An *in vitro* comparative evaluation of casein phosphopeptide-amorphous calcium phosphate fluoride, tricalcium phosphate and grape seed extract on remineralization of artificial caries lesion in primary enamel

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Objectives: *To evaluate and compare the remineralization potential of casein phosphopeptide-amorphous calcium phosphate fluoride, tricalcium phosphate and grape seed extract on artificial caries lesions in primary enamel.* ***Study design:*** *A sample of 40 non-carious, primary molar teeth was collected and cut in longitudinal sections into three equal halves. Those 120 samples were divided into four equal groups. Group A: Sections treated with casein phosphopeptide-amorphous calcium phosphate fluoride (CPP-ACPF), Group B: Sections treated with tricalcium phosphate, Group C: Sections treated with grape seed extract Group D: Sections treated with deionized water (control group). Samples were evaluated for change in surface characteristics, mineral content using Scanning electron microscopy-energy dispersive X-ray analysis (SEM-EDX) and microhardness using Vicker's microhardness tester. Cavitated lesions were evaluated for Cone beam computer tomography to obtain baseline data post remineralization.* ***Results:*** *The remineralization potential of grape seed extract was found to be greater compared to tricalcium phosphate followed by CPP-ACPF.* ***Conclusion:*** *All the three groups viz. CPP-ACPF, tricalcium phosphate and grape seed extract showed remineralization under the in vitro pH cycling model, while grape seed extract group showed significantly greater remineralization compared to the CPP-ACPF and tricalcium phosphate groups.*

Keywords: *Carious lesions, CPP-ACPF, Tricalcium phosphate, Grape seed extract, Remineralization*

INTRODUCTION

Acknowledged as a worldwide health problem, dental caries is a pathological process of localized devastation of tooth tissue by microorganisms, affecting several urban and rural communities. Numerous agents have been established to prevent or reverse the formation of carious lesions[1]. Dental caries development is dynamic process, in which demineralization of the enamel is followed by remineralization, which have a crucial impact on the hardness and strength of tooth enamel[2].Despite of the widespread success of fluoride, many new agents have been planned and suggested for personal and professional applications[3].

Introduced as a remineralizing agent in the year 1998, Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP)[4,5] is a milk product, that helps in remineralization. It can deliver amorphous calcium phosphate and also helps the ACP bind with the dental enamel. Casein phosphopeptides

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are used alone as CPP-ACP or further complexed with fluoride, *i.e.*, Casein phosphopeptide with amorphous calcium phosphate fluoride (CPP-ACPF). With respect to its interaction with fluoride, the CPPs have been shown to keep fluoride ions in solution, hence enhancing the efficacy of the fluoride as a remineralizing agent, with the end result being the formation of fluorapatite [6].

Tricalcium Phosphate (TCP) is a smart calcium phosphate system that controls the transfer of calcium and phosphate ions to the teeth, works synergistically with fluoride to advance performance, but does not interact with fluoride during product storage [7].

Currently, a variety of naturally occurring vegetables and food supplements have been shown to promote health. Antimicrobial compounds of plant origin can be considered as an alternative to the commonly used chemicals for controlling dental diseases [8].One of these is grape seed extract (GSE) [8–10], which contains about 98% proanthocyanidin (PA). It is widely available in fruits, vegetables, nuts, seeds, and flowers and is a natural plant metabolite, antioxidant, and free radical scavenger. Flavin is found in the large molecule structure of PA, a bioflavonoid. PA from fruits and vegetables has been found to stop acid production by *Streptococcus mutans* as well as increase collagen synthesis by preventing the conversion of soluble collagen to insoluble [11].

Though these promising agents are supposed to bring about remineralization changes on the enamel surface, scant data is available to determine its remineralization potential on the primary enamel surface.

Thus, the aim of the present *in vitro* study was to evaluate the ability of CPP-ACPF, TCP, and grape seed extract in bringing about remineralization changes on remineralization of artificial caries lesions in primary enamel surfaces that has been exposed to artificial caries challenge in a simulated oral environment.

MATERIALS AND METHOD

An ethical clearance was obtained, prior to conducting the study, from the ethical committee of the institution (PDCH/20/EC-215, dated 30.05.2020).

