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Evaluating The Potential Of Newer Biomimetic Remineralizing Agents To Achieve Oriented And Ordered Remineralization Of Enamel – A Scanning Electron Microscopic Study

Jagrit Thaploo[1], Manisha Agarwal[2], Sapna Konde[3], Preetha Peethambar[4], Sahana N Prasad[5], Swathi Kakathkar[6]

Department of Pediatric and Preventive Dentistry, AECS Maaruti College of Dental Sciences & Research Centre, Bengaluru, Karnataka

Abstract

Background: *Smart Nano-biomaterials have been introduced aiming to remineralize lost dental tissues with biologically similar ones adopting the concept of minimally invasive dentistry. Novel Self-Assembling Peptide and Synthetic Nanohydroxyapatite are introduced as a biomimetic approach in guided enamel regeneration.*

Aim: *The aim of this study is to assess the remineralizing potential and orientation of enamel prisms formed in primary teeth following the use of Self-Assembling Peptide and Synthetic Nanohydroxyapatite in vitro and their effect in combination with CPP-ACPF and SDF using SEM.*

Methodology: *A total of 105 extracted primary teeth were collected and randomly assigned to seven groups(N=15): G1-Control group (Artificial saliva); Study groups: G2-(Self-Assembling peptide), G3-(Self-Assembling Peptide+CPP-ACPF Varnish), G4-(Self-Assembling Peptide+SDF),G5-(SyntheticNanohydroxyapatite), G6-(Synthetic Nanohydroxyapatite+CPP-ACPF Varnish), G7-(Synthetic Nanohydroxyapatite+SDF). Artificial enamel lesions were created on the buccal surface of teeth in study groups. All products were applied according to manufacturer's instructions and specimens were stored artificial saliva. Samples were subjected to SEM to assess the orientation of enamel prism and remineralizing potential of the agents used at 7days, 1month, 3months, 6 months.*

Results: *G3-(Self-Assembling Peptide+CPP-ACPF Varnish) showed a significantly better mean Ca/P ratio and well-defined orientation compared to other study groups over a period of 6-months.*

Conclusion: *Added benefits can be obtained through combining self-Assembling Peptide with other remineralizing agents allowing faster and enhanced regeneration of non-cavitated caries.*

Keywords: *Enamel regeneration, Biomimetic remineralization, Self-Assembling Peptide, Synthetic Nanohydroxyapatite, CPP-ACPF, SDF*

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I. Introduction

Dental caries is a dynamic disease process caused by organic acids produced by bacteria on enamel surface leading to the loss of mineral ions from the hydroxyapatite (HAP) lattice, this process is called demineralization. It has been established that the process of dental caries occurs by the imbalance between the demineralization and remineralization processes. Thus, if this balance is not restored by early intervention measures, caries are likely to gradually develop from enamel to dentin, leading to tooth cavities and eventually the loss of teeth structure[1].

The improved understanding of the dental caries process has led to the development of approaches for detecting early non-cavitated caries lesions and the use of non-invasive methods for treating such lesions. Therefore, the focus of restorative dentistry has been directed towards minimally invasive dentistry, which approaches treating dental caries at microscopic level using remineralizing agents[2].

The remineralization process focuses on delivering ions directly to where and when they are most needed. There are several mechanisms of remineralization. Biomimetic science is a new concept that helps focus dental research on simulating natural tooth enamel. This system is known as Biomimetic Regenerative System which primarily focus on two strategies: Tissue engineering and Nanotechnology.

Under tissues engineering, several investigations have utilized the proteins that mimic the naturally occurring ones to imitate natural protein-mediated biomineralization because therapeutic use of extracellular matrix proteins in their extracted purified form for the remineralization of demineralized hard tooth tissues is technically not possible at the moment[3]. An exciting development in this field is a monomeric peptide consisting

of 11 amino acids called P11-4 which is commercially available as Curodont™ Repair (Self-Assembling Peptide) Varnish. This rationally designed Peptide Self-Assembles into hierarchical 3-dimensional fibrillar scaffolds in response to local conditions such as high ionic strength and acidic pH found in the lesion body (Kirkham et al., 2007)[4]. The P11-4 fibrillar matrix has a high affinity for Ca^{2+} ions and acts as a nucleator for de novo HA formation resulting in remineralization of the lesion body (Kind et al., 2017; Kirkham et. al., 2007) [4,5].

