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Evaluation of remineralizing efficiency of two calcium-based non-fluoridated remineralizing agents for the prevention of white spot lesions (WSL): A comparative FESEM & EDS in-vitro study

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ABSTRACT

Background: This research work was designed to appraise and equate the remineralizing efficiency of two calcium-based phosphate delivery agents, Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP) and Calcium Sucrose Phosphate (CaSP) for preventing the development of White Spot Lesions (WSL) around the orthodontic brackets.

Materials and Methods: Forty orthodontically removed premolar teeth were randomly allocated to Group 1 (n=20) - (CPP-ACP) and Group 2 (n=20) - (CaSP). All the specimens were subjected to FESEM and EDS analysis before the initial demineralization phase and after 14 days of the pH cycling phase, to observe and record changes in surface topography and mineral content (Ca/P ratio % wt.). Statistical data for the Intragroup comparison were analyzed by using Paired sample t-test, whereas for the Intergroup comparison, an Unpaired t-test was performed. Furthermore, the One-way ANOVA test was applied for comparing Intragroup data and the Post-hoc Tukey test was applied to compare Intergroup data.

Results: Group 1 samples showed an 11.3% increase in remineralization and Group 2 samples showed a 21.3% increase in remineralization, indicating a statistically significant ($p < 0.05$) 11% of greater remineralizing efficiency of CaSP.

Conclusion: The use of Calcium Sucrose Phosphate (CaSP) tooth cream around the orthodontic brackets seems to produce significantly better remineralization effects.

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1. Introduction

Fixed orthodontic appliances and bonding of attachments to the teeth cause debris accumulation on tooth surfaces that generally are not susceptible to caries, creating stagnation areas for plaque and making it difficult to achieve good oral hygiene. Since orthodontic brackets, bands, and arch wires also have significant rough surfaces,

thereby impeding the innate self-cleaning capacity of salivary flow and perioral muscles.^{1,2} The demineralization and remineralization process of the enamel surface is a dynamic and continuous mechanism that can lead to initial demineralization, progress to non-cavitated lesions, and eventually, produce cavitated lesions.³ According to Fejerskov and Kidd, white spot lesions (WSL) are the forerunner of caries that can be easily perceived with a bare eye.³ The development of minute lines around the

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brackets should be suspected of developing into White spot lesions (WSL), but they may also present clinically as large and visible decalcified areas with or without cavity formation.⁴ Patients susceptible to acquiring White spot lesions (WSL) include patients with poor dental hygiene (pre-treatment and intra-treatment), preadolescent age when the orthodontics treatment is started, a high DMFT index, greater duration, and larger area for etching.⁵ A significant increase has been reported in the prevalence of these lesions around the bracket bases or underneath the bands, facially and lingually, especially in cervical areas and gingival margins of the teeth.⁶ Experimentally, White spot lesion (WSL) can be induced in just a span of 4 weeks, i.e., within two clinical appointments.⁶ The incidence of White spot lesions (WSL) has a wide variation and ranges from 0 to 97%. This variation has been attributed to several factors, such as individual differences in tooth structure, salivary composition and flow, frequency of tooth brushing, the area between the free gingival margin and the bracket, use of bands or bonded attachments, differences in sample size and methodology to assess.^{7,8} Although some remineralization of White spot lesions (WSL) occurs from bioavailable ions present in saliva, once the appliances are removed. However, the remineralizing potential of saliva is insufficient and progresses gradually.^{9–11} Consequently, remineralization of teeth using an exogenous source of critical ions, such as casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), calcium sucrose phosphate (CaSP), etc have been emphasized. Therefore, with this aim in focus, the current in-vitro investigation was undertaken to measure and relate the effectiveness of two calcium-based phosphate systems on demineralized enamel surfaces around orthodontic brackets and attachments.