In the current *in vitro* study, a sample of forty sound intact primary molars was collected, thoroughly cleaned free of debris and calculus using hand scalers and stored in normal saline, till they were sectioned. The surface enamel of each tooth was examined under a microscope to rule out any cracks or white spot lesions. Each tooth was cut in longitudinal sections, into three equal halves with a diamond disc using micromotor handpiece, to obtain a sample of 120 sectioned teeth. The teeth were coated with an acid resistant nail varnish, leaving a narrow 'window', approximately 2 × 4 mm wide, on the intact surface on buccal or lingual surfaces.

De/Remineralizing solution preparation

The buffered remineralizing and demineralizing solutions were made from high-grade chemicals and deionized water. The demineralizing solution consisted of 2.2 mM Calcium Chloride ($CaCl_2$), 2.2 mM Monopotassium phosphate (KH_2PO_4), 0.05 M acetic acid and the pH was adjusted to 4.4 with 1 M Potassium hydroxide (KOH). The remineralizing solution consisted of 1.5 mM $CaCl_2$, 0.9 mM Monosodium phosphate (NaH_2PO_4), 0.15 M Potassium chloride KCl and pH was adjusted to 7.0.

Each tooth was immersed in the demineralizing solution (10 mL) for 96 hours to produce artificial caries-like lesions.

120 samples were randomly divided by a blinded operator into four equal groups of 30 each, as follows:

- Group A: Sections treated with CPP-ACPF (GC Tooth Mousse Plus, Recaldent^{TM} GC company, Australia)
- Group B: Sections treated with tricalcium phosphate (3M ESPE Clinpro Tooth Crème 0.21% sodium fluoride anticavity paste with tri-calcium phosphate, USA)
- Group C: Sections treated with grape seed extract (Grape Seed Extract, AVR Creative Company, India, containing 97.8% Proanthocyanidin)
- Group D: Sections treated with deionized water (control group)

pH cycling

These sections were placed in the pH cycling system for 10 days. The total time for each cycle was 8 hrs/day. Each cycle is completed with three hours of demineralization twice a day with two hours of remineralization in between. After the completion of each cycle per day, the sections were kept in deionized water, until required for use the next day.

After the completion of the pH cycle, the samples were examined for change of mineral content, surface characteristics using Scanning electron microscope energy dispersive X-ray (SEM-EDX) and microhardness using Vickers's microhardness tester. Cavitated lesions were evaluated using Cone beam computed tomography (CBCT). The obtained results were noted and compared with pre-experiment baseline data. Thus, the potential of the remineralizing agents were assessed.

Quantitative and Qualitative analysis by Scanning electron microscope energy dispersive X-ray (SEM-EDX)

The samples were then ensured to be moisture-free, and to avoid any direct contact with air or moisture. These samples were analyzed using a Scanning electron microscope (Carl Zeiss EVO 40, Germany) for surface morphology and an energy dispersive X-ray analyzer (Bruker XFlash 6160 EDX detector, Bruker Corporation Berlin, Germany) for quantification of calcium and phosphorous content from the amount of calcium ions and phosphate ions present. The images were obtained with a magnification of 2000 × and a 20 kV voltage for comparison.

Microhardness test

Microhardness using Vicker's microhardness tester (Micro Vickers, Matsuzawa Co. Ltd, Toshima, Japan). A load of 100 g for 10 s was applied, the diamond pyramid indent was measured for length and depth digitally, and the hardness value was calculated.

Cone beam Computed tomography (CBCT)

The specimens were mounted on rubber base mold with elastomeric impression material, Aquafil soft putty (DENTSPLY, Konstanz, Germany) to ensure that the vertical orientation of the teeth and specimens were repositioned in the same position reproducibly and was consistent for CBCT imaging. All the teeth were assessed at baseline, demineralization and remineralized period. The area of interest was imaged using CBCT (Carestream 9300®, Carestream Health, Rochester, NY, U.S.A.) The cavitated lesion of all the groups of specimens were scored in different categories. The tooth surfaces were scored in one of the following categories as per the criteria used by Wenzel *et al*.[12], 0: sound, 1: lesion in enamel without cavitation, 2: lesion in enamel with cavitation, 3: lesion one-third or less into the dentine without cavitation, 4: lesion one third or less into the dentine with cavitation, 5: lesion more than one-third into the dentine without cavitation, 6: lesion more than one-third into the dentine with cavitation and 7: surface not recordable.

