried out in the department of oral pathology on a total of 45 maxillary first premolars teeth, which were included 15 teeth in each study group. **Group1:** Artificial Saliva. **Group2:** CPP-ACP (GC Tooth mousse); **Group3:** Grape seed extract (Vista nutrition).

An invitro study was done to assess the efficacy of CPP-ACP and GSE on enamel remineralization for a period of 3 months i.e from June 2017 to August 2017.

The extracted teeth were washed thoroughly under running tap water to remove blood, saliva and were disinfected in 5.25% sodium hypochlorite solution.[8,9] They were stored in 10% formalin solution and samples were cut into buccal and lingual halves by using a diamond disc (Dentarum Germany) with micromotor handpiece (Marathon).[10,11] Samples showing discoloration, fracture and dental caries were excluded from the study. Buccal halves were taken into the study and the samples were mounted in dental stone. A 5 x 5 mm window was created using a stick-on paper on the buccal surface and the remaining surfaces were coated with black color nail varnish. After this stick-on paper was removed.

Teeth were kept in demineralized solution and P^H of the solution was checked with digital P^H meter (HM 80) and it was adjusted to 4 by adding 50% KOH. Teeth were checked with a DIAGNOdent to assess the demineralization. The teeth with sub-surface lesion with the DIAGNOdent score >9 were taken in for further evaluation by using three types of remineralizing agents.

Group1: Artificial saliva.
Group2: CPP-ACP (GC Tooth mousse)
Group3: Grape seed extract (Vista nutrition)

The samples in Group 1 were stored in artificial saliva. The samples in Group 2 were applied with CPP-ACP (GC Tooth Mousse) by applicator tips for 3 minutes daily twice with 8 hrs gap for 14 consecutive days. Group 3 were applied with Grape Seed Extract by applicator tips on the tooth surface for 14 consecutive days, 5 minutes daily twice with 8 hrs gap. After application of each remineralizing agent, samples were washed under running tap water, dried and placed back in artificial saliva. One of the existing salivary substitutes namely Xialine I artificial saliva was prepared in the department of oral pathology.[6,9]

After application of remineralizing agents, DIAGNOdent scores were recorded. Those indicated

score <7 were considered as remineralization. 4 samples in each group were randomly selected and compared using scanning electron microscopy.

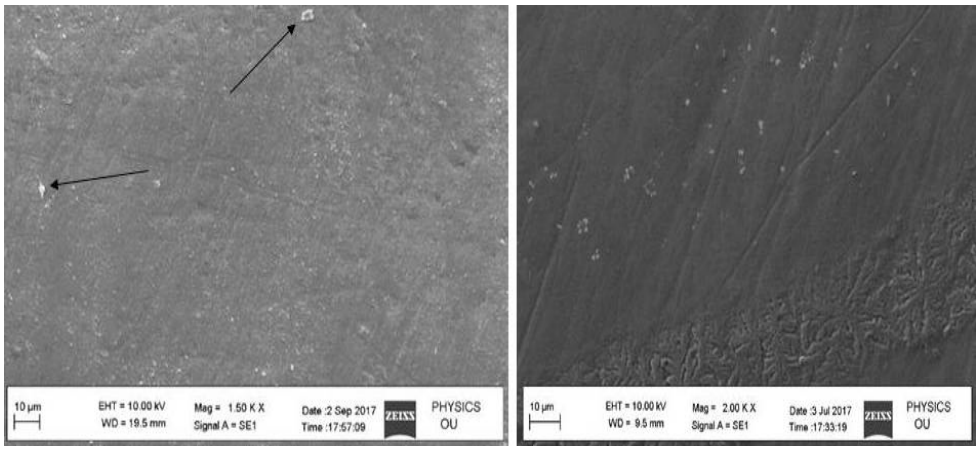
Statistical analysis:

Statistical analysis was done by using ANOVA to know the significant difference among three study groups. Tukeys post hoc test was used to identify which among the three groups differ significantly with respect to DIAGNOdent values after remineralization. Repeated measures of ANOVA were done to compare the scores over different time intervals in each study group. Bonferroni post hoc test was used for pair-wise comparison of scores over different time intervals in each study group. Fishers exact test was used to know the frequency of remineralization scores among the three groups. Statistically Significant difference was observed at $p<0.05$.

