ORIGINAL RESEARCH

Comparative Evaluation of Zwitterionic Material, Self-assembling Peptide, and Bioactive Glass Incorporated with MI Varnish for Fluoride, Calcium, and Phosphorus Ion Release, Enamel Remineralization, and Microhardness

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Abstract

Background: White spot lesions occur when the pathogenic bacteria have broken through the enamel layer. White spot lesions (WSLs) can be treated using a complex approach. The most crucial step is to stop demineralization and biofilm formation and use assorted strategies for remineralization of lesions, thinning, microabrasion, erosion infiltration, adhesive composite resin restorations, and the bonded facets.

Aim: To evaluate and compare the fluoride, calcium, and phosphorus ion release, remineralizing efficacy, and microhardness of zwitterionic material, self-assembling peptide, and bioactive glass (BGA) incorporated with MI Varnish.

Materials and methods: The original study was conducted on 60 extracted premolars; the sample size calculated was 10 per group. All samples were divided into four groups—group A, MI Varnish (control), group B, MI Varnish + zwitterionic material, group C, MI Varnish + self-assembling peptide, and group D, MI Varnish + BGA. All these groups were further evaluated and compared for fluoride, calcium, and phorphorus ion release, remineralizing efficacy, and surface microhardness (SMH).

Results: Zwitterionic material, when incorporated with MI Varnish showed high fluoride and calcium ion release and high remineralizing efficacy under polarized light microscopy (PLM). BGA, when incorporated with MI Varnish showed high phosphorus ion release and higher values in the evaluation of SMH, followed by zwitterionic material and self-assembling peptide.

Conclusion: MI varnish alone had remineralizing properties of WSLs, but when novel materials like zwitterionic ion, self-assembling peptide, and BGA are incorporated, its efficacy increases. Among all zwitterionic ions showed superior results for fluoride and calcium ion release and remineralization and BGA for phosphorus ion release and SMH.

Keywords: Casein phosphopeptide–amorphous calcium phosphate, Dentinal caries, Incipient lesions, *In vitro* study.

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Introduction

Dental caries is described as an irreversible microbial ailment affecting the hard tissues of the teeth. It involves the demineralization of the tooth's inorganic component and the degradation of its organic substance, often resulting in the formation of cavities.[1]

The advancement of caries occurs gradually through repeated instances of prolonged exposure to an acidic environment, typically below the critical pH level of 5.5, leading to demineralization. These periods of demineralization are interspersed with resting phases where the plaque's pH returns to approximately 7.0, facilitating remineralization. When plaque accumulates in areas where it can be retained, followed by the consumption of acidic foods, the balance shifts toward demineralization. This imbalance can result in the development of clinically noticeable white spot lesions (WSLs).[2]

White spot lesions can be treated using a complex approach. Halting demineralization and biofilm formation stand out as the pivotal measure, alongside employing a variety of tactics for remineralizing lesions. These strategies encompass thinning, microabrasion, erosion infiltration, and adhesive composite resin restorations and bonded facets.[3,4]

Researchers are unceasingly developing new remineralization therapies to reinforce the offered treatment ways. Most novel methods are developed to reinforce the effect of existing fluoride therapies. Locally applied fluorides are commercially available in the form of varnishes, toothpastes, creams, mouthrinses, and chewing gums/mints. Since varnishes can cling to the tooth surface in the form of a thin film for longer periods of time, they were initially designed to extend the duration (12 hours or more) when fluoride and dental enamel are in touch, and so they are thought about as fluoride reservoirs which slowly release fluoride as they stop the instant loss of fluoride after application and also decrease the chances of acute toxicity.[5,6]

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The active ingredient of fluoride varnish is typically 5% sodium fluoride (22,600 ppm fluoride). To reinforce the remineralization potential of the varnishes, the incorporation of adjunctive active agents has been suggested in research. These methods include adding agents like casein phosphopeptide–amorphous calcium phosphate (CPP-ACP), self-assembling peptide, zwitterionic ion, and bioactive glass (BGA) which give extra remineralizing ions.[7]

Casein phosphopeptide–amorphous calcium phosphate helps prevent demineralization. CPP, the casein protein, carries phosphate and calcium ions in the form of APP. Thus, phosphate and calcium ions are delivered by the CPP-ACP complex. The anticariogenic properties of CPP-ACP rely on its integration into the nano complexes that are found on the surface of teeth and in dental plaque. This allows it to function as a calcium and phosphate reservoir.[8]