Statistical analysis

Data collected was analysed using the Statistical Package for Social Science software version 22 for Windows (SPSS Inc., Chicago, IL, USA). Results were expressed as a mean with a standard deviation. One-way One way Analysis of variance (ANOVA) test and Chi-square test were used to compare the remineralization potential between the groups. For all the tests, a p-value of 0.05 or less was considered to be statistically significant.

RESULTS

Microhardness evaluation

The microhardness remineralization values of all the groups at the baseline, after demineralization, and after remineralization are summarized (Table 1). These values show that there was a significant amount of remineralization that occurred in the grape seed extract group compared to other experimental groups.

One-way ANOVA test shows that there was significant difference between tested groups (p-value < 0.05) (Table 2).

Scanning electron microscope evaluation

A comparison of the mean and standard deviation of scanning electron microscope within the group was done and the significant differences between various Ca/P ratios of the baseline, demineralized and remineralized specimens between the groups were estimated. In Groups A, B, C, D mean remineralization changes were 0.181, 0.199, 0.2, 0.187 respectively. The higher mean value was observed in grape seed extract group followed by Tricalcium phosphate and CPP-ACPF groups (Table 3).

SEM images at 2000 × magnification of surfaces of the samples before and after remineralization have been presented as Fig. 1. SEM evaluation of samples treated with grape seed extract showed the interprismatic substances, with porosities and areas of remineralization, which was much better than other groups. There were coating depositions of some insoluble complexes on the enamel surface. The reaction products of GSE were seen as amorphous clumps. Spherical globular agglomerates were observed on the surface of the enamel. SEM-EDX analysis of samples treated with different remineralization agents showed better deposition of mineral content after being treated with grape seed extract (Fig. 2).

Cone beam computed tomography evaluation

The intergroup comparison of the cavitated lesion of all the groups using Cone beam computed tomography was done using Chi-square test. The highest remineralzation percentages were found in grape seed extract group followed by Tricalcium phosphate, CPP-ACPF and deionized water groups respectively (p-value < 0.05) (Table 4, Fig. 3).

DISCUSSION

The non-invasive innovative approach in dental caries management can change a lesion from an active to an inactive state. Toothpastes and different professionally applied delivery systems like gels, varnishes, foams and other fluoride releasing materials are used to remineralize high risk areas. Adding traditional diagnostic methods with advanced, more sensitive methods will improve caries diagnostic efficiency and hence the dental care and treatment of patients[4,5].

In the present *in vitro* study, artificial caries were created using the demineralizing solution and subjected to CPP-ACPF, TCP and grape seed extract remineralizing agents to evaluate their efficacy over a period of 10 days. Microhardness measurement is the most appropriate for the enamel, which has a fine homogeneous microstructure, that is prone to cracking. Identification of surface microhardness is a simple, rapid and nondestructive method used in demineralization and remineralization studies. Scanning electron microscopic analysis (SEM) was chosen, as it is extremely sensitive to changes in hard tissue surface characteristics. With respect to demineralization and remineralization, SEM can quantitatively reveal the areas of mineral loss and mineral gain represented by the visualization of areas with different porosities. For comparison, the surfaces of the sound demineralized enamel and remineralized enamels were examined, using the images obtained at 2000 × magnification. Majority of the studies showed that CBCT was more accurate than intraoral film, charge-coupled device (CCD) sensors or photostimulable storage phosphor systems for overall detection of lesions in approximal surfaces[13–18].

When CPP-ACP or CPP-ACPF applied over the tooth surface, the sticky CPP part of the CPP-ACP/ACPF multifaceted binds to the enamel, biofilm, and soft tissues, bringing the calcium and phosphate ions precisely, where it is needed. The free calcium and phosphate ions come out from CPP, enter into the enamel rods and reorganize the apatite crystals. In an *in vitro* study, it was found that the efficacy of CPP-ACPF containing paste was more than CPP-ACP containing paste in the remineralization of artificial caries lesion[19]. In the current *in vitro* study, the samples treated with the CPP-ACPF showed better results and lower caries score. This was possibly due to the capability of CPP-ACP to interact with fluoride in order to build an additive anticariogenic effect through the formation of a stabilized amorphous calcium fluoride phosphate phase[20].

Table 1: Microhardness of the four different groups (in gms).