RESULTS

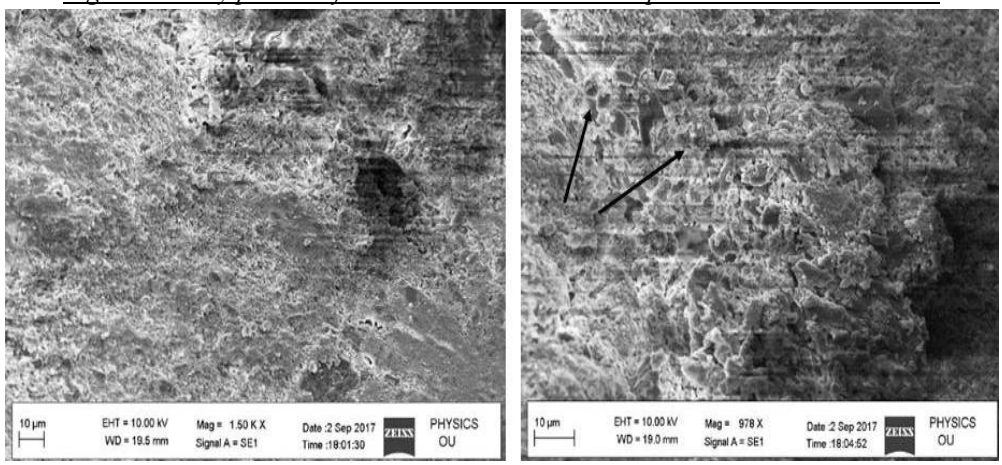
SEM evaluation: At the baseline the SEM photomicrograph of sound enamel, revealing an orderly smooth appearance. There are also some spherical particles on the surface. Inter-prismatic tissue is intact. [Figure 1]

Figure 1: At the baseline the SEM photomicrograph of sound enamel, revealing an orderly smooth appearance, arrow shows some spherical particles.



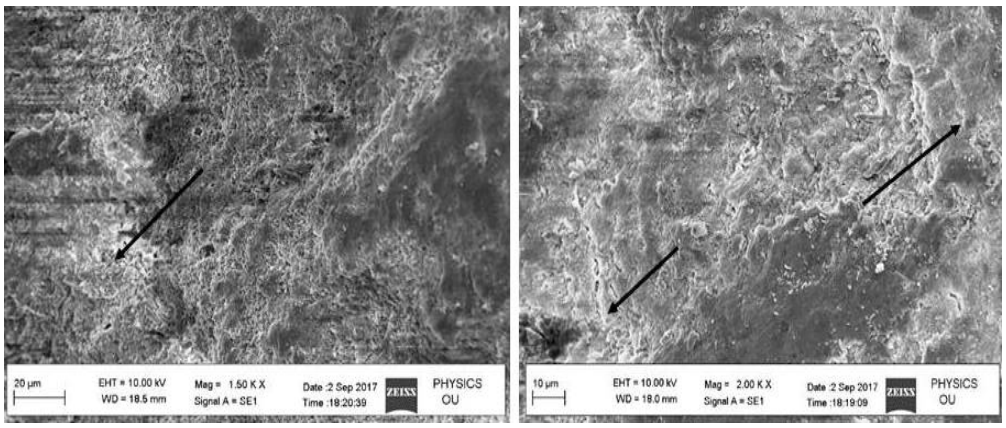
After demineralization the photomicrograph of demineralized enamel (×1000 magnification) before treatment, the enamel surface is rough and disorganized with porosities. Discontinuous and broken enamel crystals were visible after the demineralization process. Early pattern of demineralization with inter-prismatic tissue dissolution is seen. [Figure 2]

Figure 2: Early pattern of demineralization with inter-prismatic tissue dissolution