2. Materials and Methods

Forty therapeutically removed premolar teeth (Figure 1) were collected from patients reporting to the Post-graduate Clinic of Orthodontics and Dentofacial Orthopedics, Career Post Graduate Institute of Dental Sciences & Hospital, Lucknow for orthodontic treatment and were arbitrarily allocated into two sets of twenty teeth each, designated as Group 1 and Group 2, respectively. Permission for this in-vitro study was acquired from the University ethics board, and informed consent was obtained from each patient.



Fig. 1: Extracted tooth samples placed in 10 ml storage containers

2.1. Composition of remineralizing agents

Agent 1: Tooth Mousse® topical tooth cream by GC containing glycerol, CPP-ACP (casein phosphopeptide - amorphous calcium Phosphate), D-glucitol, colloidal silica, propylene glycol, sodium carboxyl methyl cellulose, guar gum, titanium dioxide, xylitol, phosphoric acid, zinc oxide, ethyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate, magnesium oxide, sodium saccharin. **Agent 2:** Toothmin (Abbott Healthcare) tooth cream containing calcium sucrose phosphate, colloidal silica, sodium saccharine, sorbitol, glycerine, distilled water, cocamidopropyl betain, titanium dioxide, propyl hydroxybenzoate, sodium carboxy methyl cellulose, methyl hydroxybenzoate. (Figure 2).



Fig. 2: a) Wax sheet, b) LED curing light, c) Acid resistant nail varnish, d) Remineralizing agent 1, e) Remineralizing agent 2

2.2. Sample preparation

All the sample teeth were immersed in 10% formalin immediately after therapeutic extraction and were subjected to careful washing with normal saline to eliminate all the soft tissue attachments and remnants. A window cover of nearly 4mm × 4mm on the enamel surface was created with the help of wax sheets (Figure 3 a), at the buccal aspect, in the centre of each sample tooth crown and the remaining uncovered buccal surfaces were coated with an acid impervious nail paint. Wax was removed and Orthodontic brackets were bonded (Figure 3 b & c) onto the centre of exposed surfaces of each sample, leaving only a few mm of an uncoated tooth surface adjacent to the bracket margins, and represents the delineated area studied for the changes induced by demineralization and remineralization process. 37% Orthophosphoric acid Tooth Conditioner Etching Gel (Dental Products of India) was used for etching. Conventional Transbond XT Light Cure Adhesive Paste and Adhesive Primer were used to bond MBT 0.022 slot 3M Unitek Gemini Metal Brackets

(Figure 4). Group 1(n=20) -(CPP-ACP) and Group 2(n=20) -(CaSP), were subjected to FESEM and EDS analysis before the initial demineralization phase to observe untreated surface topography and record the reference mineral content (Ca/P ratio % wt.).



Fig. 3: a): Wax window of 4×4 mm² created on the buccal surface of the sample tooth, b): Peripheral varnish coating with bracket positioned at the centre, c): LED Light cure application

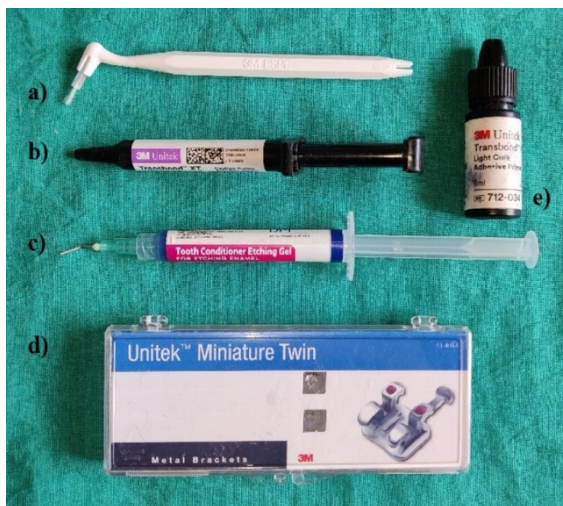


Fig. 4: a): Applicator brush, b): Light cure adhesive paste, c): Etching gel, d): Metal brackets, e): Light cure adhesive primer

2.3. Artificial saliva preparation

400 ml of artificial saliva (Figure 5 a) was made as per the recommendation of Göhring et al. (2003).¹² The pH was adjusted between 6 and 7. Small container plastic boxes filled with 10 mL of artificial saliva were used to store the samples till they were subjected to the experimental procedure. The artificial saliva, therefore, served as a storage medium and a vehicle for the mobility of remineralizing ions in this process.