Groups	Baseline	After demineralization	After remineralization
CPP-ACPF	280.3 ± 7.631	243.7 ± 6.601	249.1 ± 9.183
Tricalcium phosphate	280.7 ± 8.512	241.8 ± 8.203	251.4 ± 6.204
Grape seed extract	281.1 ± 7.724	243.3 ± 5.638	259.7 ± 7.134
Deionized water	281.1 ± 7.688	241.4 ± 5.562	243.2 ± 5.712

CPP-ACPF: Casein phosphopeptide with amorphous calcium phosphate fluoride.

Table 2: Intergroup comparison of microhardness of all experimental groups after remineralization (100 gm load).

	Sum of squares	Df	Mean square	F	*p*-value
Between group	14.02	3	467.30	9.06	0.000*
Within group	1857.00	36	51.58		

**One-way ANOVA; *p-value < 0.05 indicates statistically significant difference.*

Table 3: Intergroup comparison of the calcium-phosphorous weight% ratio of all the experimental groups by using Scanning electron microscope (SEM).

	Stages							
	Baseline		Demineralization		Remineralization		Remineralization change	*p*-value
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation		
Group A	1.737	0.030	1.457	0.030	1.638	0.031	0.181	0.09
Group B	1.844	0.032	1.533	0.031	1.732	0.032	0.199	0.00*
Group C	1.935	0.033	1.645	0.033	1.845	0.034	0.200	0.00*
Group D	1.645	0.029	1.353	0.028	1.540	0.027	0.187	0.12

**One-way ANOVA; *p-value < 0.05 indicates statistically significant difference.*

Table 4: Intergroups comparison of the cavitated lesion of all the experimental groups by using Cone beam computed tomography (CBCT).

	CBCT Demineralization and Remineralization					*p*-value
	0	1	2	3	4	
CPP-ACPF	2 (20%)	3 (30%)	1 (20%)	4 (30%)	0 (0%)	
Tricalcium phosphate	0 (0%)	1 (10%)	5 (50%)	3 (40%)	1 (10%)	0.017*
Grape seed extract	0 (0%)	0 (0%)	2 (20%)	7 (70%)	1 (10%)	
Deionized water	0 (0%)	6 (60%)	3 (30%)	1 (10%)	0 (0%)	
Total	2 (5%)	10 (25%)	11 (27.5%)	15 (37.5%)	2 (5%)	

**Chi square; * p-value < 0.05 indicates statistically significant difference.*
CPP-ACPF: Casein phosphopeptide with amorphous calcium phosphate fluoride.

