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A Comparative Evaluation of Remineralization Effect of Grape Seed Extract, CPP-ACFP and Combined Effects of Both on Coronal Dentin-An Invitro study

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Abstract: *Thepresentinvitrostudywasundertakentoanalyseandcomparethedemineralizingeffectsof Grape seed extract (GSE), Casein phosphopeptide amorphous calcium phosphate (CPP-ACP) with 900ppmfluoride (CPP –ACFP) separately and evaluate the combined effects of GSE with CPP-ACFP using pH cycling model. Thirty freshly extracted third molar teeth were fragmented into dentin slabs of 1mm thickness and were kept in 4 separate jars labelled as A,B,C,D and subjected to demineralization solution . Sample-A was the control group, Sample-B was immersed in GSE with phosphate buffer, Sample-C was immersed in CPP-ACFP with acidic buffer and Sample-D was immersed in GSE+CPP-ACFP in neutral buffer. This pH cycling procedure was repeated 6 times per day for 8 days. After remineralization procedures, the dentin slab samples were subjected to Vickers's microhardness testing and confocal laser scanning microscopy (CLSM). The values were tabulated and statistical analysis done using one way ANOVA followed by TUKEY-HSD post hoc test with "SPSS statistics V-17" software. The results revealed significant regain in microhardness value (62.89%) of demineralized dentin and wider precipitation band in CLSM analysis for GSE+CPP-ACFP treated samples. Thus the benefits of fluoride incorporated into CPP-ACP and proanthocyanidins (PA) of GSE seems to enhance remineralization in coronal dentin, thereby providing a novel non-invasive material for treating dental caries that has invaded the coronal dentin.*

Keywords*: Grape seed extract, proanthocyanidin, remineralization, calcium phosphopeptide amorphous calcium phosphate, coronaldentin.*

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I. Introduction

Grape seed extract (GSE) is a dietary supplement made by removing, drying and pulverizing the bitter tasting seeds of grapes. It is a potent anti-oxidant, improves blood circulation and promotes collagen synthesis. It plays a promising role in medical field and now it has been a boon to dentistry as the proanthocyanidin (PA) with its demineralizing properties seems to prevent the progression of dental caries.

Dentin, the yellowish mineralized tissue structure beneath the enamel forms the major bulk of the tooth. It contains 70% minerals, 20% organic material (mainly fibrillar type 1 collagen) and 10% fluid[1]. Several natural and synthetic agents namely GSE can increase and preserve the inter and intra-molecular collagen cross links.

Calcium and phosphate ions are the building blocks for the demineralization process which are found in saliva. Casein phosphopeptide amorphous calcium phosphate (CPP-ACP) releases calcium and phosphate ions via a pH or concentration gradient mechanisms. The inclusion of fluoride into CPP-ACP as CPP-ACFP (GC tooth mouse) forms a better material enhancing fluorapatite formation and erosion resistance[2]

In this study, an in vitro pH cycling model is used to evaluate and compare the remineralization effects of GSE, CPP- ACFP and the combined efficacy of GSE with CPP-ACFP on coronal dentin.

II. Materials And Methods

Materials used:

1. Grape seed extract (Dry Creek Nutrition Inc., Modesto,California)
2. GC Tooth Mousse plus (CPP-ACFP) Tokyo,Japan.

3.Demineralization solution (2.2 mM $CaCl_2.2H_2O$, 2.2 mM KH_2PO_2, 50mM acetate, pH 4.6)
4.Buffering solutions

a)Phosphate buffer (0.025 M KH_2PO4, 0.025MK_2HPO_4, pH7.4)
b)Acidic buffer (50mM acetate; 2.25mM CaCl..$2H_2O$; 1.35mM KH_2PO_4; 130mM KCl; pH5.0)
c) Neutral buffer (20mMHEPES; 2.25mM$CaCl_2.2H_2O$; 1.35mM KH_2PO_4; 130mM KCl, pH7.0)
5. Deionized water (dH_2O)
6. Rhodamine B solution

Dentin slab preparation

Thirty freshly extracted third molar teeth without caries were obtained from the Department of Dental Surgery, Government Erode medical College and Hospital, Perundurai, Erode. All the organic remnants and stains were removed and kept in deionized water prior to experimental use. In all the teeth, enamel part of the crown was removed, and the dentin was sectioned horizontally to 1mm thickness dentinal slabs. Therefore, each tooth provided four dentin slabs which were kept in four separate jars labeled as A,B,C,and D.