Zwitterionic particles (ZPs) show antifouling properties; because ZPs are salt-responsive, their presence in a solution containing low-molecular salt (LMS) affects its viscosity; this is regarded as antipolyelectrolyte effect (APE). The combination of the material's steric repulsion properties and surface hydration causes resistance to the growth of biofilm. The purpose of the self-assembling peptides is to mimic the three-dimensional matrix that is formed by the enamel matrix proteins. Around this matrix, enamel crystals made of salivary calcium phosphate develop. This peptide consists of a combination of fluoride, calcium phosphate, and protein molecules.[9,10]

Bioactive glass is a bioactive substance consisting of an amorphous composition of sodium–calcium–phosphosilicate; when it comes into contact with saliva, calcium, sodium, and phosphorous ions are released into the saliva. Silanols that form after BGA dissolution act as a site of nucleation to form an apatite structure by attracting released phosphate and calcium ions, called hydroxycarbonate apatite.[11,12]

Plenty of clinical studies are available on commercially available fluoride varnish, but very few literature aims at trials after the addition of novel remineralizing agents to fluoride varnish; hence, the present study aims at comparative evaluation of zwitterionic material, self-assembling peptide, and BGA incorporated with MI Varnish for fluoride, calcium, and phosphorus ion release, enamel remineralization, and microhardness.

Materials and Methods

The present *in vitro* study was done at the Department of Paedodontics and Preventive Dentistry, School of Dental Sciences, Krishna Vishwa Vidyapeeth (Deemed to be University), Malkapur, Maharashtra, India. The ethical clearance was received from the committee before starting the study. A sample size of 120 was calculated with the level of significance (α) = 5% and power (β) = 80%. The teeth samples selected for the study included 60 human premolars that were extracted for orthodontic purposes. Inclusion criteria were as follows—nonhypoplastic, noncarious, no cracks and nonrestored teeth, and exclusion criteria were carious and fractured teeth.

After extraction of premolars, the teeth were cleaned and later stored in thymol solution (0.1% wt/vol), which is the antifungal storage media. Polishing of tooth surfaces was done with pumice slurry using a slow-speed handpiece and rubber cups; later, they were washed in distilled water, followed by drying. With the help of a diamond disk mounted on a straight handpiece, the teeth were decorated at the cementoenamel junction.

The 60 decoronated samples obtained were further sliced buccolingually with the help of a diamond disk and water coolant jet to obtain two enamel surfaces; the mesial and distal halves were used as two individual specimens in further procedures. Specimens obtained were later mounted in an autopolymerizing acrylic resin disk.

After mounting the specimens, a label of 3 × 4 mm was placed on the center of the tooth surface, and the rest of the exposed enamel around the label was covered with a nail varnish. Later, the sample was dried, and the label was peeled off the tooth surface. Thus, an enamel window of 3 × 4 mm was made, which was used further in the study. These samples were stored in distilled water to prevent dehydration.

Artificial Caries Induction

Artificially, caries were formed on the exposed enamel surface. To induce these lesions, all the samples were placed in a demineralizing solution, Modified Ten Cate's solution, at 37°C for 96 hours. The solution was prepared according to the composition specified by John Hicks, which consisted of 2.2 mmol phosphate, 2.2 mmol calcium, 5.0 mmol fluoride, and acetic acid at pH 3.75. After demineralization, the samples were removed and washed with distilled water.

Varnish Application

All samples after demineralization were divided randomly into four groups. Each group consisted of 30 samples. The groups were labeled as:

- Group A: Control group—MI Varnish.
- Group B: MI Varnish + zwitterionic material [2-methacryloyloxyethyl phosphorylcholine (MPC)].
- Group C: MI Varnish + self-assembling peptide.
- Group D: MI Varnish + BGA.

Preparation of Experimental Varnishes

- Around 0.44 gm (0.4 mL), MI Varnish was mixed with 13.2 mg zwitterionic material (2-methacryloyloxyethyl phosphorylcholine).
- Around 0.44 gm (0.4 mL), MI Varnish was mixed with 13.2 mg self-assembling peptide.
- Around 0.44 gm (0.4 mL), MI Varnish was mixed with 13.2 mg BGA particles.