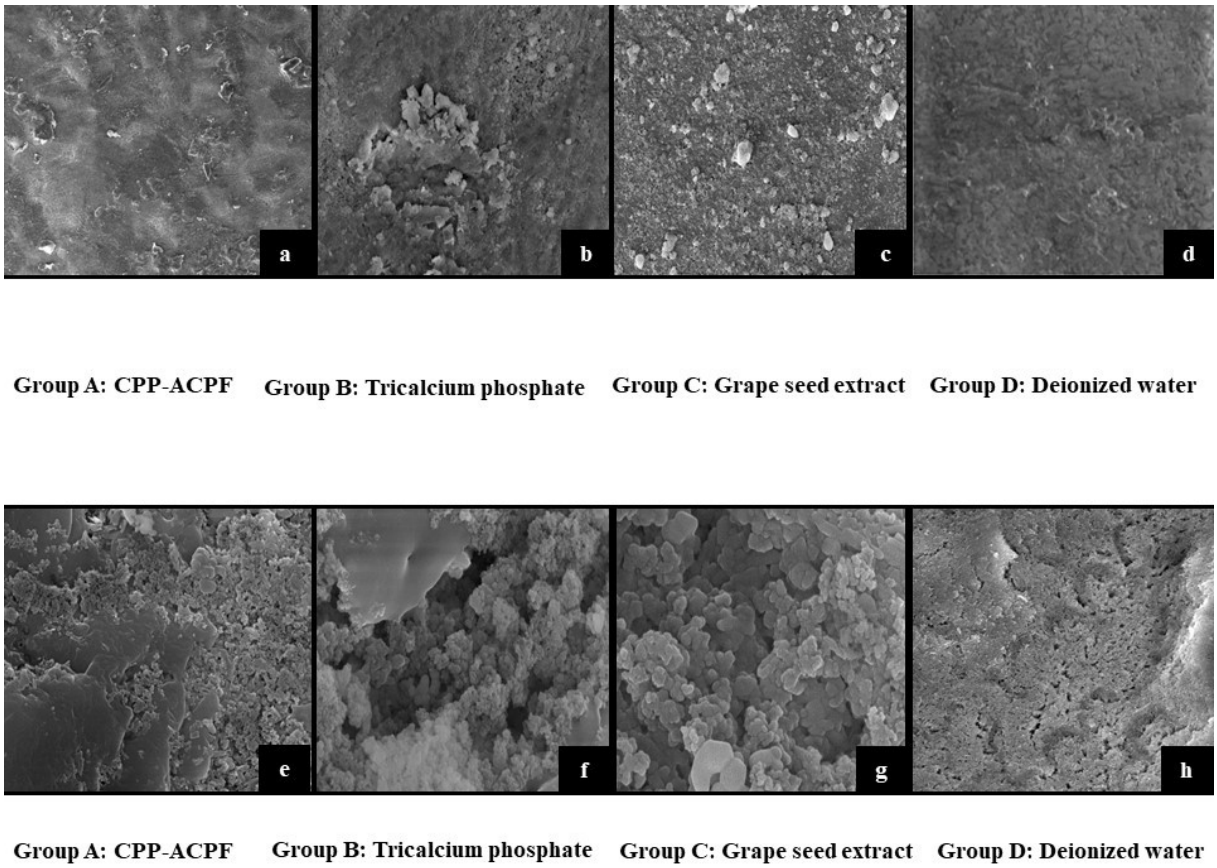

Figure 1: SEM images at 2000 × magnification of surfaces of the samples before remineralization (a to d) and after remineralization (e to h). a. SEM image at 2000 × magnification of surfaces of the samples before remineralization treated with CPP-ACPF; b. SEM image at 2000 × magnification of surfaces of the samples before remineralization treated with Tricalcium phosphate fluoride; c. SEM image at 2000 × magnification of surfaces of the samples before remineralization treated with Grape seed extract; d. SEM image at 2000 × magnification of surfaces of the samples before remineralization treated with Deionized water; e. SEM image at 2000 × magnification of surfaces of the samples after remineralization treated with CPP-ACPF; f. SEM image at 2000 × magnification of surfaces of the samples after remineralization treated with Tricalcium phosphate fluoride; g. SEM image at 2000 × magnification of surfaces of the samples after remineralization treated with Grape seed extract; h. SEM image at 2000 × magnification of surfaces of the samples after remineralization treated with Deionized water. SEM: Scanning electron microscope, CPP-ACPF: Casein phosphopeptide with amorphous calcium phosphate fluoride.

Tricalcium phosphate is stable in an aqueous environment and it does not affect fluoride action in dentifrices. It has been suggested that the combination of fluoride to TCP provides greater fluoride uptake and remineralization[21]. Previous *in vitro* studies[22–25] have revealed that TCP provide superior surface and sub-surface remineralization compared to 5000-ppm fluoride and CPP-ACP. The potential of TCP is promising; however, more studies are required, supporting its efficacy in boosting remineralization.

Grape seeds are the leftover products of the winery and grape juice industry. These seeds contain lipids, proteins, carbohydrates and 5–8% polyphenols depending on the variety. The biologically active constituents of GSE are polyphenols, mainly proanthocyanidins, which represents a variety of polymers of flavan-3-ol such as catechin and epicatechin[26].Proanthocyanidins (PACs), also known as condensed tannins, which are spread over to the plant kingdom, as well as in fruits, seeds of some plants, flower, nuts or barks. They can be easily found in fruits like berries or grapes, as well as in tea, cocoa, chocolate, wine, peanut, almond and avocado or some cereals[27,28].Scientific studies have shown that the antioxidant power of proanthocyanidins is 20 times greater than the vitamin E and 50 times greater than vitamin C[29,30].