2.4. Demineralizing solution preparation

2.2 mM Potassium dihydrogen phosphate, 2.2 mM Calcium chloride and 0.05 M Acetic acid (Ten Cate et al. 1982)¹³ were used to formulate 400 ml of demineralizing solution (Figure 6 b). The pH of the prepared solution was regulated at around 4.4-5.5 a) by the addition of 1 M Potassium



Fig. 5: a): Chemicals used for preparation of artificial saliva, b): Chemicals used for preparation of demineralizing solution

hydroxide.

2.5. PH cycling model

A 24-hour Model for pH Cycling was formulated, similar to the recommendations of Itthagarun and Wefel (2000).¹⁴ Each prepared tooth sample was subjected to a 14-day pH cycling process, that consisted of immersion in the 10mL of demineralizing solution for up to 6 hours incubated at 37° C and then in the 10mL of remineralizing solution for up to 18 hours (overnight) incubated at 37° C (Figure 6 b). For Group 1 specimens, CPP-ACP and for Group 2 specimens, CaSP remineralizing agent was meticulously applied with the help of a 3M applicator brush over the exposed areas adjacent to bracket margins for 1 to 2 minutes, before the onset and 2-3 times during the remineralizing phase for a period of 14 days. Samples were thoroughly rinsed for 30-60 seconds with distilled water before placing them in counter solutions, to prevent any cross-reaction of chemical ingredients and affect the process.

2.6. FESEM and EDS analysis

During the pH Cycling procedure, FESEM (Field Emission Scanning Electron Microscope) analysis to qualitatively evaluate the superficial variations in the enamel configuration and EDS (Energy Dispersive Spectroscopy) analysis for quantitatively measuring the inorganic elements (Ca/P ratio) of each sample was performed on day 1 (T0) and day 14 (T1). Each specimen was dried with the aid of Critical Point Drying (CPD) machine (Figure 6 c), then subjected to gold sputtering (Figure 6 d & e) and followed by examination by means of the Scanning Electron Microscope (Model: JSM 7610F, JEOL India Private Ltd.) at 15kV. Three SEM photomicrographs were captured at 1500x magnifications at the Occlusal, Middle, and Cervical 1/3rd of each specimen. The image with the best clarity was selected for observation (Figure 7 a to f). The EDS study at the three spots was carried out in the same manner, and the average value of the three measurements was noted. EDS Detector LN2 free, Peltier cooled, Octane Plus Model with T-EAM Software support (30 mm² and 127eV resolution) was used that operates as an inbuilt



Fig. 6: a): Demineralization solution pH adjusted at 4.4, b): Incubator, c): Critical Point Dryer (CPD), d): Gold sputter coater machine, e): Samples inside gold sputter machine

component of the scanning electron microscope imaging. The inorganic elements were electronically identified and the digital output for each was interpreted and recorded.

2.7. Statistical analysis

Paired sample t-test was applied for Intragroup assessment, whereas for Intergroup comparison, an Unpaired t-test was done. Two-tailed ($\alpha=2$) P value < 0.05, was regarded as statistically considerable. Furthermore, the One-way ANOVA test was applied to equate Intragroup data, and the Post-hoc Tukey test was applied to compare Intergroup data. The outcome measure of the study was the Calcium and Phosphate Ratio (Ca/P). Data Analysis was performed on the latest SPSS⁶ Statistics v22.0 software. Substantial variations in the calcium and phosphate ratios of the reference, demineralization, and remineralization groups of the samples were reflected.

