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Comparative evaluation of remineralizing efficacy of silver containing fluoride, nano hydroxyapatite and self assembling peptide on early enamel carious lesions under atomic force microscope: An in vitro study

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Abstract---Aim: To evaluate and compare the efficacy of 3 different materials on remineralization of early enamel carious lesions. Materials and Methods: 80 maxillary single rooted premolars were divided into four groups (n=20). Group 1: Control, Group 2: Silver diamine fluoride, Group 3: Nano-hydroxyapatite and Group 4: Self assembling peptide. Each group except control group were subdivided into two subgroups. All the materials were demineralized in demineralizing solution for 96 hours, which was daily renewed after 24 hours until uniform early carious lesion were formed. After the formation of lesions, half of specimens from each tested subgroups had the material applied and the half of the specimens didn't. All the specimens were immersed together in 20 mL of artificial saliva at 37°C for 24 h. After 24 hours experimental materials were rubbed off using cotton pellet soaked in acetone. All the specimens were then washed with distilled water and again immersed in fresh artificial saliva for 48 hours at 37°C in an incubator. Saliva was exchanged after 24 hours with fresh saliva. The pair of negative control group was only immersed in artificial saliva for 24 and 48 hours continuously and saliva was not exchanged during this period. Specimens were then stored in distilled water and stored in frozen form until further tests were carried out. Statistical Analysis: One way ANNOVA test was carried out for the statistical analysis. Results: Self assembling peptide group showed highest surface microhardness values as well as highest depth of remineralization (absolute depth profile) followed by Nano-hydroxyapatite and Silver containing fluoride. Conclusion: Self assembling peptide can be used to reverse early enamel caries lesions clinically with promising results.

Keyword---early enamel caries, remineralization, self assembling peptide, depth of remineralization, atomic force microscope.

Introduction

Caries prevention is the greatest challenge dentists are facing worldwide. Dental caries is one of the most common diseases affecting the world population. Goal of modern dentistry is to manage and prevent non-cavitated lesions noninvasively to prevent further disease progression and to preserve healthy tooth structure. Considering its globally high prevalence, dental caries can be called as "pandemic". (1). Dental caries results in the dissolution of hydroxyapatite crystals and the loss of calcium, phosphate, and other ions, which leads to demineralization of the tooth substrate and finally results in cavitated lesions. Dental caries is a disease with multi factorial etiology which is the result of

countless demineralization and remineralization events which are unidirectionally unbalanced in favor of mineral loss. (2,3)

The progression of the early enamel lesion is a slow process and it is reversible via the process of remineralization. Demineralisation occurs when a low pH persists for a longer period of time in oral cavity which releases large amounts of calcium ions from the enamel of the tooth (mainly through hydroxyapatite; HAP) (4). White spot lesions (WSLs) are defined as enamel surface and subsurface demineralization, but initially does not result in cavitation. White spot lesions are areas of demineralized enamel that develops because of prolonged plaque accumulation at the area in oral cavity. When these conditions are maintained, acids diffuse into the enamel and begin to demineralize the subsurface enamel. (5)

Initially enamel crystal dissolution begins with subsurface demineralization, which leads to the formation of pores between the enamel rods. Thus, the refractive index is altered in the affected area that results in both surface roughness, loss of surface shine and alterations in internal reflection, that finally results in greater visual enamel opacity which may be called as demineralization. (6) Remineralization can be defined as natural repair process for non cavitated lesions which depends on calcium and phosphate ions assisted by fluoride to rebuild a new surface on existing crystal remnants in subsurface lesions remaining after demineralization. Generally, fluoride is used commonly as remineralizing agent. But due to certain limitations of fluoride it urged manufactures to introduce newer systems.