Over the last decade, PACs are gaining attention not only from the food industry but also from public health organizations, because of their health benefits. Recent evidence focus on the impact of proanthocyanidins on chronic diseases, with an emphasis on oxidative stress, inflammation and metabolic

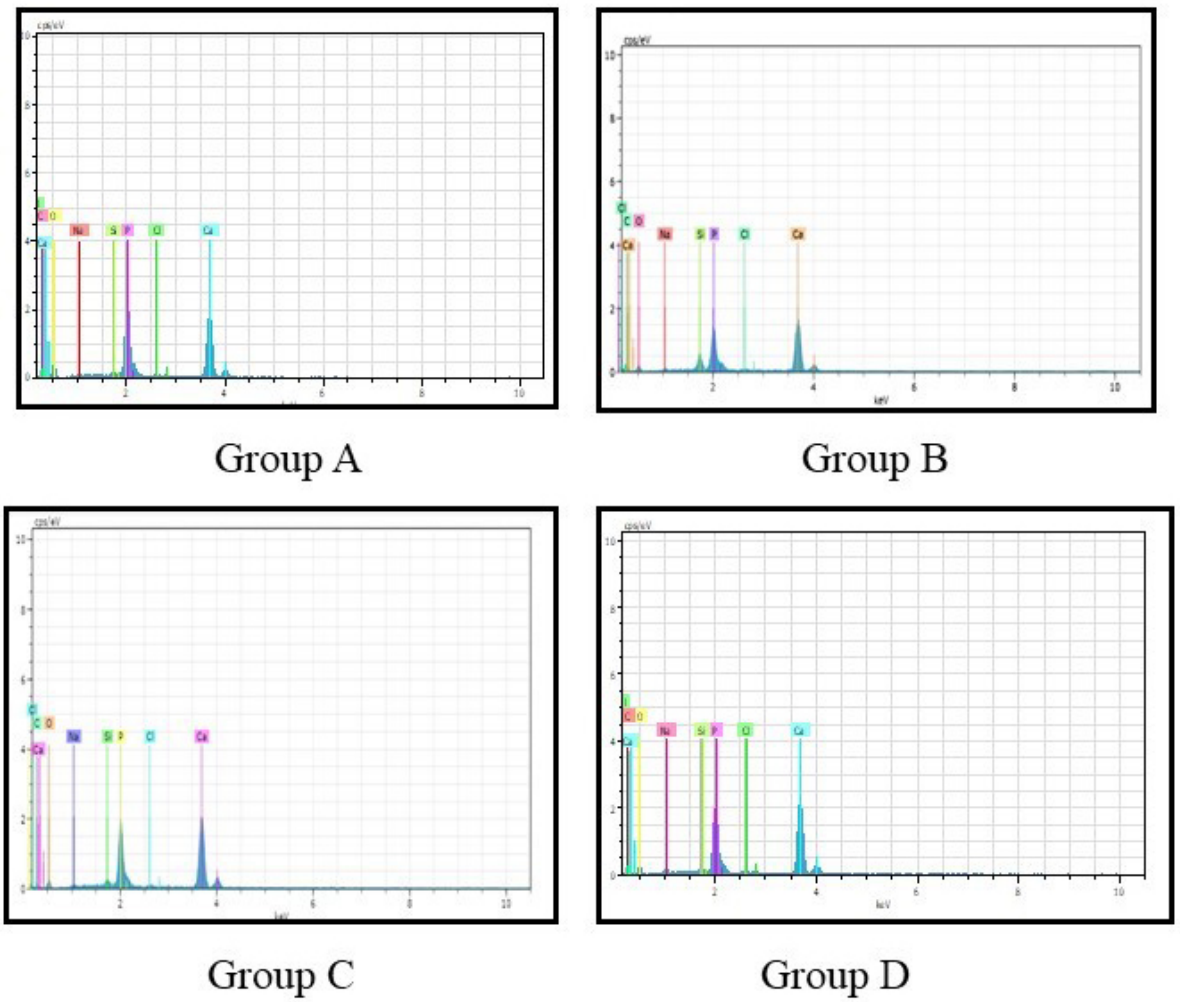

Figure 2: Shows EDX analysis of samples treated with CPP-ACPF, Tricalcium phosphate, Grape seed extract and deionized water after remineralization.

syndrome related disorders, such as obesity, diabetes and cardiovascular risk disease *in vivo* to offer new perspectives in the field that allow further research [31].