Whereas Nanotechnology has minimized hydroxyapatite to Nano-HA, with particles reduced to the size of 20-50 nanometers (nm). It has increased the degree of remineralization, especially in an acidic environment, by increasing the supply of calcium and phosphorus ions to the demineralized zone (Huang, S et.al.) [6]. Nano-hydroxyapatite has been used in toothpastes since 1980, in Japan and was clinically approved as an anti-caries agent in 1993 as Apagard[7].

As these Biomimetic regenerative systems are dependent on adequate salivary or plaque calcium and phosphate ions[8]. The bioavailability of Ca2+ and PO4 3– ions is often the limiting factor for net remineralization, and this is especially exacerbated under hyposalivation conditions (Reynolds et al., 2008; Vogel et al., 2008)[9,10]. The presence of extrinsic sources of Ca^{2+} and PO_4^{3-} ions can increase diffusion gradients. Therefore, there have been numerous attempts to combine various remineralizing agents for a complementary mode of action in order to get additional advantages of accelerating or enhancing the remineralization capacity of the agents together rather than when used alone. Hence an in vitro Scanning Electron Microscope Study was conducted with a purpose to assess the remineralizing potential and orientation of enamel prisms formed in primary teeth after using Self-Assembly Peptide and Synthetic Nanohydroxyapatite in vitro and also their combination effect with CPP-ACPF and SDF under Scanning Electron Microscope.

II. Materials And Method

A total of 105 samples (extracted or exfoliated deciduous teeth) with no evidence of caries on visual inspection which were collected from the children who underwent extraction procedures. The inclusion criteria considered in our study were:

a) The extracted or exfoliated deciduous teeth were selected.

b) Healthy teeth that are free from dental caries, previous restorations, developmental defects or white spot lesions.

Sample Preparation:

Samples were thoroughly cleaned with the ultrasonic scaler followed by polishing with pumice slurry using a rubber cup. The teeth were sectioned horizontally using diamond disc at CEJ. Tooth blocks were then obtained with dimension: 4mm x 4mm x 2mm from each tooth.

Demineralization:

Before demineralization 15 samples were randomly selected for Control group.

The remaining 90 samples were then kept in 15ml of freshly prepared demineralizing solution (pH-4.5) containing the following components: $CaCl_2$: 2.2 mMol/L, NaH PO : 2.2 mMol/L, Acetic acid: 0.05 M, NaOH: 50%.

Demineralization was done for 2 days at 37^0c to create an artificial lesion. Samples with no lesion formation were re-immersed in the demineralizing solution for another day.

Post Demineralization Evaluation was done by visual inspection using International Caries Detection and Assessment System II[11].

Finally, the samples were thoroughly rinsed with deionized water and kept for washout period of 24 hours.

Sample Grouping:

Group 1: Control Group

Post demineralization, 90 samples were further divided randomly into 6 study groups (n=15 each).

Group 2: Curodont Repair, **Group 3**: Curodont Repair + CPP-ACP varnish, **Group 4**: Curodont Repair + SDF, **Group 5**: Apagard Apa-kids, **Group 6**: Apagard Apa-kids + CPP-ACP varnish, **Group 7**: Apagard Apa-kids + SDF.

Application of Remineralizing agents:

Further, Application of Remineralizing agents was done as per the manufacturer's guidelines, where all the agents were applied only once except the Synthetic nanohydroxyapatite which was applied once daily for 6 months.

pH Cycling

After food is comsumed, oral bacteria decompose sugar and produce acid, causing pH decline. To imitate the oral environment in vitro, besides the remineralizing process, all the specimens were immersed in the demineralization solution for 3 hours and back in artificial saliva for the rest of 21 hours, every day. The

3- hour demineralization time consists of total duration of pH fall during and after 3 meals.

