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Assessing the effect of diverse remineralising agents on enamel: An in-vitro study

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Abstract
Introduction:*Recent scientific advancements in restorative materials have led to evolution of preventive techniques. There is an ever-increasing range of agents to promote remineralisation and prevent demineralisation. This study was conducted to generate evidence and compare enamel remineralisation after treatment with four different remineralising agents using surface microhardness assessment.*
Materials and Methods: *In this in-vitro study, 50 enamel slabs were created and divided into 6 groups,ten samples each forcasein phosphopeptide amorphous calcium phosphate, sodium flouride, calcium sucrose phosphate and green tea extract, and 5 samples each in positive and negative control groups. A pH cycling model was adopted for 7 days and each of the demineralised sample was treated with respective remineralising agents. Vicker's microhardness test was used to assess microhardness. One-way analysis of variance with tukey-kramertest was done to assess significant differences in the values.*
Results:*Remineralisation achieved by all the four experimental groups was statistically significant against demineralised enamel, while remineralisation achieved using calcium sucrose phosphate was significant in comparison to sodium flouride and green tea extract as per the tukey-kramer comparison test.*
Conclusion: *Calcium sucrose phosphate showed the maximum enamel remineralisation potential with higher mean microhardness values and also showed higher hardness values than sound enamel, thus can be used for effective remineralisation in clinical settings.*
Key Words: *Amorphous calcium phosphate, Analysis of variance, Calcium sucrose phosphate, Cariostatic agents,Dental Caries, Surface hardness,Tooth demineralisation, Tooth remineralisation*

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I. Introduction

Dental caries is the localized destruction of the tissues of the tooth particularly by lactic acid, a result of fermentation of dietary carbohydrates by bacteria in dental plaque.[1] Sugars and other fermentable carbohydrates provide substrate for the action of oral bacteria, which in turn lower plaque and salivary pH. The resultant action is the beginning of tooth demineralisation.[2,3]

Scientific advances in restorative materials and greater understanding the pathogenesis have led to more efficient oral health management and preventive techniques. To ensure the long-term success of the caries management regimen, efforts have been concentrated on lowering the risk of caries in patients and have emphasised the significance of a partnership approach between patients and dentists.[4]

Remineralisation 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 demineralised enamel to produce net mineral gain.[3] A substantial literature now exists demonstrating an anticariogenic effect of dairy products such as cheese, attributed to casein phosphopeptide - amorphous calcium phosphate (CPP-ACP) clusters following enzymatic digestion of the multiphosphoseryl-containing sequences of casein.[4] Various preparations containing compounds based on bioavailable phosphate and calcium in form of casein phosphopeptides with CPP-ACP to prevent demineralisation and promote remineralisation of early enamel lesions has been demonstrated in several in vitro, in situ and in vivo studies.[5-7]

In other studies, the preventive effects of 2% sodium fluoride (NaF) solution against initial dental caries have been reported to createa superficial reserve of calcium fluoride, from which fluoride is released when the oral pH drops, thus maximizing remineralisation in the aftermath of a carious attack.[5, 8, 9]

Other methods based on mixture of calcium sucrose phosphates (CaSP) and inorganic calcium phosphates are also proven to have potential of re-mineralisation in many in vitro studies. These work by creating an aqueous solution containing high concentration of calcium (10-12%) and phosphate (8-10%) by weight without precipitation leading to remineralisation.[10, 11]

Furthermore, a considerable interest has arisen in recent times, concerning the health promoting potential of green tea *(Camellia sinensis)*, leading to its wide consumption in the world today, 2nd only to water. A variety of dried and processed leaves are extracted in water to make tea. They have numerous medicinal properties, mainly attributed to their antibacterial and antioxidant properties, and their bioactive chemicals, including; polyphenols, alkaloids, minerals and volatile oils. From dental point of view, tea has preventive effect against tooth decay.[12] It reduces both dental plaque and caries as a result to its high fluoride and organic constituents that inhibit bacterial activity and re-mineralise enamel.[13]

Nevertheless, due to emergence of a number of such agents, there is a need to quantitively compare their remineralising potential, so that specific indication-based application may be carried out by the clinicians. Hence, the purpose of this studywas to evaluate and compare the enamel remineralisation potential of four different remineralising agents - casein phosphopeptide-amorphous calcium phosphate, sodium fluoride, calcium sucrose phosphate and green tea - using surface microhardness assessment.

II. Methodology

This *in-vitro* study was conducted using twenty-five freshly extracted human molar teeth, collected and stored in 10% formalin solution after cleaning with ultrasonic scaler. The teeth were observed to have been extracted for orthodontic needs or from periodontally compromised individuals, intact teeth not having any attrition / abrasion or surface irregularities to be included in the study. Primary teeth, and permanent teeth having decay, cracks or fractures were excluded.