Step: 1

pH cycling procedure

In pH cycling procedure, all the dentin slabs are subjected to demineralization by immersing it in demineralized buffer solution of pH3.5for 120hrs at room temperature and it is kept for Micro hardness test and CLSM analysis.(Fig 2).

Control group – Microhardness and CLSM analysis

Following pH cycling procedure, dentin slabs "A" (n= 10) were rinsed with deionized water for three minutes and sectioned into two halves. One half was embedded perpendicular to the demineralized surface in self-cure resin for microhardness evaluation using Vickers microhardness testing.

Vickers microhardness testing

This test was performed at MSME testing center, Guindy, Chennai. Measurements of Vickers microhardness were carried out on the dentin slab, in a micro hardness tester (NEOPHOT 21 with microhardness attachment, Germany). Three indentations with a load of 300 gm. were placed on the center of the dentin slab, for 15 seconds using a square-based diamond pyramid indenter. This procedure resulted in well-defined indentations.

CLSM analysis

The other half was sectioned using hard tissue microtome (Leica SP 1600) at Saveetha dental college and hospital, Chennai, to obtain 50µm thickness of specimen and it was stained with freshly prepared 0.1% Rhodamine B solution for confocal laser scanning microscope (CLSM) analysis (Fig1) (Leica TCS SP2, Germany) performed at National center for ultrafast processes, Madras university,Taramani,Chennai. Samples were analyzed with a CLSM using an argon laser with a 529-nm excitation wavelength and the specimens were viewed at 10 and 20 x magnification and images were captured with an image-analysis system. The images of stained specimens were qualitatively analyzed for their optical density. The optical density is directly related to the porosity of the demineralized dentin, where increased porosity corresponds with decreased optical density. If remineralization occurs, the optical density will increaseaccordingly.

Step 2:

The demineralized dentin slabs B, C and D were subjected to three different in-vitro remineralization protocols as follows.

Before proceeding into this protocol, the buffering solutions i.e., acidic buffer with a pH 5.0, neutral buffer with a pH 7.0 and phosphate buffer for GSE with a pH 7.4 were prepared at the institute of Biochemistry, MMC,Chennai.

First, the softened dentin slabs B, C and D were washed with deionized water (dH_2O) and kept in the 6.5 %(w/v) GSE in phosphate buffer, CPP-ACFP and GSE + CPP-ACFP respectively for 10 mins, and then slabs were

rinsed with dH_2O. Then it was kept in an acidic buffer and in a neutral buffer for 30mins and 10mins respectively. Then it was washed in dH_2O and placed it in treatment solutions. Then once again the same procedure was repeated. This pH cycling procedure was done 6 times per day for 8 days. Then the slabs were kept in a neutral bufferovernight.

The GRAPE SEED EXTRACT (GSE) used in this study was purchased in the trade name of Activin from Dry Creek Nutrition, Inc., California. It is in a brown powder form with proanthocyanidin (PA) content greater than90%.

GC Tooth Mousse Plus contains 900 ppm fluoride ions (0.2% sodium fluoride) specially incorporated into CPP-ACP to form CPP-ACFP. It is commercially available as GC Tooth Mousse Plus (CPP-ACFP 900 ppm F-).

III. Results

Statistical Analysis: The Micro hardness values of all the dentin slabs were tabulated(Table.1) and compared statistically using One-way ANOVA at .05% significance level, followed by Turkey-HSD post hoc test (Table.2). All statistical analyses were done using "SPSS statistics v 17" software.

Table 1: Micro hardness values of all the dentin slabs

			AFTER REMINERLIZATION PROTOCOL		
S.No	Control	Demineralized	GSE	CPP-ACFP	GSE+CPP -ACFP
1	63.25	46.58	52.11	54.82	59.19
2	62.47	45.86	50.76	52.63	57.02
3	64.74	47.75	52.89	55.11	58.90
4	64.08	46.32	51.94	54.97	59.03
5	66.33	48.27	53.13	56.19	58.96
6	67.59	45.92	52.78	55.98	58.01
7	65.41	46.95	53.56	57.04	59.66
8	62.92	44.87	53.15	56.89	59.83
9	65.69	47.16	52.21	55.91	58.95
10	68.15	44.93	51.87	55.09	59.05