Several authors studied the grape seed proanthocyanidin composition along with its degree of polymerization. Gu *et al.* [32] reported that the total proanthocyanidins content in grape seeds is 35.3 mg/g of seed d.w. and in which, monomers (catechin and epicatechin) and polymers were the most abundant. Travaglia *et al.* [33] determined that the mean of proanthocyanidins content in seeds of 37 grape cultivars was 159 mg/g of seed. Escribano-Bailon and co-workers [34] documented that the abundance of PACs in grape seeds are in the following order: (+)-catechin > (−)-epicatechin-3-O gallate > epicatechin 3-O-gallate—(4–8)-catechin (B1-3-O-gallate) > epicatechin-(4–8)-epicatechin (dimer B2). Kuhnert *et al.* [35] analyzed several commercial grape seed extracts and determined a total content of proanthocyanidins ranging from range of 76 to 99%. The GSE used in this study consisted of 97.8 percent proanthocyanidin, as per the manufacturer, which is measured by Vanillin HCl assay technique.

Some studies have evaluated the effect of GSE on demineralized dentin [36,29], but the effect of this agent on the remineralization of enamel defects is not well understood. Using grape seed extract, Alrafee *et al.* [37] could produce remineralization of sound and carious affected dentin of white rabbits. Silva APP *et al.* [38] found that grape seed extract could inhibit demineralization of artificial carious lesions in extracted bovine central incisors, though in a smaller scale compered to fluoride. According to Cheng *et al.* [39] gallic acid, one of the major constituents of grape seed extract and galla chinensis, facilitates mineral deposition, predominately on the surface layer.

In the SEM investigation of the current study, irregular and broken enamel crystals were observed after the demineralization process. After treating with grape seed extract, there were scaffolding deposits on the enamel surface with cluster-like structures reminiscent of initiation of remineralization process. Spherical particles were also noticeable on sound enamel surface as well as on treated enamel surface to a more extent.