The teeth were washed under tap water immediately after extraction and stored in normal saline. Thereafter, the samples were placed in 3% hydrogen peroxide to remove external stains and then in 3% sodium hypochlorite for 1 hour to remove adherent soft tissue. Then the remaining calculus was removed by scaling.

The radicular part of each tooth was removed and the coronal part of was sectioned buccolingually. Enamel samples (2 mm thickness) were prepared from the buccal and lingual surfaces of the teeth using a double-faced diamond disc mounted on a straight hand piece. Sample preparation windows were created (dimension of 5 mm × 5 mm) using adhesive tape and the sample was made completely resistant to acid attack by coating nail varnish. (Colorama nail varnish, Maybelline).

A total of 50 enamel slabs were randomly divided into 6 groups, ten samples each for CPP-ACP, NaF, CaSP and Green Tea Extract,and 5 samples in both positive and negative control groups.The materials were procured from commercially available preparations such as GC Tooth Mousse (CPP-ACP), Colgate PhosFlur (NaF) and Anticay® Technology based Toothmin. (Table 1)

A slurry of respective ‘remineralising agents’ were prepared in deionised water in the ratio of 1:3. While green tea solution was prepared by brewing 2g crushed dried leaves in 100 ml deionized water for 30 seconds.

'Demineralising solution' was preparedin accordance with pH cycle method used by Ten Cate and Duijsters,[14] using 2.2g calcium chloride, 2.2g potassium hydrogen orthophosphate, 3g acetic acid and 56g potassium hydroxide in 1050 ml distilled water – maintained at pH of 4.5 and the 'remineralising solution' was prepared using 0.1665g calcium chloride, 0.108g sodium dihydrogen phosphate and 11.25g potassium chloride in 152 ml distilled water – maintained at pH of 7.

After drying the samples, the adhesive tape was removed from the enamel using a sharp tipped instrument exhibiting a rectangular area on the enamel surface. Each of the enamel samples (except Group VI) were then immersed in 40 ml of demineralising solution for a period of 72 hours at a constant temperature of 37°C, in an incubator to create artificial caries while replicating a demineralization hotspot.

A pH cycling model was adopted to simulate the dynamic process of demineralisation and remineralisation that occurs in the oral cavity.Each of the enamel samples were treated with the respective remineralising agents for a period of 2 min, following which the samples were individually immersed in 20 ml of demineralising solution for a period of 3hrs. This was followed-up with treatment of the samples again with the respective remineralising agents for 2 min. Then all the enamel samples were individually immersed in 30 ml of remineralising solution for a period of 17hrs. This pH cycle continued for 7 days.

Enamel specimens were prepared. Custom made cylindrical moulds were made and cold cure acrylic resin was poured in it; then each enamel blocks were embedded on top of partially set acrylic and were allowed to set.

The microhardness was assessed using Vicker's microhardness tester. A load of 25 grams was applied for 10 seconds, for all the specimens. The micro vicker's hardness number (VHN) of three indentations at spacing of 100 microns were taken, and the average value was considered to be the mean microhardness value.

Statistical analysis

The quantitative data was described in mean and standard deviation (SD) and for multiple comparisons, statistical analysis was done using one-way analysis of variance (ANOVA) followed by Tukey-Kramer test and the *p-value* of 0.05 or less was regarded as significant.

III. Results:

Out of the total 50 samples, 10 specimens each were divided into 4 experimental groups while 5 specimens each were divided into positive and negative control groups. The Vicker's hardness values of all 50 samples are shown in table 2.

The maximum mean hardness values were observed in samples treated with Calcium Sucrose Phosphate (352.40, SD = 4.624) followed by casein phosphopeptide amorphous calcium phosphate (335.50, SD = 21.15), sodium flouride (320.10, SD = 22.63) and green tea extract (309.60, SD = 16.77). Moreover, the mean value obtained from controls of sound enamel was 320.50 (SD = 3.96) and demineralised enamel was 282.60 (SD = 2.41).

Statistically significant results (F = 15.561, $P < 0.0001$) were obtained upon statistical analysis using One-way ANOVA, and on comparison using Tukey-Kramer Test, most significant hardness results were obtained after treatment with calcium sucrose phosphate (CaSP - Negative control: q = 11.176, $P < 0.001$). An important finding inferred was that the treatment with all 4 remineralising agents produced significant results of varying level of significance when compared with negative controls of demineralised samples, however, results with CaSPin comparison with positive control of sound enamel also yielded significance results (q = 5.060, $P < 0.05$), as described in table 3).