Many remineralizing agents have been used in dentistry since many years. Most commonly used agents include various fluorides, casein phosphopeptides–amorphous calcium phosphate (CPP-ACP), Amorphous calcium phosphate, Sodium calcium phosphor silicate (bioactive glass), xylitol etc. Recently new bioactive materials were introduced for enamel remineralization apart from the conventional fluoride which have given promising results. For our study we have selected materials like Silver diamine fluoride, Nano hydroxyapatite and Self assembling peptide. Atomic Force Microscope (AFM) was used to check the absolute depth profile or depth of penetration in this study. AFM provides a number of advantages over conventional microscopy techniques which are available. AFM can make measurements in three dimensions, x, y, and z (normal to the sample surface), that enables the presentation of three-dimensional images of a sample surface. This provides a great advantage over any microscope available. AFMs do not require vacuum environment or any special sample preparation, and they can be used in either an ambient or liquid environment.

Microhardness tests are commonly used to study the physical properties of materials in various fields, and they are widely used to measure the hardness of teeth in the field of dentistry. This method is relatively easy, quick, and requires only a tiny area of specimen surface for testing. Hence we have used Vickers microhardness tester in this study. Aim of this study was to evaluate and compare the efficacy of 3 different materials on remineralization of early enamel carious lesions using Vickers microhardness tester and under Atomic Force Microscope.

Materials and Methods

After institutional ethical committee approval, the study was conducted in-vitro. The teeth samples included in the study were therapeutically extracted premolars. Inclusion criteria were non-carious, non-hypoplastic, no cracks, and non-restored teeth extracted for orthodontic therapies. While the exclusion criteria was carious, non-therapeutically extracted teeth. The study included eighty extracted single rooted human maxillary premolars.

Sample Preparation

The primary steps included cleaning of the extracted premolars by removing all the blood and tissues attached with the help of an ultrasonic scaler. The teeth were then placed in 0.1% thymol solution, used as an antifungal agent. This was followed by polishing of the teeth with the help of a pumice slurry and polishing cup, cleaning them with distilled water and drying the teeth samples with oil-free air by a three-way syringe. Decoronation of the teeth at CEJ was done with a tooth cutting disc mounted on a slow-speed handpiece. Only the crowns were used for this study to create the artificial lesions. All the specimen were divided into four groups i.e. one negative control group and three treatment group. Each group contained 20 specimens. Three treatment groups were divided again into two subgroups containing 10 specimens each.

- **Group.1 - Control group-Without application of tested materials(n=20)**
- **Group. 2 - Silver diamine fluoride (Fagamin, Argentina)**
 - Subgroup A-With material applied(n=10)
 - Subgroup B- Without application of materials(n=10)
- **Group. 3 - Nano hydroxyapatite (Ultrananotech, 30-50nm particle size)**
 - Subgroup A-With material applied(n=10)
 - Subgroup B- Without application of materials(n=10)
- **Group 4 - Self assembling peptide P11 4 (Curodont Repair, Switzerland)**
 - Subgroup A-With material applied(n=10)
 - Subgroup B- Without application of materials(n=10)

The buccal surface of each specimen was coated with an acid-resistant nail varnish leaving window of 3 mm × 3 mm each to expose the enamel for demineralization and to limit the area of study. All specimens were demineralized in demineralizing solution (pH 4.4 with 1M potassium hydroxide) for 96 hours, which was daily renewed after 24 hours until uniform white spot lesions were created. Demineralizing zones were formed on the specimens in the form of white spots due to surface demineralization of hydroxyapatite crystals which gave them white frosty appearance. In each treatment groups materials were applied to half of the specimens and half were not. After this specimens were paired together so that each pair contains one specimen on which material was applied and one on which material was not applied. Specimens were mounted on self cure acrylic resin moulds with standard dimensions of 4mm height and 10 mm width .