Table 2: One-way anova followed by post hoc tests

A Group	B Group	Mean Difference A - B	P Value
Control	Demineralized GSE CPP-ACFP GSE + CPP-ACFP	17.8020(*) 11.8230(*) 8.8000(*) 5.3910(*)	P <0.001** P <0.001** P <0.001** P <0.001**
Demineralized	Control GSE CPP-ACFP GSE + CPP-ACFP	-17.8020(*) -5.9790(*) -9.0020(*) -12.4110(*)	P <0.001** P <0.001** P <0.001** P <0.001**
GSE	Control Demineralized CPP-ACFP GSE + CPP-ACFP	-11.8230(*) 5.9790(*) -3.0230(*) -6.4320(*)	P <0.001** P <0.001** P <0.001** P <0.001**
CPP-ACFP	Control Demineralized GSE GSE + CPP-ACFP	-8.8000(*) 9.0020(*) 3.0230(*) -3.4090(*)	P <0.001** P <0.001** P <0.001** P <0.001**
GSE + CPP-ACFP	Control Demineralized GSE CPP-ACFP	-5.3910(*) 12.4110(*) 6.4320(*) 3.4090(*)	P< 0.001** P< 0.001** P< 0.001** P< 0.001**

***** The mean difference is significant at the .05% level.
** Highly significant

Calculation of Surface Microhardness Changes in Percentage

1. Sound dentin on treating with demineralizing solution for 96hrs causes 28.31% reduction inthe microhardnessvalue.
2. GSE treatment results in 48.04% regain in microhardness value of the demineralizeddentin.
3. CPP-ACFP treatment results in 52.43% regain in microhardness value of the demineralizeddentin.

4. GSE + CPP-ACFP treatment results in 62.89%regain in microhardness value of the demineralizeddentin.

Fig 1. Confocal Laser Scanning Microscope (CLSM) Fig 2. CSLM Image of Formation Of Lesion

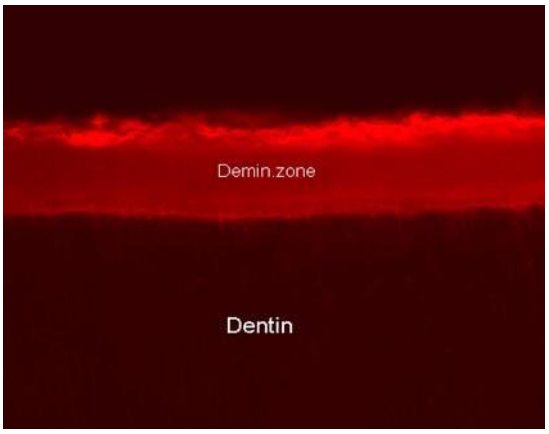


Fig 3. GSE+CPP-ACFP With MoreWider And HomogenousPrecipitationBand

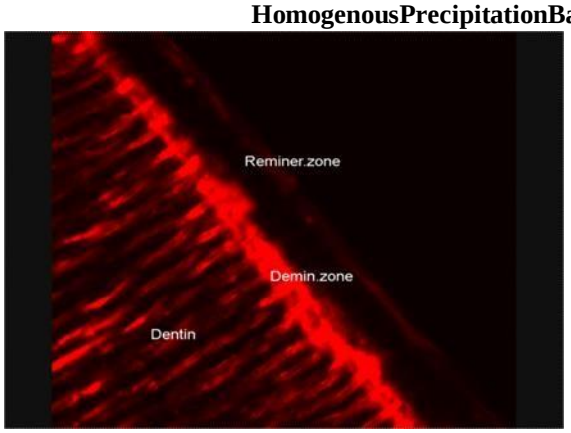
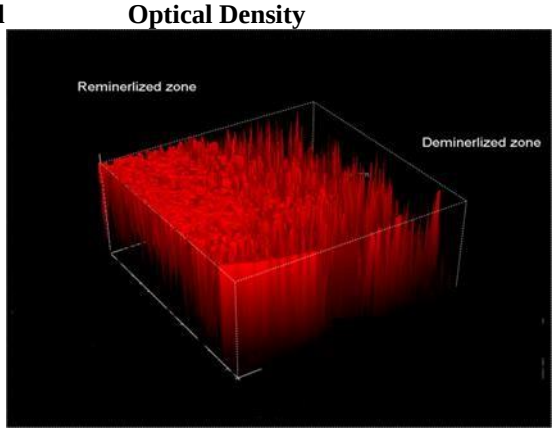