Scanning Electronic Microscope Examination

Enamel specimens in each group were dehydrated in a series of ethanol solutions, critical point dried in a desiccator and finally sputter coated with gold.

An elemental assessment was carried out on the surfaces of each specimens in each group. The levels of calcium(Ca) and Phosphorus(P) ions were evaluated by energy-dispersive X-ray spectroscopy (EDX) under SEM. The mean weight percentage ratio of Ca and P was calculated.

The prism morphologies of the specimens were then observed under Scanning Electron Microscopy (SEM) (JEOL SEM IT 300) at 5 kV in high-vacuum mode. And later divided in 3 categories: Well-defined prism, Shallow prism, Microporosity.

Statistical Analysis:

Statistical Package for Social Sciences [SPSS] for Windows Version 22.0 Released 2013. Armonk, NY: IBM Corp., will be used to perform statistical analyses. Descriptive analysis of all the explanatory and outcome parameters were done using frequency and proportions for categorical variables, whereas in Mean & SD for continuous variables.

One-way ANOVA test followed by Tukey's post hoc test was used to compare the mean Ca/P ratio values between 7 groups at different time intervals.

Repeated Measures of ANOVA test followed by Bonferroni's post hoc test was used to compare the mean Ca/P ratio values between different time intervals in each group.

Chi Square Test was used to compare the Orientation of Enamel Prisms between 7 groups at different time intervals.

The level of significance was set at $P<0.001$.

III. Results

The present study was carried out to assess the remineralizing potential and orientation of enamel prisms formed in primary teeth after using Self-Assembly Peptide and Synthetic Nanohydroxyapatite in vitro and also their combination effect with CPP-ACPF and SDF and an intergroup comparison was done at the end of 7 Days, 1Month, 3 Months and 6 Months.

Figure 1(a&b) showed the remineralizing potential of all the study groups at different time points, i.e., 7days, 1month, 3 months and 6 months. Curodont repair+ CPP-ACP varnish (Group 3) showed the maximum amount of remineralization at day 7 (2.2309/ 71.60%) itself, which gradually continued up till 6 months (3.0921/99.25%) where complete remineralization of the tooth structure was seen. This was followed by Curodont Repair (Group 2) which attained 93.06% of remineralization at the end of 6 Months and the difference was statistically significant. The lowest remineralizing potential was shown by the synthetic nanohydroxyapatite combined with SDF(Group 7), which reached a Ca/P ratio of 69.66% at the end of 6 Months.