Each group (A, B, C, and D) was further divided into three subgroups. Each subgroup had 10 samples that were randomly selected from the group. The subgroups were based on the testing parameters that were used. The subgroups were labeled as:

- Subgroup a: Fluoride, calcium, and phosphorus ion release.
- Subgroup b: Enamel remineralization.
- Subgroup c: Microhardness.

Evaluation of the Parameters

Remineralizing Protocol

The remineralizing solution was prepared with the help of a composition containing calcium phosphate (calcium = 1.5 mmol/L, phosphate = 0.9 mmol/L), with potassium chloride at 150 mmol/L and cacodylate buffer to pH 7.0 (20 mmol/L).

Assessment of Fluoride, Calcium, and Phosphorus Ion Release

Test varnish was applied on 40 prepared samples at 3 × 4 mm window created on the proximal surface.

Samples were immersed in 5 mL deionized water; they were removed after 24 hours, and the test tube was sealed, and the same was repeated at 7-day and 28-day intervals. Fluoride, calcium, and phosphorus ion release were checked at three intervals—24 hours, 7 days, and 28 days using ion electrode.

Assessment of Remineralization

Test varnish was applied on 40 prepared samples at 3 × 4 mm window created on the buccal surface, and the samples were subjected to a pH cycling process.

pH Cycling Procedure

Samples were kept in demineralizing solution at pH 4.3 for 6 hours; then samples were washed and kept in water for 15 minutes; then they were placed in remineralizing solution (at pH 7 for 17.5 hours and samples were washed and kept in water for 15 minutes. Then again, the cycle continued for 9 days and night.

After pH cycling, samples were cut buccolingually and were sectioned and polished to their final thickness (100 µm) using Arkansas stone. The samples were then mounted on microscopic slides and were observed under a polarized light microscope (BA310 POL Trinocular microscope) equipped with a Moticam 5 digital camera under 100× magnification. The lesion depths were quantified using image focus 4.0 at three different points along the window, and the mean measurements were received in microns. Images obtained were analyzed using ImageJ software (Fig. 1).

Assessment of Surface Microhardness

Three times, surface microhardness (SMH) was measured—prior to demineralization (SMH sound), following enamel lesion formation (SMH lesion), and following varnish application and pH cycling (SMH treatment). It was calculated using the following formula:

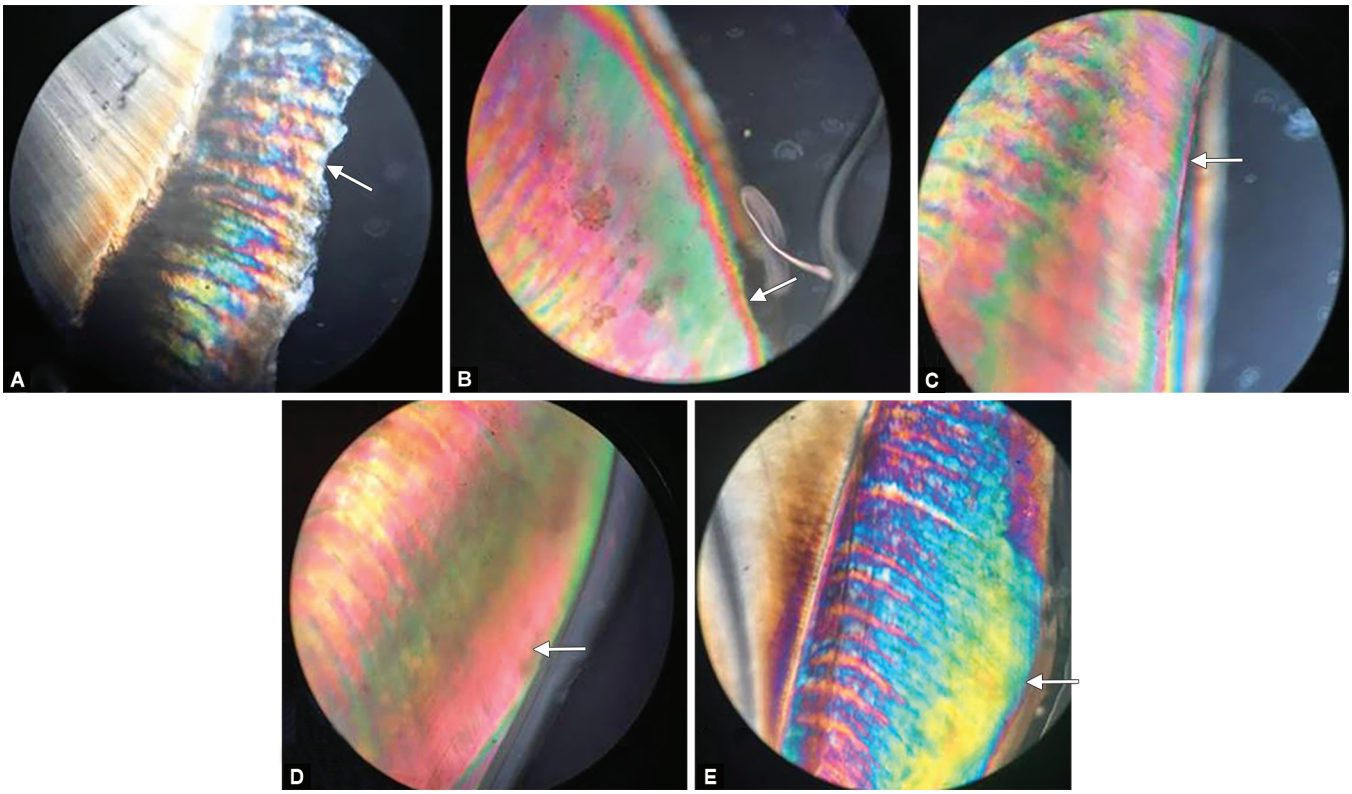
$$\%SMHR = 100[(SMH_{treatment} - SMH_{lesion}) / (SMH_{sound} - SMH_{lesion})]$$