3. Results

Demineralization and remineralization of Group 1 and Group 2 demonstrated a statistically substantial difference (P value < 0.05) in the ratio of both the calcium and phosphate ions, signifying the net increase of both calcium and phosphate particles after the remineralization phase. Gain in the mean value of Ca & P ion ratio was observed from 1.55 to 1.663 for Group 1 and from 1.5605 to

1.7730 for Group 2, as described in Tables 1 and 2, respectively. A graphic representation of the Ca/P ratio between demineralization and remineralization for both groups is shown in Graph 1 and Graph 2. Results of Group 1 and Group 2 were compared and a statistically considerable difference (P value < 0.05) in the proportion of calcium and phosphate particles was recorded, specified by a relatively greater net gain of calcium ions in Group 2, as evident statistically by the greater mean value 1.7730 of remineralization in Group 2 in contrast to the mean value of 1.6630 for the remineralization in Group 1. A graphic representation of the disparity in calcium and phosphate ion ratio between remineralization in Group 1 and Group 2 is depicted in Graph 3. The intra-group assessment was done by using Paired t-test and for the inter-group assessment, an Unpaired t-test was further used to statistically calculate and compare the amount of remineralization (Percent Change) achieved by Group 1 Agent (CPP-ACP) and Group 2 Agent (CaSP), after 14 days of pH cycling procedure. Group 1 samples showed an 11.3% increase in remineralization (Table 3) and Group 2 samples showed a 21.3% increase in remineralization (Table 4), indicating a statistically noteworthy ($p < 0.05$) 11% of greater remineralizing efficiency of Group 2 Agent than Group 1 Agent, as depicted in Graph 4, respectively.

