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ORIGINAL ARTICLE

Caries-preventive Efficacy of Resin Infiltrant, Casein Phosphopeptide-amorphous Calcium Phosphate, and Nanohydroxyapatite using Confocal Scanning Electron Microscope: An *in vitro* Study

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ABSTRACT

Introduction: White spot lesion is the early sign of demineralization occurring under intact enamel which may lead to the development of caries.

Aim: The aim of this study is to compare and evaluate the caries-preventive efficacy of a resin infiltrant, casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), and nanohydroxyapatite (nano-HA) on white spot enamel lesions.

Settings and designs: A total of 40 freshly extracted human maxillary incisors were used in this study. Enamel samples (2 mm thickness) were prepared and sample preparation windows were created (dimension of 5 × 5 mm) using adhesive tape, and the sample was made completely resistant to acid attack by coating nail varnish.

Materials and methods: The samples were divided into four groups, which are resin infiltrant, CPP-ACP, nano-HA, and control of 10 enamel samples in each group. The samples were evaluated using confocal laser scanning microscopy before and after the application of resin infiltrant and remineralizing agents.

Statistical analysis: Statistical analysis was done using analysis of variance and *post hoc* Bonferroni test was used for comparing intragroups and Tukey test for comparing intergroups.

Results: The mean value after demineralization is 245, 246, 250, and 247 µm for Groups I to IV. After remineralization, group I > group II > group III > group IV. After acid challenge for a period of 14 days, group I > group II > group III > group IV.

Conclusion: The resin infiltrant showed higher caries inhibition potential and superior acid resistance than CPP-ACP and nano-HA.

Clinical significance: The inhibition of caries progression by resin infiltration technique should be considered as an alternative approach to the more invasive therapies and warrants a place in the range of minimally invasive dentistry techniques.

Keywords: Casein phosphopeptide-amorphous calcium phosphate, Confocal laser scanning microscope, Nanohydroxyapatite, Resin infiltrant.

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INTRODUCTION

White spot lesions are initiated by the pathogenic bacteria that have breached the enamel layer and by the organic acids produced by them. These cause the removal of a certain amount of calcium and phosphate ions which fail to be replaced naturally during their mineralization process.[1] White spot lesions are commonly reversed by the process of remineralization mainly through the application of fluorides.[2] Deep enamel lesions show a tendency to remineralize only superficially. Consequently, the arrested lesions show a thick and highly mineralized surface layer[3] but the underlying lesion body is still porous and the whitish appearance often persists.[4] The goal of caries management is, therefore, to stop or arrest the progression of the lesion. But, remineralization brought about by the topical application of fluoride requires multiple treatment sessions and a strict long-term follow-up, which requires strong motivation and cooperation from the patient but is often seen to be difficult to achieve. In addition, the monitoring systems used for assessing the status and progression of the lesions over time are still being studied and are difficult to apply in everyday clinical practice.[5]

A new microinvasive treatment method suggested for the management of white spot lesions is the infiltration of a resin into the lesion. The resin infiltrant prevents further progression of the initial enamel caries lesion by occluding the microporosities within the lesion as it has a low viscosity.

Remineralization of enamel subsurface lesions has been studied widely both *in vitro* and *in situ* as well as in numerous clinical studies.[6] One such system that has been developed uses casein phosphopeptide (CPP) in order to stabilize the calcium and phosphate ions at higher concentrations and to form an amorphous nano

complexes namely casein phosphopeptide-amorphous calcium phosphate (CPP-ACP).[7]

In current practice, nanohydroxyapatite (nano-HA) has been widely used as an effective anticaries agent mainly because of its unique potential to bring about remineralization.[8] The size of the calcium phosphate crystal also plays an important role in the formation of hard tissues and also has a significant impact on its intrinsic properties, solubility, and biocompatibility.[9]

Confocal microscopy is a useful tool to study the infiltration of low-viscosity resin into initial enamel carious lesion. The images obtained are high-resolution optical images with depth selectivity.[10] Currently, there are no studies available in the literature that compare the depth of penetration of resin infiltrant with tooth remineralizing agents like CPP-ACP and nano-HA.