Vickers microhardness testing (VMT) machine was used to assess the SMH of every tooth sample at the working window. Using VMT, the indentations were created for 10 seconds at a rate of 100 gm load. To avoid any discrepancy, the average microhardness of the specimen was determined from three indentations.

Results

Fluoride, Calcium, and Phosphorus Ion Release Estimation

The fluoride, calcium, and phosphorus ion release intragroup analysis at different time intervals was done, and it was assessed by analysis of variance (ANOVA) *F*-value, and it was found that all the groups showed statistically significant results with ($p < 0.0001$, S) and the ANOVA value was higher in group B for fluoride and calcium ion release and higher in group D So, we could state that group B was more effective for fluoride ion release and group D was more effective for phosphate ion release.



Figs 1A to E: Polarized light microscopy (PLM) images of the enamel surface for measurement of the depth of remineralization; (A) Demineralized surface; (B) MI Varnish group (control); (C) MI Varnish + zwitterionic group; (D) MI Varnish + self-assembling peptide group; (E) MI Varnish + BGA

The intergroup analysis of fluoride, calcium, and phosphorus ion release was done at different time intervals. It was observed that statistically significant results were obtained in fluoride and calcium ion release and no significant results for phosphorus Ion release for days 1, 7, and 28, respectively, with ($p < 0.05$) (Tables 1 to 4).

Polarized Light Microscopy

The images of the samples were analyzed using ImageJ software, and the depth was recorded using micrometers. This provided results at two different time intervals, out of which the range for mid-testing (after induction of artificial lesion) was 250–270, and the range for posttesting (after remineralization) was 130–165. The values obtained were analyzed using mean and standard deviation as descriptive statistics by using measures of central tendencies. After running an ANOVA, Tukey's *post hoc* analysis was done. The probability level of 5% was designated as the importance. The statistical package for the social sciences, Statistical Package for the Social Sciences (SPSS) 25.0 (SPSS Inc., Chicago, Illinois, United States of America), was used to conduct the analysis. Intragroup analysis between demineralization and remineralization was done, and it was found that all the study groups were showing statistically significant results with ($p < 0.0001$), and the mean difference of PLM was higher in group B, that is, reduction in results by –118.54 mm. So,