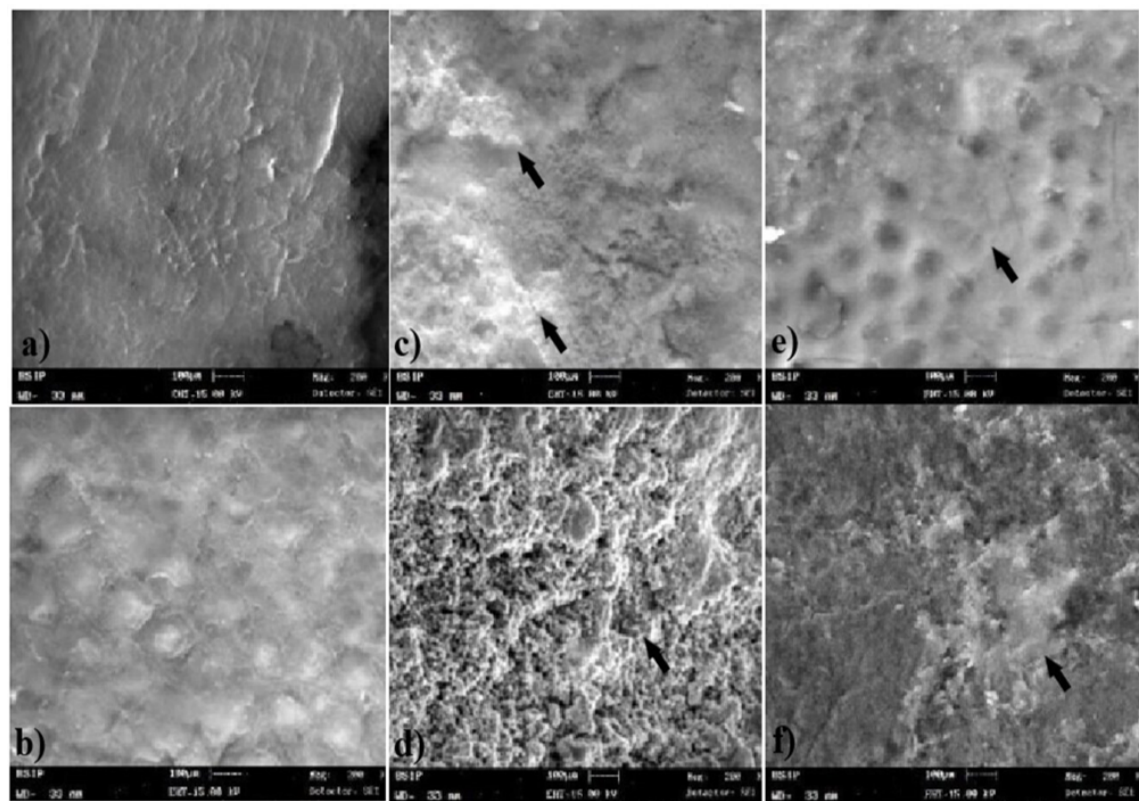


Fig. 7: a): Reference Group 1, b): Reference Group 2, c): Demineralization Group 1, d): Demineralization Group 2, e): Remineralization Group 1, f): Remineralization Group 2

Table 1: Demineralization versus remineralization (Group 1)

Ca/P Ratio	n	Mean	S.D.	S.E. Mean Diff.	95% Confidence Interval of the Difference		T value	df	P Value
					Lower	Upper			
Demineralization	20	1.55	.06044	.01518	-.14478	-.08122	-7.442	19	.000
Remineralization	20	1.663	.01809						

P Value: Significant < 0.05, non-significant > 0.05

Table 2: Demineralization versus remineralization (Group 2)

Ca/P Ratio	n	Mean	S.D.	S.E. Mean Diff.	95% Confidence Interval of the Difference		T value	df	P Value
					Lower	Upper			
Demineralization	20	1.5605	.04662	.01256	-.23879	-.18621	-16.915	19	.000
Remineralization	20	1.7730	.02203						

P Value: Significant < 0.05, non-significant > 0.05

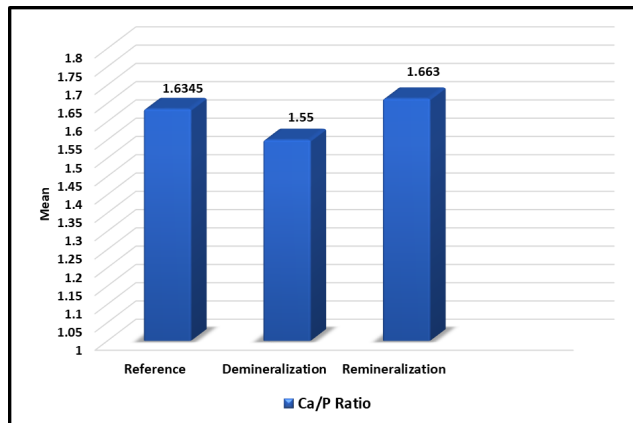
Table 3: Percent change in remineralization (Group 1)

Ca/P Ratio	n	Mean Difference and Percent Change	S.E. Mean Diff.	95% Confidence Interval of the Difference		T value	df	P Value
				Lower	Upper			
Demineralization	20							
Remineralization	20	.113 (11.3%)	.014	.0844	.01416	8.010	19	.000

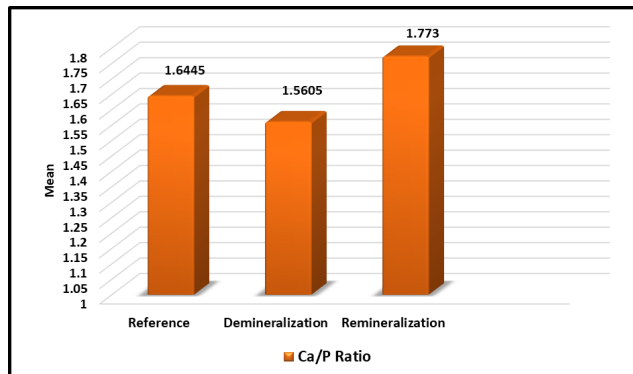
Table 4: Percent change in remineralization (Group 2)