MATERIALS AND METHODS

A total of 40 human maxillary incisors extracted for periodontal reasons were included in this study. Teeth with any visible caries, hypoplastic lesions, and white spot lesions were excluded from this study.

Enamel Sample Preparation

The teeth were thoroughly cleaned of all debris including calculus and tissue debris. Occupational Safety and Health Administration and Centers for Disease Control and Prevention recommendations and guidelines were followed during the collection, storage, sterilization, and handling of the extracted teeth. Then the radicular portions were removed by decoronating the teeth 2 mm coronal to cementoenamel junction using a diamond disk (Axis Dental, Texas) attached to a slow-speed micromotor straight handpiece rotating at 1,500 rpm. The 40 enamel samples were prepared by sectioning at the middle third region of the crown. Following sample preparation windows were created with approximate dimension of 5 × 5 mm using adhesive tape and the sample was made completely resistant to acid attack by coating nail varnish and then stored in 10% formalin at room temperature of 37°C and humidity.

The 40 incisors are divided into four groups of 10 samples each: Group I, resin infiltrant (Icon); group II, CPP-ACP (GC Tooth Mousse); group III, nano-HA (Acclaim); group IV, control. All the samples in four groups were kept separately in artificial saliva for 24 hours.

Composition of artificial saliva[11]

- Ascorbic acid – 0.002 gm
- Glucose – 0.030 gm
- Sodium chloride (NaCl) – 0.580 gm
- Potassium chloride (KCl) – 1.270 gm
- Calcium chloride ($CaCl_2$) – 0.170 gm
- Ammonium chloride (NH_4Cl) – 0.160 gm
- Sodium thiocyanate (NaSCN) – 0.160 gm
- Dihydrogen potassium phosphate (KH_2PO_4) – 0.03 gm
- Urea – 0.02 gm
- Sodium hydrogen phosphate (Na_2HPO_4) – 0.3 gm
- Mucin – 2.7 gm
- Distilled water to make up a volume of 1 L

Demineralization of Enamel Samples

In order to create the artificial enamel lesions, the samples were demineralized by placing in a beaker containing the prepared demineralizing solution.

Demineralizing Solution[12]

Composition of the Demineralizing Solution

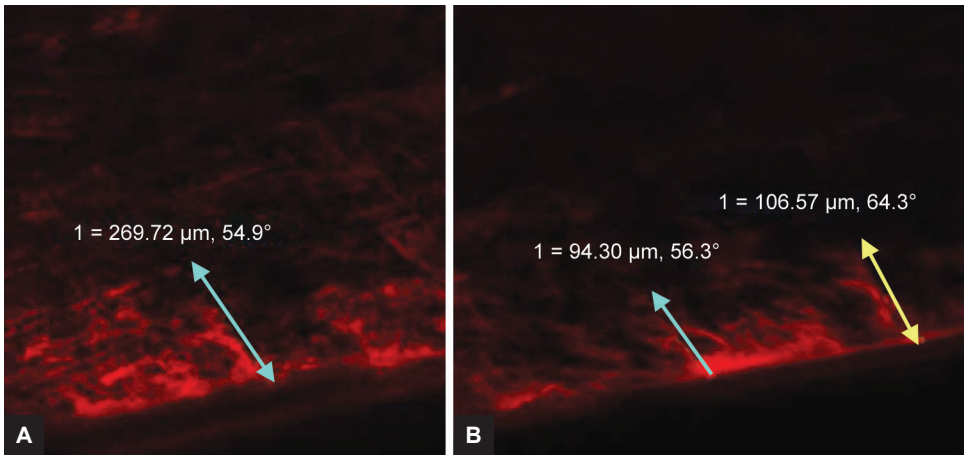
- Methylhydroxydiphosphonate – 6 µM
- Calcium chloride dihydrate ($CaCl_2 – 2H_2O$) – 3 mM
- Potassium dihydrogen phosphate ($KH_2 PO_4$) – 3 mM
- Acetic acid – 50 mM
- Traces of thymol – 0.2 mM

The enamel samples were then stored for a period of 14 days all the while maintaining a pH of 5.0 and at 37°C temperature. The pH was checked daily using a pH meter and any variation in pH was corrected by adding either glacial acetic acid or potassium hydroxide solution. The depth of demineralization of all the enamel samples were measured using confocal scanning electron microscope equipped with argon/krypton laser.