Table 1: Intergroup comparison of fluoride ion release at 1, 7, and 28 days

	1 day		*7 days*		*28 days*	
Fluoride release	*Mean*	*Standard deviation (SD)*	*Mean*	*SD*	*Mean*	*SD*
Group A MI Varnish (control)	0.965	0.0455	0.821	0.0962	0.685	0.0913
Group B MI Varnish + zwitterionic material	1.096	0.036	0.943	0.047	0.818	0.025
Group C MI Varnish + self-assembling peptide	0.971	0.054	0.854	0.075	0.763	0.07
Group D MI Varnish + BGA	0.915	0.049	0.853	0.051	0.764	0.039
ANOVA *F*-value		26.97		5.57		7.52
p-value		<0.0001*		0.003*		0.0005*

*, significant when $p < 0.05$

Table 2: Intergroup comparison of calcium ion release at 1, 7, and 28 days

	1 day		*7 days*		*28 days*	
Calcium release	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Group A MI Varnish (control)	2.252	0.314	1.949	0.177	1.466	0.681
Group B MI Varnish + zwitterionic material	2.511	0.051	1.998	0.241	1.839	0.119
Group C MI Varnish + self-assembling peptide	1.959	0.231	1.537	0.372	1.147	0.524
Group D MI Varnish + BGA	2.672	0.291	1.717	0.436	1.319	0.41
ANOVA *F*-value		14.778		8.417		4.774
p-value		<0.0001*		0.0014*		0.0168*

*, significant when $p < 0.05$

Table 3: Intergroup comparison of phosphorus ion release at 1, 7, and 28 days interval

	1 day		*7 days*		*28 days*	
Phosphorus release	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Group A MI Varnish (control)	0.905	0.043	0.829	0.06	0.642	0.121
Group B MI Varnish + zwitterionic material	0.941	0.037	0.842	0.061	0.595	0.106
Group C MI Varnish + self-assembling peptide	0.918	0.032	0.792	0.048	0.575	0.102
Group D MI Varnish + BGA	1.014	0.063	0.903	0.063	0.553	0.069
ANOVA *F*-value		2.295		2.019		0.9713
p-value		0.12		0.1524		0.3914

Table 4: Intergroup comparison of PLM results at demineralization and remineralization phase

	Demineralization		*Remineralization*	
PLM	*Mean*	*SD*	*Mean*	*SD*
Group A MI Varnish (control)	259.37	8.36	161.73	9.52
Group B MI Varnish + zwitterionic material	256.94	13.61	138.4	0.25
Group C MI Varnish + self-assembling peptide	263.34	5.38	154.9	20.53
Group D MI Varnish+ BGA	264.59	13.63	157.51	14.84
p-value		0.38		0.0054*

*, significant when $p < 0.05$

Table 5: Intragroup comparison of microhardness results at three different time intervals

	Pre		*Mid*		*Post*				
Study group	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean difference*	*ANOVA F-value*	*p-value*
Group A MI Varnish (control)	234.1	5.96	214.3	8.8	346.28	22.15	112.18	251.84	<0.0001*
Group B MI Varnish + zwitterionic material	234.97	9.03	210.68	7.64	325.21	12.04	90.24	383.36	<0.0001*
Group C MI Varnish + self-assembling peptide	236.88	13.24	211.86	11.89	332.76	11.72	95.88	244.97	<0.0001*
Group D MI Varnish + BGA	240.85	11.27	216.48	11.86	354.83	29.19	113.98	146.11	<0.0001*

*, significant when $p < 0.05$; MD, mean difference pre-post

we could state that group B was more effective for PLM compared to other study groups.

The intergroup analysis between demineralization and remineralization was done, and it was observed that statistically significant results ($p < 0.05$) were obtained.

The highest depth was observed in group D at demineralization, and at remineralization, the highest depth was observed in group A.

Surface Microhardness Testing

The study groups' surface microhardness descriptive statistics are displayed in Table 5. An intragroup analysis of the pre-, mid-, and posttime intervals was conducted, and the results indicated that all study groups exhibited statistically significant results ($p < 0.0001$, S). Group D showed the highest mean difference in microhardness, or a 113.98 mm increment in results. Thus, we could conclude that group D performed better.

The intergroup analysis of microhardness was done, and it was found that there were no statistically significant results for the pre- and mid-study phases with ($p > 0.05$), but results were statistically significant for the poststudy phase with ($p < 0.05$).