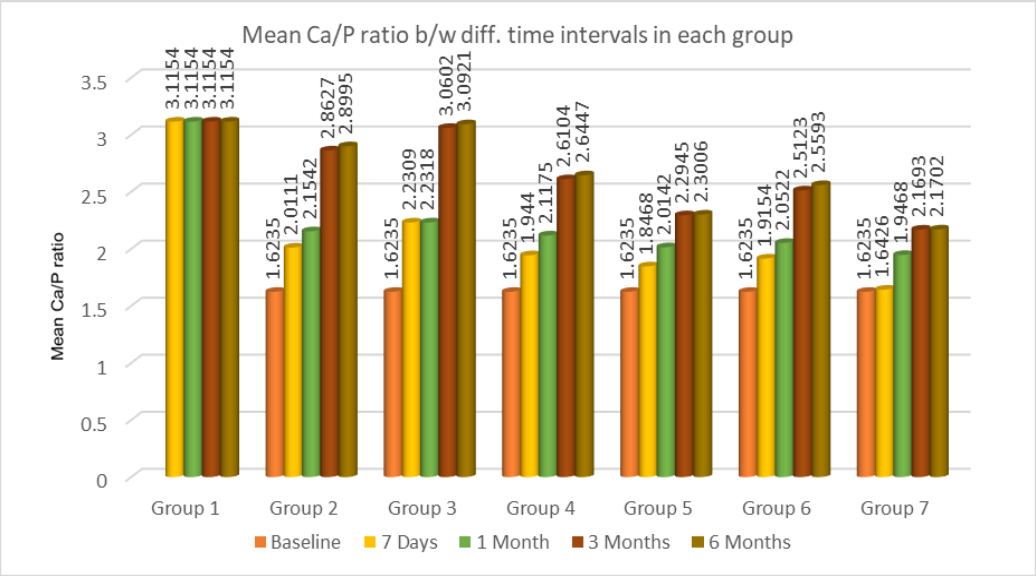


Figure 1(a)

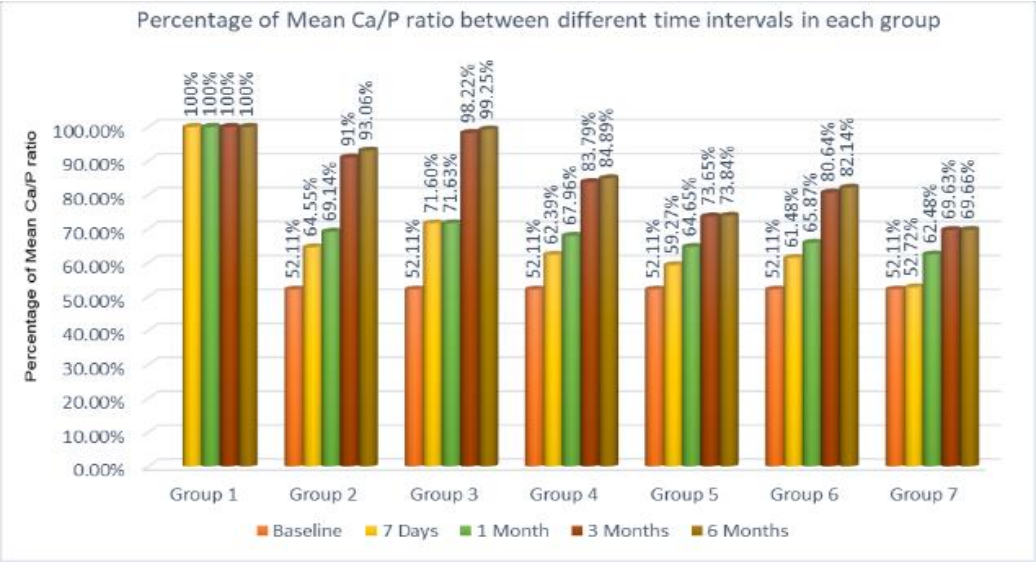


Figure 1(b)

Figure 2(a), 2(b), 2(c), 2(d) Comparison of Orientation of enamel prisms between different groups at different time intervals. All the samples of Group 1 showed well-defined orientation. In Group 3 all the samples at 7days and 1 month time interval showed sallow prism pattern out which 86.7% (n=13) showed well-defined orientation at the end of 3 months. At the end of 6 months all the samples (n=15) showed well-defined orientation. Group 2 showed microporosities in all the samples at 7 days time interval among which 66.7% (n=10) sample showed sallow prism pattern at 1 month interval followed by 100%(n=15) at 3 months interval and was maintained same at 6 months time interval. Whereas, Group 4, Group 5, Group 6 and Group 7 100% sample continued to show microporosities. A significant difference was noted in the orientation of enamel prisms wherein Group 1 and Group 3 attained well-defined orientation with 100% samples showing this orientation. Both groups showed a significant difference in orientation pattern compared with Group 2, Group 4, Group 5, Group 6 and Group 7.