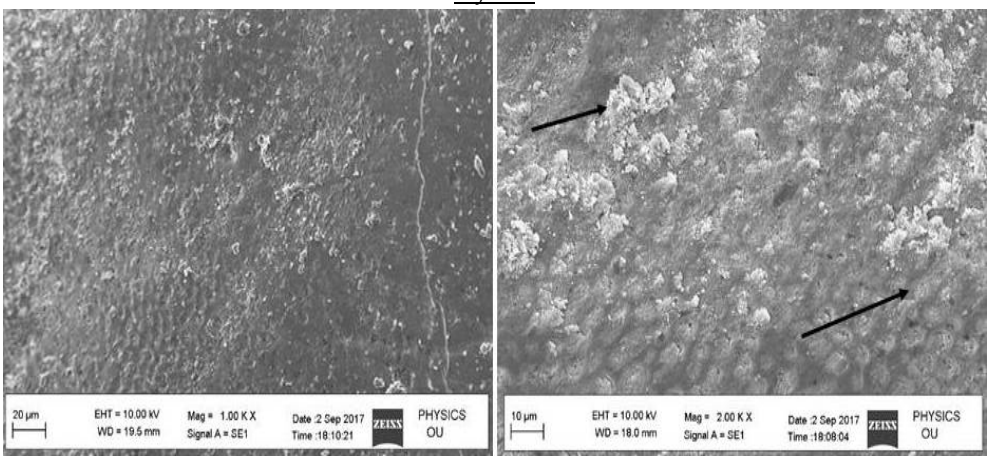
In group 1 (control; artificial saliva), the configuration of sound enamel topography is apparent with certain porous defects [1000× magnification], but the enamel rods are barely discernable at this magnification. At a higher magnification of 2000×, the porosities are more evident and faint lines of mineralization can be seen in and around the porosities. [Figure 3]

Figure 3: (a) In group 1 (control; artificial saliva), the configuration of sound enamel topography is apparent with certain porous defects [1000x magnification] (b) At magnification of 2000x, the porosities are more evident and faint lines of mineralization can be seen in and around the porosities.



After treating with CPP-ACP, enamel rods and prismatic substance are not discernable at 1000, but the areas of calcified deposits are more evident and smooth surface was observed. At a higher magnification of 2000×, areas of mineralized deposits are discernible and are seen profusely scattered along the porous defects. Spherical globular agglomerates were observed on the surface of the enamel. [Figure 4]

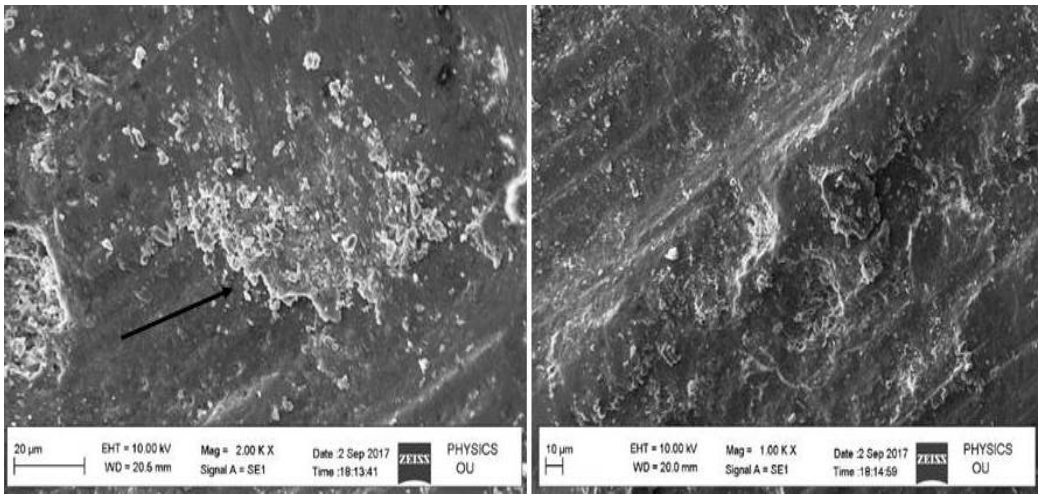
Figure 4: (a) After treating with CPP-ACP, enamel rods and prismatic substance are not discernable at 1000x. (b) At a higher magnification of 2000×, areas of mineralized deposits are discernible and are seen profusely scattered along the porous defects.