IV. Discussion

The study provided a comprehensive overview and comparison of hardness values after demineralisation and treatment with remineralising agents. Numerous studies conducted globally have shown findings of effective remineralisation following fluoride application (Abou Neel*et al*, 2016; Dai*et al*, 2019; Byeon*et al*, 2016), sodium fluoride (Puig-Silla *et al*, 2009; Zhou *et al*, 2011), CPP-ACP (Llena*et al*, 2015; Jose *et al*, 2016), CaSP (Sargod*et al*, 2015; Raghu and Ananthakrishna, 2016) and green tea extract (Chatterjee*et al*, 2012, Singh*et al*, 2010). However, this study provided a comparative analysis and evidences towards relative effectiveness of such products for clinical use.[7, 9, 13, 15-22]

For the purpose of generalisability of the results, demineralisation similar to enamel subsurface lesion was sought. An intermediate pH 4.0-5.0 was therefore employed for 4 days using the composition similar to the one employed by Ten Cate and Duijsters (1982, 2015).[14] Similarly, for assessment of enamel remineralisation, most commonly used microhardness tests are vicker's microhardness test and knoop microhardness test.[23] As this study focuses on evaluation of surface microhardness of enamel, vicker's surface microhardness test was used.

Effective remineralisation from all agents were observed in comparison to demineralised sections, however, the results from surface microhardness indicated greater remineralisation of enamel in the samples of CaSP followed by CPP-ACP, NaF, whilegreen tea being the least. The results of the present study are in accordance with previous studies conducted by Kaur *et al* (2015), Kshirsagar*et al* (2015), Esfahani*et al* (2015) and George *et al* (2015).[10,11,24, 25]

One-way ANOVA showed a significant difference between all the six groups,however, multiple group Tukey-Kramer test showed significant differences between CaSPand all other groups except CPP-ACP.

V. Conclusion

The study provided evidence that Casein Phosphopeptide Amorphous Calcium Phosphate (CPP-ACP), Sodium Flouride (NaF), Calcium Sucrose Phosphate (CaSP) and Green Tea Extract, all showed effective remineralisation potential and increased surface microhardness as compared to demineralised enamel. Calcium Sucrose Phosphate showed maximum enamel remineralisation potential when compared to Sodium Flouride and Green tea and while showinga higher mean microhardness value than Casein Phosphopeptide Amorphous Calcium Phosphate. Moreover, CaSPalso showed higher hardness values than sound enamel, thus can be used for effective remineralisation in clinical settings.

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Table 1: Division of the samples in different groups

Group I	Demineralised and treated with GC tooth mousse (CPP-ACP)
Group II	Demineralised and treated with Colgate PhosFlur (NaF)
Group III	Demineralised and treated with Toothmin (CaSP)
Group IV	Demineralised and treated with green tea extract
Group V	Demineralised and not treated with any solution (negative control)
Group VI	Sound enamel (no treatment) (positive control)

Table 2: Vicker's microhardness values (VHN) of all samples and intergroup comparison

	A	B	C	D	E	F
Specimen	**CPP-ACP**	**NaF**	**CaSP**	**Green Tea**	**Demineralised Enamel**	**Sound Enamel**
I	342	332	358	300	281	322
II	320	330	350	318	286	327
III	343	340	359	313	284	317
IV	338	328	355	322	282	320
V	365	330	349	320	280	318
VI	343	338	352	325	-	-
VII	285	295	345	318	-	-
VIII	347	302	357	279	-	-
IX	330	275	349	319	-	-
X	342	321	350	282	-	-
Mean	335.50	320.10	352.40	309.60	282.60	320.50
SD	21.152	22.625	4.624	16.768	2.408	3.962

Table 3: Tukey-Kramer Multiple Comparisons Test

Dependent Variable	**Comparisons**		**Mean Difference**	**q-value**	***P-value***	**Significance**
Surface microhardness	A (CCP-ACP)	B (NaF)	15.400	3.020	>0.05	Ns*
		C (CaSP)	-16.900	3.314	>0.05	Ns
		D (Green tea)	25.900	5.079	<0.05	+†
		E (Demineralised)	52.900	8.470	<0.001	+++
		F (Sound enamel)	14.700	2.354	>0.05	Ns
	B (NaF)	C (CaSP)	-32.300	6.334	<0.001	+++
		D (Green tea)	10.500	2.059	>0.05	Ns
		E (Demineralised)	37.500	6.004	<0.01	++
		F (Sound enamel)	-0.700	0.1121	>0.05	Ns
	C (CaSP)	D (Green tea)	42.800	8.393	<0.001	+++
		E (Demineralised)	69.800	11.176	<0.001	+++
		F (Sound enamel)	31.600	5.060	<0.05	+
	D (Green tea)	E (Demineralised)	27.000	4.323	<0.05	+
		F (Sound enamel)	-11.200	1.793	>0.05	Ns
	E (Demineralised)	F (Sound enamel)	-38.200	5.297	<0.01	++

*Ns: Not Significant
†+ : Level of significance

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