Pairs were then immersed in container of 2ml of artificial saliva (pH 7) at 37°C for 24 hours for remineralization. Artificial saliva was prepared according to the

formulation of ten Cate and Duijsters and contained 1.5 mM CaCl_2 , 0.9 mM NaH_2PO_4 and 0.15 M potassium chloride at pH 7.0. After 24 hours materials were rubbed off using cotton peltate soaked in acetone. All the specimens were then washed with distilled water and again immersed in fresh artificial saliva for 48 hours at 37°C in an incubator. Saliva was exchanged after 24 hours with fresh saliva. The pairs of negative control group were immersed in artificial saliva for 24 and 48 hours continuously and saliva was not exchanged during this period. Specimens were then stored in distilled water and stored in frozen form until further tests were carried out. All the samples were then subjected to measure the depth of remineralization under atomic force microscope and surface microhardness under Vickers microhardness tester. The data was then subjected for the statistical analysis.

Statistical Analysis

Statistical analysis was carried out using One-way ANNOVA test for intra and intergroup comparison.

Results

Assesment of depth of remineralization / Absolute depth profile (ADP)

Atomic force microscopic analysis was performed after remineralization of samples to check the depth of remineralization or Absolute depth profile. The evaluations of absolute depth profile in enamel were carried out at a scanning rate of 49.5 lms by using an AFM in the contact mode with a silicon nitride (SiN), probe which is having a tip height of 14 μm and a tip radius of <10 nm. The conditions used for the short cantilever contact mode were as follows: Source Z height of 119, Data width and height 256pxl, X scan size 30 μm , Y scan size 30 μm , Scan rate 0.5 Hz, Z servo gain 1, Set point 863 nm, Amplitude 19.973 Hz, Sel. frequency 256.283 Hz, Drive 0.44 percent. The Smart Scan application and XEI software (Version 1.6) was used for image analysis and to measure the AFM parameters. A calibration grid of silicon oxide on silicon material (Park Systems, Korea) with an XY periodicity of 10 μm and a Z height of 118 nm was used for calibration prior to each evaluation session.

In absolute depth profile Self assembling peptide (SAP) showed highest depth profile than any other group tested. That means SAP can remineralize the white spot lesions more deeply than other materials tested. The mean absolute depth profile value for SAP for the groups on which material is applied (A) is 1025.2 nm (Fig.6), followed by nano hydroxyapatite (755.7 nm.) (Fig 4), SDF (703.6 nm.) (Fig.2), respectively. (Table 1., Graph 1.) The mean absolute depth profile value for SAP for the groups on which material is not applied (B) is 678.8 nm. (Fig.7) followed by nano hydroxyapatite (555.6 nm) (Fig.5) and SDF (496.6 nm) (Fig.3) respectively. (Table 1., Graph 1.) Control group showed mean absolute depth value of 640.3 nm. (Fig.1) Control group showed more depth values than nanohydroxyapatite and silver diamine fluoride from subgroups on which material was not applied. This difference in depth profile may be due to less penetration of the particles because of their particle sizes. Nanohydroxyapatite used in this study has particle size of around 30-50nm. Due to larger particle size

they may not have penetrated the surface more deeply as compared to control group.

Assesment of surface microhardness (SMH)