Ca/P Ratio	n	Mean Difference and Percent Change	S.E. Mean Diff.	95% Confidence Interval of the Difference		T value	df	P Value
				Lower	Upper			
Demineralization	20	.213 (21.3%)	.012	.01892	.02358	18.430	19	.000
Remineralization	20							

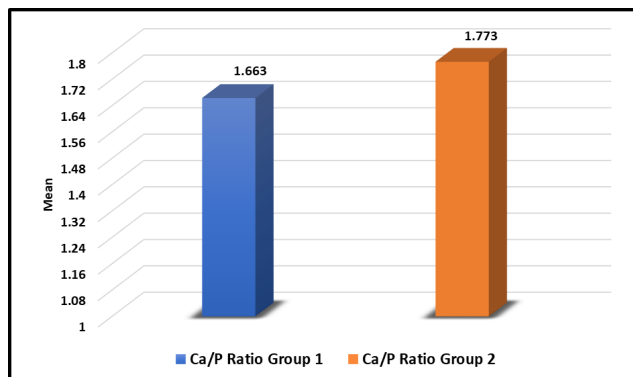
P Value: Significant < 0.05, non-significant > 0.05



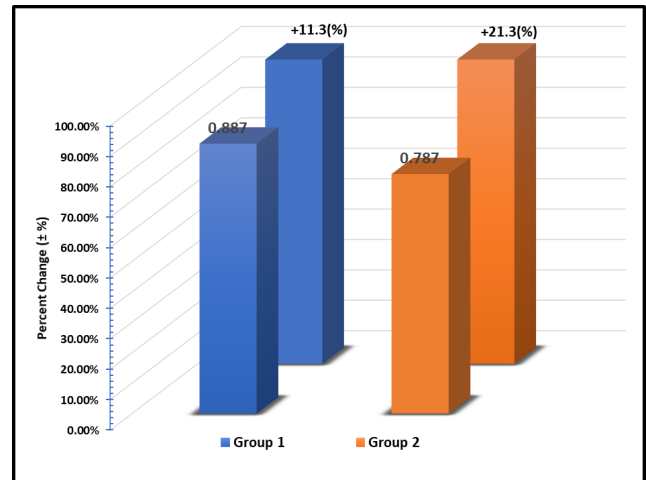
Graph 1: Mean Values of Ca/P Ratio of Group 1 (CPP-ACP)



Graph 2: Mean Values of Ca/P Ratio of Group 2 (CaSP)



Graph 3: Mean Values of Ca/P Ratio of Group 1 (CPP-ACP) and Group 2 (CaSP)



Graph 4: Amount of remineralization (Percent Change) of Group 1 (CPP-ACP) and Group 2 (CaSP)

4. Discussion

Due to the lack of any residual cellular components, the enamel surface of the tooth has a distinctive composition in contrast to both cementum and dentin, incapable to repair and restore its structure when it gets damaged by a cariogenic episode. Therefore, loss of tooth enamel due to demineralization and its subsequent repair through remineralization is a part of a dynamic and constant process, governed by the bioavailability of apatite mineral ions in the saliva.¹⁵ Although direct bonding has many advantages, enamel demineralization in adjoining tooth surfaces of orthodontic attachments is an irrefutable problem. The present study focused on a non-invasive approach of using topical remineralizing agents to prevent demineralization and perhaps at the same time promote remineralization of white spot lesions (WSL). An ideal remineralization agent should have these properties: not merely deliver inorganic ions of calcium and phosphorous onto the superficial layer, but be able to penetrate into the deeper layers of the lesion, not promoting the growth of calculus by delivering an excess of calcium, should be effective even at acidic pH values and must sufficiently saturate the saliva with free ions to enhance its remineralizing capability.¹⁶ Although Fluoride containing dentifrices and topical creams have been widely used for caries prevention and remineralization, mainly due to their ability to form calcium fluoride