Remineralization of Enamel Samples

- Group I (resin infiltrant): Enamel samples were then subjected to 15% HCl etching for 2 minutes, rinsing for 30 seconds, ethanol desiccation for 30 seconds, resin infiltrant applied and light cured for 40 seconds, and stored in artificial saliva for 30 days.
- Group II (CPP-ACP): Enamel samples were then brushed with (CPP-ACP) twice daily for 1 minute and stored in artificial saliva for 30 days.
- Group III (nano-HA): Enamel samples were brushed with nano-HA toothpaste twice daily for 1 minute and stored in artificial saliva for 30 days.
- Group IV (control): Untreated enamel samples were stored in artificial saliva for 30 days.

The treated enamel samples were then subjected to confocal laser scanning microscope (CLSM) analysis and the depth of penetration was measured for all the four groups. The treated enamel samples in all the groups were subjected to acid challenge by placing in demineralizing solution for 14 days. The depth of the lesion was analyzed using CLSM after subjecting to acid challenge (Figs 1A and B).



Figs 1A and B: Confocal laser scanning microscope images after demineralization and after acid challenge for 14 days (group I, resin infiltrant)

Data were analyzed using Statistical Package for Social Sciences version 20.0. Data were statistically analyzed using analysis of variance followed by *post hoc* Bonferroni test used for comparing intragroups and Tukey test used for comparing intergroups.

RESULTS

The mean value after demineralization of enamel samples in demineralizing solution is 245 µm for group I (resin infiltrant), 246 µm for group II (CPP-ACP), 250 µm for group III (nano-HA), and 247 µm for group IV (control). After remineralizing the enamel samples for a period of 30 days, the results are group I (resin infiltrant) 158 µm > group II (CPP-ACP) 28.8 µm ≥ group III (nano-HA) 26.3 µm > group IV (control) 23 µm. After acid challenge for a period of 14 days, there was in group I (resin infiltrant) 114 µm (72%) > group II (CPP-ACP) 16.4 µm (57%) ≥ group III (nano-HA) 13.8 µm (50%) of remaining material. The untreated control group had least resistance with mean of 8 µm. The p-value was 0.993 after demineralization and <0.001 after remineralization and after acid challenge for 14 days when comparison was done between all the four groups. After *post hoc* Tukey intergroup comparison, group I showed statistically significant difference which was greater when compared with group II (CPP-ACP) and group III (nano-HA) where there was no difference (Table 1).

DISCUSSION

Carious lesions formed on the enamel surface are unique in that the enamel is both acellular and avascular. Thus, in contrast to other tissues enamel cannot heal by the cellular repair mechanism.[13] It is now a well-established fact that the formation of incipient enamel white spot lesions is a reversible process, where periods of demineralization alternates with periods of remineralization.[14]

Table 1: Intergroup comparisons for all the study groups

Phases	*Groups*	*N*	*Mean*	*Std. deviation*	*p-value*
Demineralization	I: Resin infiltrant	10	245.00	71.37538	0.853
	II: CPP-ACP	10	246.00	73.36363	
	III: Nano-HA	10	250.50	69.21986	
	IV: Control	10	230.50	54.93412	
	Total	40	247.17	68.87875	
Remineralization	I: Resin infiltrant	10	158.10	53.45289	<0.001
	II: CPP-ACP	10	28.80	2.34758	
	III: Nano-HA	10	26.30	1.15950	
	IV: Control	10	23.00	0.90711	
	Total	40	71.06	69.33921	
Acid challenge	I: Resin infiltrant	10	114.00	41.15013	<0.001
	II: CPP-ACP	10	16.40	1.71270	
	III: Nano-HA	10	13.80	2.09762	
	IV: Control	10	8.502	0.696	
	Total	40	48.06	52.70211	