After treating with GSE, there were scaffolding deposits on the enamel surface with cluster-like structures resembling remineralization process initiation. Interprismatic substances are evident with porosities and areas of remineralization also seen at 1000×. (2000×) of the same group displays certain areas of calcifications are evident along the porosities. The SEM pictures of the three groups suggest remineralization in the order, with the CPP-ACP showing the greatest amount of mineral

deposits, followed by GSE and then artificial saliva. [Figure 5]

Figure 5: (a) After treating with GSE, there were scaffolding deposits on the enamel surface with cluster-like structures resembling remineralization process initiation at 1000x. (b) at 2000x magnification displays certain areas of calcifications are evident along the porosities.



The comparison of mean ±S.D. of diagnodent scores of the study groups at the baseline of Artificial saliva, GC tooth mousse plus and Grape seed extract were (3.53±1.19), (3.67±1.05), (3.87±1.19) respectively and noted no significant difference between the groups. After demineralization mean ± S.D. of Artificial saliva (13.87±2.87), tooth mousse (13.33±2.26) and grape seed extract (12.60±1.64) showed no statistically significant difference between the groups. After remineralization mean ±S.D. of artificial saliva (12.40±2.06), tooth mousse (5.67±1.35) and grape seed extract (8.27±0.88), showed statistically significant difference among groups.[Table 1]

Table 1:- ANOVA test for mean of the scores of study groups during different time intervals

Time interval	Study group	N	Mean	SD	Min	Max	ANOVA	
							F	p-value
Base line	Artificial saliva	15	3.53	1.19	2	6	0.32	0.73
	Tooth Mousse	15	3.67	1.05	2	6		
	Grape seed Extract	15	3.87	1.19	2	6		
DM after 96hrs	Artificial saliva	15	13.87	2.07	10	17	1.51	0.23
	Tooth Mousse	15	13.33	2.26	10	17		
	Grape seed Extract	15	12.60	1.64	11	16		
RM	Artificial saliva	15	12.40	2.06	9	16	75.77	<0.001 *
	Tooth Mousse	15	5.67	1.35	4	8		
	Grape seed Extract	15	8.27	0.88	7	10		

**p<0.05 statistically significant, p>0.05 Non significant, NS*

After remineralization statistically significant difference (p<0.05) was observed between the study groups i.e. artificial saliva with tooth mousse and grape seed extract and also found significant (p<0.05)

difference between grape seed extract and tooth mousse. [Table 2]

Table 2. Tukeys post hoc test for comparison of Remineralization of study groups.

Study groups		Mean difference	P- Value	95% confidence interval	
				Lower Bound	Upper Bound
Artificial saliva	Tooth Mousse	6.73	<0.001*	5.39	8.07
	Grape seed Extract	4.13	<0.001*	2.79	5.47
Tooth Mousse	Grape seed Extract	-2.6	<0.001*	-3.94	-1.26

The significant difference ($p<0.05$) was observed within the study group at different intervals of time. i.e. at the baseline after demineralization and after remineralization. Artificial saliva at baseline (3.53±1.19) after demineralization (13.87±2.07) and remineralization (12.40±2.06). Tooth mousse at baseline (3.67±1.07), after demineralization (13.33±2.06) and remineralization (5.67±1.35). Grape seed extract at baseline (3.87±1.19), after demineralization (12.60±1.64) remineralization (8.27±0.88) respectively. [Table 3]

Table 3. Repeated measures ANOVA for pairwise comparison of scores over different time intervals in each study group.