Fig 4. GSE + CPP-ACFP Shows More Pronounced Optical Density



IV. Discussion

Tooth wear can result occasionally in extensive loss of enamel and dentin, leading to dentinal sensitivity and pulpal exposure[3] . Previous reports have indicated that topical fluoride can protect enamel and dentine against erosion, [4,5] Recently, Tooth Mousse (GC Corporation, Japan) whose principal ingredient is an anticariogenic remineralizing agent CPP-ACFP (casein phosphopeptide-amorphous calcium fluro phosphate nanocomplexes) has been recommended for the management of dental erosion..[6]

Studies have shown that, proanthocyanidin (PA) from grape seed extract (GSE) increased collagen synthesis and accelerated the conversion of soluble collagen to insoluble collagen during development.[7,8]

In this study the pH-cycling procedure was employed to expose the artificial caries lesions to repeated acid challenge followed by remineralization.

The Vickers indenter with 300gm load for 15 seconds was used in the present study for microhardness evaluation. In this study, base line hardness values of dentin were ranged from 63 to 68 VHN. Three treatment groups i.e., GSE, CPP-ACFP and GSE+CPP-ACFP resulted in significant increase in surface hardness from the demineralized values compared to the control group.

In this study, the remineralization effect of GSE was found to be lesser to CPP-ACFP. The treatment with GSE results in 48.04% regain in microhardness value of the demineralized dentin. The treatment with CPP-ACFP was found to be better than GSE. It results in 52.43% regain in microhardness value of the demineralized dentin. But, at the same time the remineralization effect of GSE combined with CPP-ACFP was found to be superior to GSE and CPP- ACFP when used alone. It results in 62.89% regain in microhardness value of the demineralized dentin. CLSM images after remineralization reveals that the combination of GSE and CPP-ACFP promoted enhanced and more homogenous remineralization evenly throughout the surface which was presented as precipitation band in the CLSM image (Fig 3) and there by decrease in lesion size. This may be due to the combination of 900 ppm of fluoride and CPP-ACP, which would have co-localized calcium and phosphate ions with fluoride ions at the tooth surface, presumably as CPP-ACFP nanocomplexes (Cross et

al., 2004).[9]. This increased concentration of calcium, phosphate and fluoride ions at tooth surface would drive diffusion into dentin, producing higher activities of the ions in the demineralized area resulting in higher levels of remineralization and fluoride incorporation into mineral phase forming fluorapatite.

The rationale for the CLSM analysis of demineralization and remineralization relies on the hypothesis[10] that imbibitions of a fluorescent dye into porosities of demineralized enamel decreases following remineralization and enables qualitative analysis of samples using confocal laser scanningmicroscopy..

The images of stained post-treatment lesions of CLSM were qualitatively analyzed for their optical density. The optical density is directly related to the porosity of the demineralized dentin, where increased porosity corresponds with decreased optical density. If remineralization occurs, the optical density (Fig 4) will increase accordingly. The findings of our study are similar to previous study by QianXie et al, Gonzalez-Cabegzas et al[10.11]

Based on data obtained from the previews study[11], GSE may positively affect the remineralization process through two distinct mechanisms. First, GSE may contribute to mineral deposition on the superficial layer of the lesion.. GSE formed insoluble complexes when mixed with the remineralizing solution at pH 7.4. This is consistent with reports made previously by other researchers[12,13.] Thus, it is likely that after treatment with GSE, it may combine with Ca2+ from the remineralizing solution, thereby enhancingremineralization.

Secondly, GSE may interact with the organic portion of the dentin through PA–collagen interaction, thereby stabilizing the exposed collagen matrix and inducing cross-links in the dentin collagen. This also increases the microhardness value by reducing enzymatic degradation[.14],

At the same time, the group treated with GSE+CPP-ACFP combination demonstrated increased microhardness value and more wider precipitation band than the group treated with GSE and CPP-ACFP alone.

The abovementioned study have proved the combined benefits of GSE and CPP-ACFP .The findings of our study suggest that if GSE is combined with CPP-ACP and 900 ppm of fluoride, the benefits of fluoride, CPP-ACP as well as proanthocyanidins of the GSE can be enhanced.

V. Conclusion

This in vitro study recommends utilizing GSE together with CPP-ACFP for enhanced remineralizing potential on coronal dentin .In future it may be a promising noninvasive material for treating dental caries that has invaded the coronal dentin. But the true efficacy of this combination still needs in vivo evaluation for further clinical incorporation**.**

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