*Statistically significant, p < 0.05

A favorable environment in the oral cavity leads to remineralization and helps in the repair of the carious lesion.[15] In early carious lesions, the enamel surface remains relatively unaltered, whereas the mineral loss associated with the underlying lesion body can be substantial. Clinically such enamel lesions appear as whitish discolored areas commonly referred to as white spot lesions.[16]

In this study, the mean lesion depth after demineralizing the enamel samples for all the four groups was 247 µm, which is in accordance with the results of previous studies by Meyer-Lueckel et al[17] who reported the mean lesion depth as 357 µm and Mueller et al[18] who reported the mean lesion depth as 237 µm.

In this study, CLSM was used to visualize the penetration of the material. In a study by Pioch et al,[10] it was proved that CLSM had the following advantages of nondestructive examination, since the layer visualized can be situated up to 100 µm below the surface. Moreover drying of the samples which is required for conventional scanning electron microscopy or transmission electron microscopy is not necessary leading to decreased risk of shrinkage or other artifacts.

In this study, acid conditioning with Icon (2 minutes with 15% hydrochloric acid) could have led to deeper resin penetration than etching with 37% phosphoric acid gel[19]. It could be argued that removal of surface layer by 15% HCl could additionally weaken the lesion structure. According to a study done by Meyer-Lueckel et al,[17] it was proved that no cavitation occurred after acid etching even if the complete surface layer was completely eroded and subsequent resin infiltration could ensure restrengthening of the lesion structure.

In this study, 99% ethanol was applied for 30 seconds prior to application of the infiltrant. In a study by Paris et al[20] it was proved that addition of ethanol is associated with higher penetration coefficient by decreasing the viscosity and contact angle, hence they can be used as promising tools for rapid penetration. It was also proved that mixtures containing large amounts of 2-hydroxyethyl methacrylate, triethylene glycol dimethacrylate, and ethanol are associated with higher penetration coefficients and satisfactory hardening; therefore, they might be promising tools for rapid caries penetration.

In this study, the mean penetration of the treated enamel samples for the resin infiltrant group (group I) which was observed using CLSM is 158 µm. In previous studies by Paris et al[21] and Meyer-Lueckel et al,[22] the mean penetration of resin infiltrants was 58 and 104 µm respectively. The uncharged ions penetrate the lesion and will dissociate in the form of charged ions along the diffusion path and ultimately in the lesion and promote the crystal growth.[6]

In this study, the remineralized area for group II (CPP-ACP) and group III (nano-HA) observed in CLSM is 28.8 and 26.3 µm respectively. In this study, paste-type formulation of CPP-ACP was used. The remineralization process of CPP-ACP involves diffusion of calcium and phosphate ions through the protein-/water-filled pores of the caries surface enamel into the body of the enamel lesion. Once in the body of the enamel lesion, these calcium and phosphate species increase the activities of Ca^{2+} and PO_4^{3-}, thereby increasing the degree of saturation with respect to hydroxyapatite.[22]

After acid challenge for a period of 14 days, the amount of remaining resin infiltrant which was resistant to acid attack was 114 µm (72%), amount of remaining CPP-ACP was 16.4 µm (57%), amount of remaining nano-HA was 13.8 µm (50%), and there was increased progression of depth of lesion and no resistance for untreated control group.

Within the limitations of this *in vitro* study, resin infiltrant showed greater caries-inhibiting potential than other remineralizing agents like CPP-ACP and nano-HA. In this study, CPP-ACP and nano-HA showed similar caries-inhibiting potential. However, there are few limitations in this *in vitro* study, hence further *in vivo* studies are needed to confirm the results of this study under simulated oral environment.

CONCLUSION

Within the limitations of this *in vitro* study, the following conclusions can be elucidated:

- The resin infiltrant showed higher caries inhibition potential than CPP-ACP and nano-HA.
- In addition, resin infiltrant showed superior acid resistance compared with CPP-ACP and nano-HA.
- The resin infiltrant has a promising role in the management of early enamel carious lesion.
- The resin infiltrant can be used as an alternative microinvasive approach.

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