Group		N	Mean	SD	F value (df1, df2)	p-value
Artificial saliva	Base line	15	3.53	1.19	236.12 (2, 28)	<0.001*
	DM after 96hrs	15	13.87	2.07		
	Remineralization	15	12.40	2.06		
Tooth Mousse	Base line	15	3.67	1.05	247.29 (2, 28)	<0.001*
	DM after 96hrs	15	13.33	2.26		
	Remineralization	15	5.67	1.35		
Grape seed Extract	Base line	15	3.87	1.19	197.15 (2, 28)	<0.001*
	DM after 96hrs	15	12.60	1.64		
	Remineralization	15	8.27	0.88		

The significant difference was observed within the study group during different intervals of time. i.e. artificial saliva (at the baseline with demineralization and remineralization) and artificial saliva after demineralization with remineralization. A significant difference ($p<0.05$) was observed with tooth mousse at the baseline with demineralization and remineralization and Tooth mousse at demineralization with remineralization. Statistically significant difference ($p<0.05$) was observed with Grape seed extract at the baseline with demineralization and remineralization respectively. [Table 4]

Table 4 : Bonferroni post hoc test for pair wise comparison of scores over different time intervals in each study group

Group	Time interval	Time interval	Mean Diff.	p-value	95% Confidence Interval	
					Lower Bound	Upper Bound
Artificial saliva	Base line	Demineralization after 96hrs	-10.33	<0.001*	-11.98	-8.68
		Remineralization	-8.87	<0.001*	-10.43	-7.30
	DM after 96hrs	Remineralization	1.47	0.001*	0.63	2.30
Tooth Mousse	Base line	Demineralization after 96hrs	-9.67	<0.001*	-11.04	-8.30
		Remineralization	-2.00	<0.001*	-3.06	-0.94
	DM after 96hrs	Remineralization	7.67	<0.001*	6.38	8.96
Grape seed Extract	Base line	Demineralization after 96hrs	-8.73	<0.001*	-10.29	-7.18
		Remineralization	-4.40	<0.001*	-5.19	-3.61
	DM after 96hrs	Remineralization	4.33	<0.001*	3.22	5.45

Statistically significant difference ($p<0.05$) was observed between the study groups after remineralization using Fishers exact test. [Table 5]

Table 5: Fishers exact test for comparison of study groups at baseline, demineralization and remineralization.

Time interval	DIAGNOdent Scores	Study Group			Total	Fishers exact test
		Artificial saliva	Tooth Mousse	Grape seed Extract		p-value
Base line	3 - 7	15(100.0%)	15(100.0%)	15(100.0%)	45(100%)	NS
DM after 96hrs	>9	15(100.0%)	15(100.0%)	15(100.0%)	45(100%)	NS
RM	3 - 7	0	10(66.7%)	0	10(22.2%)	<0.001*
	7 - 9	1(6.7%)	5(33.3%)	14(93.3%)	20(44.4%)	
	>9	14(93.3%)	0	1(6.7%)	15(33.3%)	

**p<0.05 statistically significant, p>0.05 Non significant, NS*

DISCUSSION

Dental caries is not life threatening but results in reduction in the quality of life and a significant financial burden on those affected. Currently, treatment consists of management through extraction and, restoration of affected tooth.[5] Despite best efforts, dental carcinogenesis remains entrenched within all age and socioeconomic groups. So, a novel approach to treat caries is required to truly prevent this disease.

Modern dentistry aims to manage non-cavitated carious lesions non-invasively through remineralization in an attempt to prevent disease progression, and to improve form, function, strength and esthetics of teeth. Remineralization is defined as the process whereby calcium and phosphate ions are supplied from a source

external to the tooth to promote ion deposition into crystal voids in demineralized enamel to produce net mineral gain.[4] Though remineralization has been a major area of investigation, it is still difficult to exactly define the efficacy of various remineralization methods. Recently, dairy products (milk, milk concentrates, and cheeses) have received a lot of attention for their anticariogenic effect in animal and human in situ caries models. The protective effect seen with dairy products is possibly best attributed to the phosphoprotein casein and calcium phosphate contents.[11] Casein is rapidly degraded by intraoral bacterial proteolytic activity, with the released phosphopeptides being relatively stable to further degradation. The recently proposed remineralization and anticariogenic mechanism of CPP-ACP involves the incorporation of nano-complexes into dental plaque and onto the tooth surface, which thereby acts as a calcium and phosphate reservoir.[12,13]