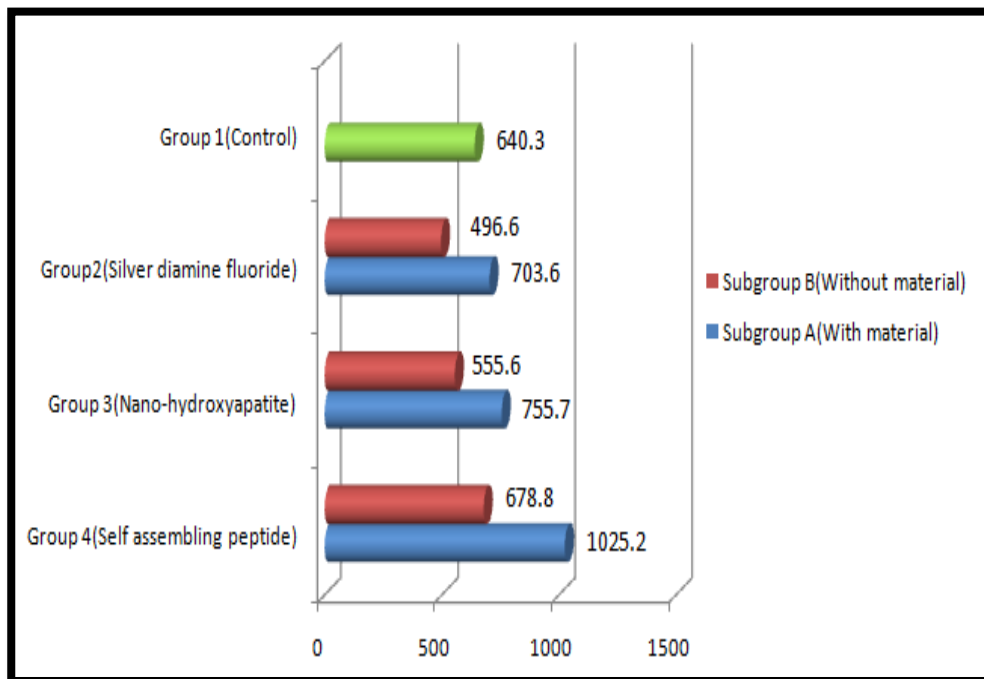
SMH was measured at baseline of sound enamel, after demineralization and after remineralization. Vicker's microhardness testing machine was used to test these samples with a load of 300g and a dwell time of 15s, oriented perpendicularly to the enamel surface. All readings were performed by the same examiner using the same calibrated machine. In each reading, three indentations were made and their average was taken to represent the specimen's hardness value. The VHN values were taken before demineralization as well as after demineralization and post remineralization to check for the microhardness values. The maximum microhardness values recovered by Self assembling peptide group, both in Subgroup A and in Subgroup B (mean 264.82 and 233.22 respectively), indicating best remineralization potential followed by Nanohydroxyapatite, and silver diamine fluoride as shown in Table 2.

For Absolute depth profile

Table 1

Intergroup comparison in relation to Absolute Depth Profile measurements under Atomic Force Measurements to check depth of remineralizing efficacy of silver diamine fluoride, nano hydroxyapatite, self-assembling peptide between with material (Subgroup A) and without material (Subgroup B)

Groups	Mean (SD)		Unpaired test	P value significance
Group 1(Control group)	640.3(13.44)		t=0.590	P=0.562
	Subgroup A(With material)	Subgroup B(Without material)		
Group 2(Silver diamine fluoride)	703.6(12.06)	496.6(32.6)	t=18.828	p < 0.001*
Group 3(Nano-hydroxyapatite)	755.7(16.49)	555.6(22.23)	t=22.856	p < 0.001*
Group 4(Self assembling peptide)	1025.2(42.73)	678.8(23.62)	t=22.433	p < 0.001*



Graph 1. Intergroup comparison in relation to Absolute Depth Profile measurements under Atomic Force Microscope

For Vickers Microhardness testing

Table 2
Comparison of microhardness among the groups at pre treatment, after demineralization and post remineralization

	Pre treatment Mean(SD)	After demineralization Mean(SD)	Postremineral ization Mean(SD)
Group1 (Control)	231.76(5.98)	173.69(5.33)	233.22(3.68)
Group2 (Silver Fluoride) Diamine	233.68(3.72)	173.54(5.49)	Subgroup-A- 199.25(5.63)
			Subgroup B- 187.56(2.99)
Group3 (Nano	234.18(4.02)	173.61(3.48)	Subgroup-A- 204.14(3.35)

hydroxyapatite)			
			Subgroup B- 192.10(2.99)
Group4 (Self Assembling Peptide)	230.96(4.19)	173.38(5.13)	Subgroup-A- 264.82(3.62)
			Subgroup B- 233.22(3.68)
OnewayAnovaF test value	F= 0.896	F= 0.006	F= 420.707
p value (overall)	p=0.455(NS)	p =0.999	p<0.001**

Discussion

Advances in the clinical management of caries disease involves replacing traditional surgical procedures with the non-invasive treatment of early carious lesions by remineralization using various remineralizing agents.. In recent years, new preparations have been developed, all of which aim to remineralize or infiltrate initial lesions and thus, counter their exacerbation and prevent further lesion progression. [7,8]. Smart nanobiomaterials aiming to induce biomimetic regeneration is now the emerging approach. Among which Self assembling peptide and nanohydroxyapatite was used in our study. These relatively new materials has potential to induce remineralization for early enamel carious lesions. From fluoride group,38% silver diamine fluoride was used as tested material.