(CaF₂) like precipitates, however, it has been noticed that the fluoride-mediated remineralization is limited to the marginal 30 µm of enamel layer only (low permeability of fluoride), thereby compromising both the structural integrity and aesthetics. Fluoride has also been classified as a neurotoxicant causing biotoxicity if consumed in higher concentrations and therefore a safety concern especially when prescribed in children, it has also been attributed to causing dental fluorosis, fluoride syndrome (occult caries) and halo effect/mottling in permanent teeth.¹⁷ Furthermore, several investigations support that topical use of agents containing fluoride has the potential to impede the process of bonding by forming globular reaction by-products such as calcium fluoride and fluorinated calcium phosphate (fluorapatite) on the etched surface, interfering with the formation of resin tags and resulting in a significant reduction in bond strength of dental resins.^{18,19} An efficient substitute of the non-fluoride calcium-based phosphate system, like Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP) and Calcium Sucrose Phosphate (CaSP) is hence needed to repair the demineralized tooth structure and overcome the shortcomings of using fluoride. A 14-day pH cycling regime with repeated acidic challenges was therefore designed, simulating the natural oral environment. The remineralizing agents were applied to samples twice a day for 2 weeks, replicating the normal recommended daily oral prophylaxis. E.C. Reynold^{20,21} in his Intra-oral caries study model observed the anti-cariogenic and sub-surface remineralization ability of a milk protein derivative, casein. He concluded that casein has a high affinity for inorganic ions of calcium and phosphate besides promoting their incorporation within the dental plaque, in this manner serving as a repository of calcium and phosphate ions. Furthermore, casein breakdown by bacteria in the biofilm produces phosphoryl residues that may raise the plaque pH, augmenting the dissolution of amorphous calcium phosphate, and also reducing the loss of tooth surface by strongly binding to the hydroxyapatite. The outcome of this study aligns with the results obtained by Reynolds, confirmed by the increase in the mean Ca/P ratio (1.6630 +/- .01809) indicating a significant (p<0.05) rise in the concentration of Calcium and Phosphorous ions after remineralization by CPP-ACP. Calcium Sucrose Phosphate (CaSP) was originally formulated by Neuberg and Pollak²² in 1910 by phosphorylation of sucrose with phosphorus oxychloride. Clinically, it has been proven to decline the prevalence of caries when introduced as a food additive.²³ M. J. Rogerson²⁴ in his study explained the potential of CaSP in promoting the process of remineralization, by forming complexes of calcium sucrose phosphate-calcium orthophosphate, known as “Anticay”, thereby providing high levels of suspended and readily available inorganic ions of calcium and phosphate. This compound works in two ways: Firstly, by producing a common ion effect that reduces the degree of dissolution in an acidic milieu

while simultaneously enhancing the remineralization ratio of enamel, and secondly, by adhering to the tooth structure and deterring plaque formation along with its bacterial acid-producing process. In this research work, results were in agreement with most of the previously discussed studies, but may disagree with some other studies, based on the disparity in sample size, methodology, inherent tooth structure, etc. The results of this study may also vary in contrast to what precisely occurs in an in-vivo environmental condition, perhaps a limitation for all the in-vitro experimental studies.

5. Conclusion

Prevention of enamel demineralization induced by fixed orthodontic appliances is still a critical and undesirable consequence arising during and after the orthodontic treatment. Therefore, special, and timely attention needs to be given to the oral hygiene of the patients and advocating for the regular application of therapeutic preventive agents to avert the development of White Spot Lesions (WSL). Several commercially available remineralizing agents can prevent or even reverse this process. In this in-vitro study, two such remineralizing agents were evaluated and compared for their effectiveness and consequently based on the results obtained, Calcium Sucrose Phosphate (CaSP) ensues to be relatively a superior source of remineralizing inorganic calcium and phosphate particles when compared alongside Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP), respectively.

6. Source of Funding

None.

7. Conflict of Interest

None.

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