Polyphenols are plant derived substances that have antioxidant and anti-inflammatory properties. They interact with microbial membrane proteins, enzymes and lipids, thereby altering cell permeability and permitting the loss of proteins, ions and macromolecules. One such polyphenol is Proanthocyanidin (PA), which is a bioflavanoid containing benzene pyran phenolic acid molecular nucleus. The PA accelerates the conversion of soluble collagen to insoluble collagen during development and increases collagen synthesis.[14]

DIAGNOdent laser fluorescence is a noninvasive method used to measure early demineralization of tooth. When the samples were evaluated with the laser fluorescence device, it was found that the test samples showed a significant change in their fluorescent readings from demineralized state to the end of fourteen days of test period of remineralization. The change in fluorescence is related to porphyrins produced by the bacterial action. The other school of thought explains that the increase in tissue porosity with respect to carious process is responsible for the change in fluorescence. This observation was in agreement with earlier reports of the studies conducted by Al-Khateeb S et al.[12] Alterations in the mineral content of enamel as in the demineralization and remineralization process directly bring about change in optical properties, including fluorescence.

A study conducted by Sridhar et al[15] showed that, there is a significant difference was observed when comparing DIAGNOdent with visual and radiographic examinations on incipient carious lesions. This is due to the fact that DIAGNOdent has good sensitivity and specificity in diagnosing caries.

Scanning electron microscope (SEM) is one of the most sensitive, time-tested techniques to assess the demineralization and remineralization of the carious lesions *in vitro* as reported in earlier studies.[16] In this study SEM was used to know the qualitative changes of study groups during different intervals i.e at the base line after demineralization and remineralization. The SEM pictures of the three groups suggest remineralization in the same order, with the CPP-ACP showing the greatest amount of mineral deposits, followed by GSE (Grape seed extract) and then artificial saliva.[16]

The surface topography of the test samples showed varying degree and extent of remineralization and is in agreement with earlier studies conducted by Stephen et al and Satyawan et al. At the base line SEM photo micrograph of sound enamel is smooth with some spherical particle on its surface which is similar to the study conducted by Mahkameh M et al.[17] This might be due to the fact that sound enamel has regularly arranged enamel rods and interprismatic tissue is intact. After demineralization there was significant porosity and interprismatic tissue dissolution is seen. This is similar to the study conducted by Jayarajan et al[11`] and Mahkameh M et al.[17] The acidic composition of demineralized solution which makes the tooth surface irregular, dissolution of interprismatic substance and results in porous defects which indicate loss of minerals from tooth surface.

After treatment with Tooth mousse there was significant reduction in porosities on the enamel and deposition of spherical particles was more visible on the tooth surface and it was homogeneous. This is similar to the study conducted by Jayarajan et al[11] and Choudary P et al.[10] Tooth mousse releases stabilized calcium and phosphate which is readily available and helps in remineralization of enamel. Calcium and phosphate is seen around the porosities which makes tooth surface homogeneous. After exposure to Grape seed extract there were some insoluble complexes on the enamel surface. Spherical globular agglomerates were observed on the enamel surface which was similar to the study conducted by Mahkameh M et al.[17] The PAs represent a variety of flavan-3-ol (catechin), which is a free radical scavenger required for the absorption of calcium. It is also a polyphenolic compound, and has the potential to give

rise to stable hydrogen, and generate non-biodegradable collagen matrices.[18]

In this study both laser fluorescence and SEM were used but no comparison between the two was considered, since laser fluorescence quantitatively evaluates the changes in fluorescence properties of enamel from baseline, after demineralization and after remineralization whereas the SEM observation of the surface morphology is a qualitative analysis of enamel.

The mean and standard deviation were calculated, and tests of significance were done for each group. At the baseline mean ± S.D. of study groups was artificial saliva (3.53±1.19), GC tooth mousse (3.67±1.05), Grape seed extract (3.87±1.19) respectively and there was no significant difference observed in between the study groups. This is similar to the study conducted by Jayarajan et al.[11] This might be due to the fact that all the teeth were caries free and without any defects.