Discussion

Fluoride varnish stands out as a preferred choice over other professionally applied fluoride treatments, such as fluoride gel, due to its ease of application, minimal requirement for application, and quicker setting time.[13] It is advised to apply fluoride varnish twice a year in children at high-risk, and it is the sole professionally recommended topically applied fluoride treatment for children under 6 years of age.[14,15] Their effectiveness might stem from their ability to facilitate prolonged contact between dental tissues and the fluoride agent, leading to increased fluoride uptake.[16]

The oral environment quickly diminishes the time fluoride varnish remains effective due to its susceptibility to removal by actions such as cheek and tongue movement, salivary flow, chewing, and oral hygiene routines. Consequently, varnishes should promptly release their ions before being lost. The addition of assorted novel chemically active compounds to varnishes has been proposed to enhance the efficacy of varnish-like remineralization potential, calcium, phosphate, and fluoride ion release. Remineralizing agents like CPP-ACP, zwitterionic ion, self-assembling peptide, and BGA have been known to aid the process of remineralization, according to the literature.[17,18]

When fluoride ions are present, unstabilized ACP has the potential to generate fluorapatite. This process of fluorapatite formation in the oral cavity could sequester available fluoride ions, consequently diminishing the capacity to remineralize subsurface enamel during acid challenges.[19] Hence, the steadiness of ACP is an issue. According to Mellanby, milk and its products facilitate the stopping of dental caries in animals, and milk protein casein was found to be an important component for the interference of caries.[20]

Georgiev et al. have researched the zwitterionic ion property. The presence of the LMS decreases the dipole interaction between polymer chains, leading to alterations in the charge interaction of the polymer.[21] This modification facilitates the elongation of the chains, resulting in subsequent swelling of the polymer that was previously compacted into globules. This fosters interaction between the polymer and water, striving for equilibrium and forming a closely bound water layer, which is the distinctive characteristic of ZPs, recognized for their exceptional superhydration capability. This capability is further shaped by the organization of water molecules within the shell, resembling free water, which enhances the affinity to water.[22,23] Consequently, a densely bound, thick, energetic layer forms on the ZP brush, exerting a potent steric effect. This effect aids in resisting protein adsorption, thereby deterring fouling and ultimately contributing to biofilm resistance. This resistance arises from the combination of surface hydration and steric repulsion.[7,24,25]

Kwon et al. explored how the incorporation of ZP (MPC) as a supplement to light-curable fluoride varnish affects its properties. They observed that higher percentages of MPC tended to elevate the film thickness, particularly noticeable beyond 5 wt.%. Subsequent studies by Kwon et al. extended this investigation, incorporating various forms of ZPs, including carboxybetaine methacrylate, MPC, and sulfobetaine methacrylate, into light varnish.[7] They employed a 3% concentration and noted that all groups exhibited resistance to protein adsorption and demonstrated protective effects against demineralization. With the advent of enamel matrix proteins, research has indicated that self-assembling peptides can be utilized in the remineralization of enamel, dentin, and cementum.[26–28]In a review article, Li et al.[26] highlighted the significance of self-assembling peptides in enamel remineralization through a biomimetic approach, aiming to mimic the natural process of enamel mineralization. These peptides facilitate the formation of a three-dimensional matrix, aiding in the remineralization of subsurface lesions. Moreover, their strong attraction to calcium ions in saliva promotes the formation of enamel crystals around the enamel matrix.[29,30]

Bioactive glass (an innovation in remineralization technology) in an aqueous environment undergoes a solution-mediated dissolution, which results in a change in composition, which in turn changes the pH and releases bioavailable sodium, calcium, and phosphate ions, contributing to the remineralization process.[31–34] BAG interacts with saliva, triggering the release of calcium ion,

phosphate, and silicon(4+) on the glass surface. The resulting precipitation of a polycondensated silica-rich layer acts as a scaffold for calcium phosphate formation. Several investigations have explored the enamel remineralization potential of BAG. Notably, the 45S5 BAG paste was found to enhance the microhardness of subsurface eroded enamel. Moreover, the application of BAG paste on incipient enamel erosive lesions was observed to repair the lesions by forming a durable layer of HAP resistant to abrasion.[27]

Conclusion

Zwitterionic material, when incorporated with MI Varnish, showed high fluoride and calcium ion release and remineralizing efficacy, followed by BGA and self-assembling peptide. BGA, when incorporated with MI Varnish, showed high phosphorus ion release and higher values in the evaluation of surface microhardness, followed by zwitterionic material and self-assembling peptide.

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