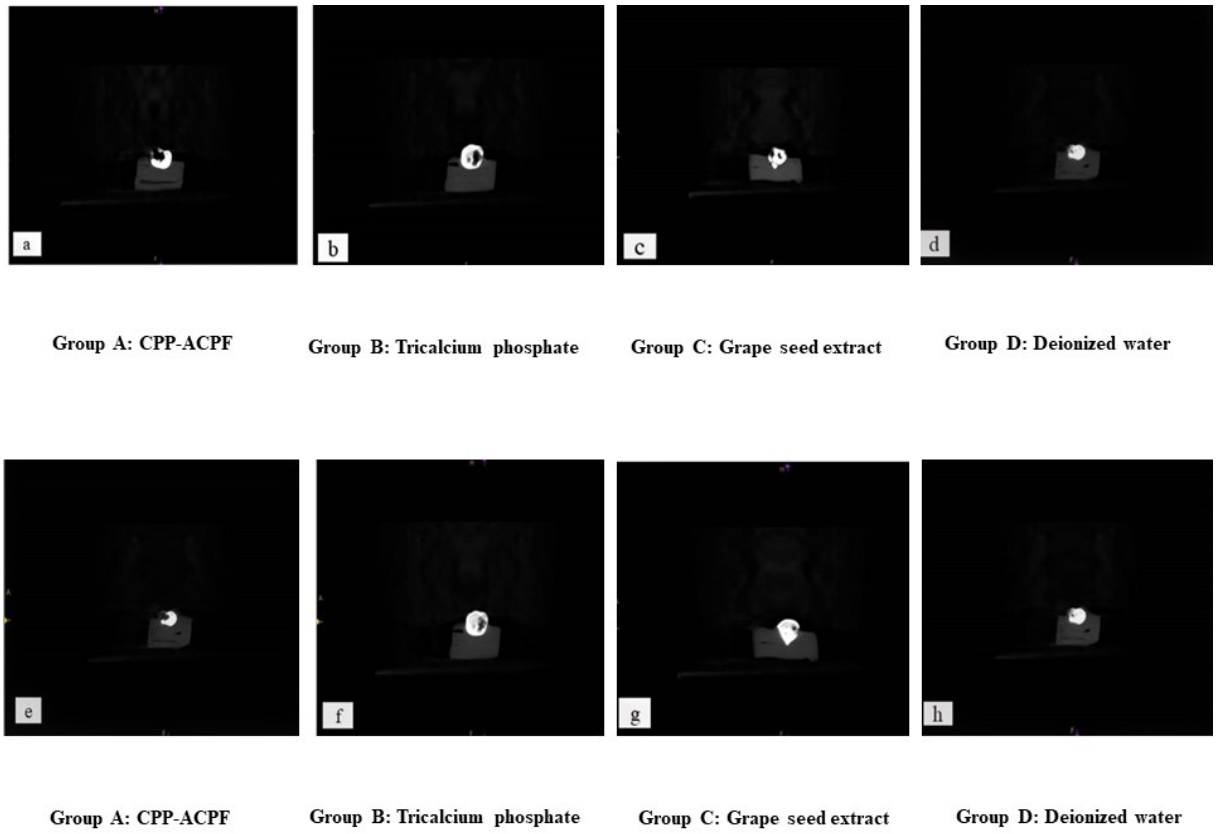

Figure 3: Shows CBCT images of the cavitated lesions of before remineralization. The scores were 6, 5, 4, 2 in Group A, B, C, D respectively (a to d); whereas scores after remineralization were 5, 4, 2, 1 in Group A, B, C, D respectively (e to h).

Similarly, grape seed extract significantly increased the micro-hardness of carious lesions compared to other groups. The study showed grape seed extract may be superior to other materials like CPP-ACPF, TCP which can noticeably increase the surface hardness of the enamel.[40] Al Ammor *et al.*[41] demonstrated that grape seed extract promoted changes in the mechanical properties of dentin matrix, resulting in increased dentin bond strength. It is believed that grape seed extract, interacts with proteins to induce cross-links by four different mechanisms: covalent interaction, ionic interaction, hydrogen bonding interaction or hydrophobic interactions[42–44]. Bedran-Russo *et al.*[45] validated that grape seed extract, as collagen crosslinking agent increased the ultimate tensile strength of demineralized dentin.

Considering these observations, it is not surprising to note the positive effects of remineralization of enamel defects produced by grape seed extract in SEM, microhardness or CBCT images. *In vitro* remineralization is quite different when compared with dynamic, complex biological system, which happens naturally in the oral cavity. However, there is a further need for long-term research under clinical conditions to show the efficacy of these agents.

There has been an increasing global interest in the potential therapeutic uses of plant-derived natural products for the prevention of oral diseases. Despite the advances made and effort spent on the identification of food components and development of food products, including fruits and nuts, with disease preventing and health promoting benefits, the general public seems less aware of natural foods that promote oral health. Out of which many of the research has been focused on establishing the harmful relationship between foods and dental plaque bacteria and the role of antimicrobials play in this system. Unfortunately, studies regarding the possible protective effects of fruits and nuts have been limited[46]. Gu *et al.*[32] demonstrated that many fruits namely, grape seeds, berries, apples and certain nuts like hazelnuts, pecans and pistachios contain substantial amounts of PAs. PAs in these fruits and nuts are different in terms of concentration, distribution of oligomers and polymers, constituent flavan-3-ol units, and interflavan linkages. According to them, PAs account for a major fraction of the total flavonoids ingested in Western diets. Remineralization capacity of PAs should be taken into account while studying the association between fruits and nuts intake and our fight against dental caries.

We must keep in mind that remineralization *in vitro* may be quite different when compared with a dynamic, complex biological system, which occurs naturally within the oral cavity. Thus, direct extrapolations to clinical conditions must then be

exercised with caution. However, there is a need for further long term research under clinical conditions to prove the efficacy of these newer agents.

The underlining hypothesis of our future research should focus on selected fruits or higher plants possessing antimicrobial phytochemicals and their mechanistic actions against oral pathogens included inhibition of metabolism and growth; acid production; acid tolerance; plaque biofilm formation, adherence and maturation; demineralization of dental enamel and dentin; and the suppression of virulence genes expression. One can assume that selected active compounds may also find application directly as oral prophylactic/therapeutic agents or serve as lead compounds for the subsequent design and synthesis of remineralization agents which are more effective than the existing ones.

CONCLUSION

From the observations of the present study, the following conclusions can be drawn:

All the three groups viz. CPP-ACPF, tricalcium phosphate and grape seed extract showed remineralization under the *in vitro* pH cycling model, while grape seed extract group showed significantly greater remineralization compared to the CPP-ACPF and tricalcium phosphate groups. The results of Vicker's microhardness, SEM-EDS, and cone beam computed tomography showed that the samples treated with grape seed extract have the higher microhardness, mineral content and remineralization potential in comparison to the CPP-ACPF and tricalcium phosphate groups. The study also showed that CPP-ACPF and tricalcium phosphate were also efficient in remineralization.

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CONFLICT OF INTEREST

The authors do not have any financial interests in the companies of the remineralizing agents included in this article.

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