Various methods are used to determine the remineralizing effects of the materials, of which absolute depth profile and surface microhardness are most commonly used methods. Absolute depth profile is the parameter which is used to check the depth of remineralization of the white spot lesions. Surface microhardness analysis (SMH) is a simple, fast and reliable method to assess demineralization and remineralization changes occurring in enamel. SMH testing measures the material's resistance against plastic deformation from a standard source, allowing repeated measurements of the same specimen over a period of time, reducing the experimental variation and reinforcing that SMH evaluations are a feasible choice to estimate mineral changes.The findings of our study indicated that all three treatment regimens significantly promoted the remineralization of enamel lesions and increased enamel microhardness compared to artificial saliva. The highest mean SMH was found in Self assembling peptide(Curodont repair, Switzerland) followed by Nanohydroxyapatie(Ultrananotech) and SDF(Fagmine,Argentina) while the lowest mean SMH was found in artificial saliva.

The artificial saliva used in many studies to reproduce real oral conditions is rich in minerals, which results in it being effective for remineralization. The artificial saliva used for this in vitro study contains1.5mM CaCl(calcium chloride), 0.9 mM

NaH_2PO_4 (sodium dihydrogen orthophosphate dehydrate), 0.15M potassium chloride and was adjusted at pH 7.0. Contents of this saliva is almost close to the saliva in oral cavity which helped in process of remineralization of the lesions. Self assembling peptides showed the highest remineralizing efficacy which is attributed to the fact that P11-4 is a rationally designed self assembling peptide that undergoes, self assembly forming three dimensional fibrillar scaffolds. Once assembled, these scaffolds serve as ideal templates for hydroxyapatite nucleation which promote guided enamel regeneration.[9-11] They are similar to biological macromolecules found in extracellular matrices, controls the deposition, morphology and growth of hydroxyapatite crystals.[9]

At neutral pH, SAP P114 exists in a low viscosity, liquid monomeric form. When the pH is decreased ($\text{pH} < 7.4$), SAP P114 changes into bioactive gel scaffolds that binds to tooth surface inducing de novo hydroxyapatite crystallization. Triggering the process of self assembly and formation of gel after its application is attributed to the low pH, the presence of cations and the high ionic strength.[6] P11-4 spontaneously selfassembles to an elastomeric nematic 3D gels that show high affinity to tooth mineral, based on matching distances of Cabinding sites on P114 and Ca spacing in the crystal lattice of hydroxyapatite.[9,,12,19] This was confirmed by a study conducted by Kirkham *et al.*[11] where the incubation of P114 in mineralizing solutions for 7 days resulted in the presence of needlelike electrondense deposits within the scaffold itself, suggested to be crystalline hydroxyapatite.

The results obtained in our study are in agreement with studies done by Kirkham *et al.*, Brunton *et al.*, Schmidlin *et al.*, Takahashi *et al.*, Bröseler *et al.*, Schlee *et al.* and Ceciet *et al.* where they also found P114 is able to induce better biomimetic remineralization of early caries lesions as compared to other experimental materials.[9,10,11-15]. Its enamel re-hardening and high SMH values are suggested to be due to its potential for subsurface remineralization which was concluded in studies investigating its remineralizing potential in deep lesions. This is attributed to the fact that the peptide is designed to be in monomeric liquid form when originally applied to the lesion's surface. This allows for diffusion because of its very low viscosity that ensures deep penetration into the subsurface lesion body micropores followed by a rapidly driven selfassembly which promotes indepth biomimetic remineralization.[11,14]