After demineralization the mean scores as measured by DIAGNOdent, for artificial saliva (13.87±2.87), Tooth mousse (13.33±2.26) and Grape seed extract (12.60±1.64) were not statistically significant different. This shows that the composition of demineralization solution that was used for the study is same for all groups which produced uniform artificial carious lesions. After remineralization mean ± S.D. of artificial saliva (12.40±2.06), Tooth mousse (5.67±1.35) and Grape seed extract was (8.27±0.88) statistically significantly different ($p<0.05$) in between the groups.

Comparison of the mean remineralization value of group A (artificial saliva; 12.40±2.06) with its mean demineralization value (13.87±2.87) showed statistically significant difference was observed, which is similar to the study conducted by Jayarajan et al.[11] This indicates that saliva by itself has some remineralization potential.

Remineralization remains imperative towards the management of non-cavitated carious lesions and prevention of disease progression within the oral cavity. The presence of fluoride in saliva speeds up crystal precipitation forming a fluorapatite- like coating which will be more resistant to caries. The process also has the ability to contribute towards restoring strength and function within tooth structure. Together, demineralization and remineralization contribute towards a dynamic process. On comparing the remineralization value of CPP-ACP (5.67±1.35) to its demineralization value (13.33 ± 2.26) it is evident that a significant amount of remineralization has occurred. It is similar to the study conducted by Reynolds et al.[8] Though saliva has some remineralization potential, it cannot by itself increase the levels of calcium and phosphate release. For mineral deposition to occur within the body of the lesion, calcium and phosphate ions must first penetrate the surface layer of enamel. This explains why the CPP supported meta stable calcium phosphate solutions are such efficient remineralizing solutions.

The results of the present study were in contrast to the study conducted by Gopala krishnan et al.[19] Here the CPP-ACP failed to remineralize carious lesions when applied for 1 min for 10 days invitro. This might be because the duration was insufficient for the cycle of remineralization and buffer to occur. Alternatively, once the surface layer has been remineralized, the agent cannot penetrate into the deeper layers of the lesion.

After demineralization, the mean scores of Grape seed extract using DIAGNOdent was (12.60±1.64) which improved after remineralization (8.27±0.88) and there was a significant difference ($p<0.05$) observed, which is similar to the study conducted by Mahkameh M et al.[17] This might be due to PA from GSE has been demonstrated to increase collagen synthesis and accelerate the conversion of soluble collagen to insoluble collagen during development. There was a significant difference was observed after remineralization in between the three groups i.e. Artificial saliva (12.40±2.06), CPP-ACP(5.67±1.35) and Grape seed extract (8.27±0.88). Comparatively more remineralization was seen in CPP-ACP when compared with two other groups. This might be due to CPPs contain the cluster sequence of -Ser (P) Ser (P) Ser (P) Glu Glu from casein. This protein nanotechnology combines the precise ratio of 144 calcium ions plus 96 phosphate ions and six peptides of CPP. The nano complexes form over a pH range of 5.0-9.0. Under neutral and alkaline conditions, the CPPs stabilize calcium and phosphate ions, forming meta stable solutions that are supersaturated, which increase as the pH increases.

The study concluded that there was a significant difference ($p<0.05$) observed in between the study groups after remineralization i.e. CPP-ACP showed significantly more remineralization than the other two groups.

This study was conducted simulating a few intraoral conditions under an *in vitro* model to demonstrate the remineralization of simulated carious lesions. The replication of the dynamics of the caries process and the

complexity of the oral environment in these *in vitro* models is limited. In this study treatment applications were done for 14 days. If the same had been extended to a scheduled routine protocol, the amount of remineralization may probably have approached the baseline value.

Given the limitations of *in vitro* studies and the dynamics of *in vivo* conditions, *in vivo* studies that evaluate the remineralization of CPP-ACP and PA with the use of suitable devices like laser fluorescence can validate the remineralizing potential of CPP-ACP and PA in clinical situations.

The effect of the remineralizing potential of natural saliva, the cyclic changes during the demineralization and the effect of bacterial assaults in a clinical situation were not determined. The researchers should focus on the standard time period for remineralization by conducting multiple studies.

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