Nano-hydroxyapatite's remineralizing potential showed a significant increase in the mean absolute depth profile and SMH compared to SDF and artificial saliva. Nano-HA precipitation and deposition of Ca^{2+} and PO_4 accelerate the remineralization effect due to surface morphological structure. Wu *et al* [16], also, demonstrated that surface hardness of the demineralized enamel increased with nano- HA. Furthermore, the higher microhardness value of nHA group can be explained by the presence of Ca/PO_4 that induce precipitation of apatite by the formation of sub-surface high mineral content. [17] Another explanation might be attributed to porous, partially demineralized and wide surface area [18]. The results of our study are in agreement with Surmeneva *et al* [19], where they found that nano-HA promote preferential remineralization of the outer layer of enamel caries lesion and full remineralization does not occur. [20].

SDF, which was used in this study showed a significant increase in mean absolute depth profile and SMH compared to artificial saliva. The results for absolute depth profile in the SDF group might be attributed to the flowability of the SDF solution. The liquidity of SDF allowed for the full contact of SDF and the enamel surface in a relatively short time. SDF at 38% concentration contains 44,800 ppm fluoride. The fluoride concentration is the highest among all of the commercially available fluoride agents in dentistry. Fluoride promotes the remineralization of hydroxyapatite in enamel and dentine. One proposed chemical reaction between SDF and hydroxyapatite of teeth involves the formation of silver phosphate and calcium fluoride. The subsequent dissolution of fluoride and calcium facilitates the formation of insoluble fluorapatite, which is a possible reaction product of fluoride ions with hydroxyapatite. It is difficult to confirm the formation of fluorapatite, primarily because its crystal structure is similar to that of hydroxyapatite.[21]

However, the mechanism of SDF on dental caries was not fully understood. The arresting action of SDF on enamel caries could be explained by its high concentration of fluoride, its alkaline property and the presence of silver [22]. Fluoride promotes the remineralisation of hydroxyapatite. The alkaline property is favorable to the remineralisation process and inactive dentine collagenases [20]. A recent published review hypothesized that silver in SDF may diffuse into the hydroxyapatite crystal and produce silver-containing hydroxyapatite [23]. This silver-containing hydroxyapatite has been shown to reduce bacterial adhesion and to minimize tissue cytotoxicity. The remineralization effect was also observed in the groups where materials were not applied i.e. indirect groups. The mean absolute depth profile value for SAP for the groups on which material is not applied i.e. subgroup B is 678.8 nm.(Fig.7) followed by nano hydroxyapatite (555.6 nm)(Fig.5)and SDF (496.6 nm) (Fig.3)respectively.(Table 1.,Graph 1.)

For microhardness testing SAP has shown the highest microhardness values i.e 233.22Mpa followed by nanohydroxyapatite (192.10 Mpa) and SDF (187.56 Mpa) respectively.(Table 2.). The remineralization effect may be due to the presence of calcium and phosphorus in the artificial saliva used in our study followed by minerals liberated from directly applied groups. This suggests that some amount remineralization may takes place from the ions and minerals liberated from the applied materials into saliva. Hence synergistic effect of both the ions and minerals liberated from materials and saliva may remineralize the early enamel carious lesions in vivo. This study found that absolute depth profile and hardness increased more in the SAP P11-4 direct and in indirect groups followed by nano hydroxyapatite and SDF groups. To summarize, as found in previous studies, the results of present study has also demonstrated that the chosen experimental agents can induce remineralization of early enamel lesions, thus avoiding the need for an invasive treatment at a later time.

Conclusion

With some limitations like exclusion of pH cycling model in methodology, the results of this in vitro study suggest that treatment with P11-4 can lead to remineralization of early enamel carious lesions more effectively than nanohydroxyapatite and Silver diamine fluoride. SAP11-4 showed superiority due

to its guided enamel regeneration potential that will undoubtedly open the doors to a new concept in enamel healing.

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