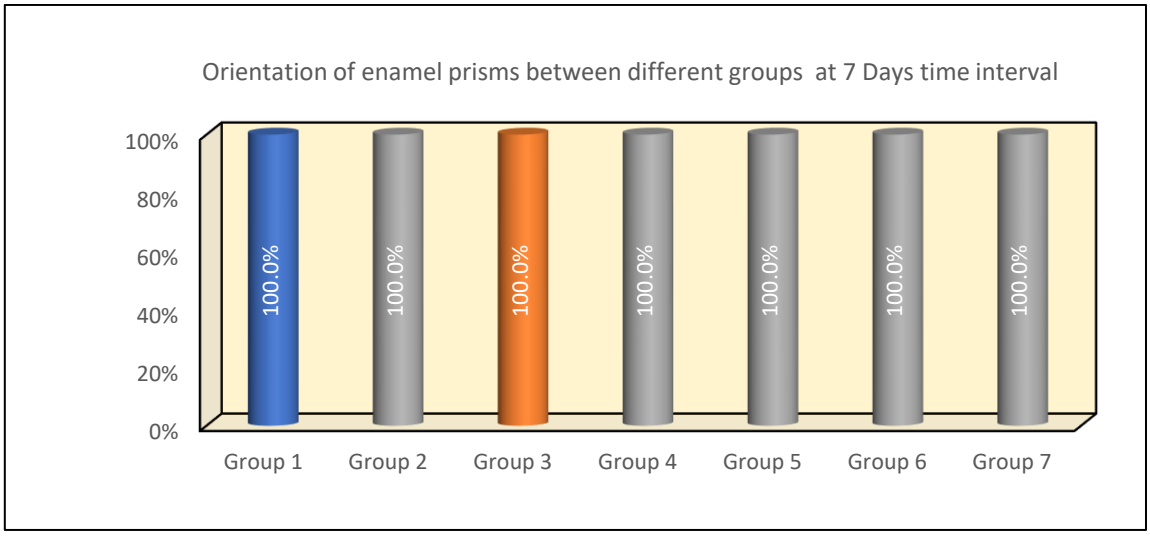


Figure 2(a)

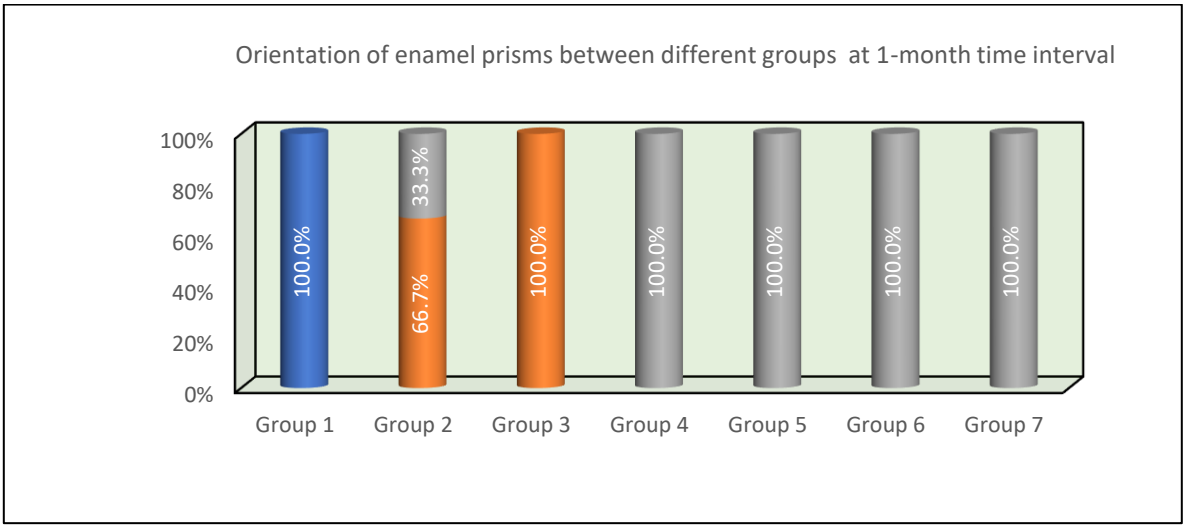


Figure 2(b)

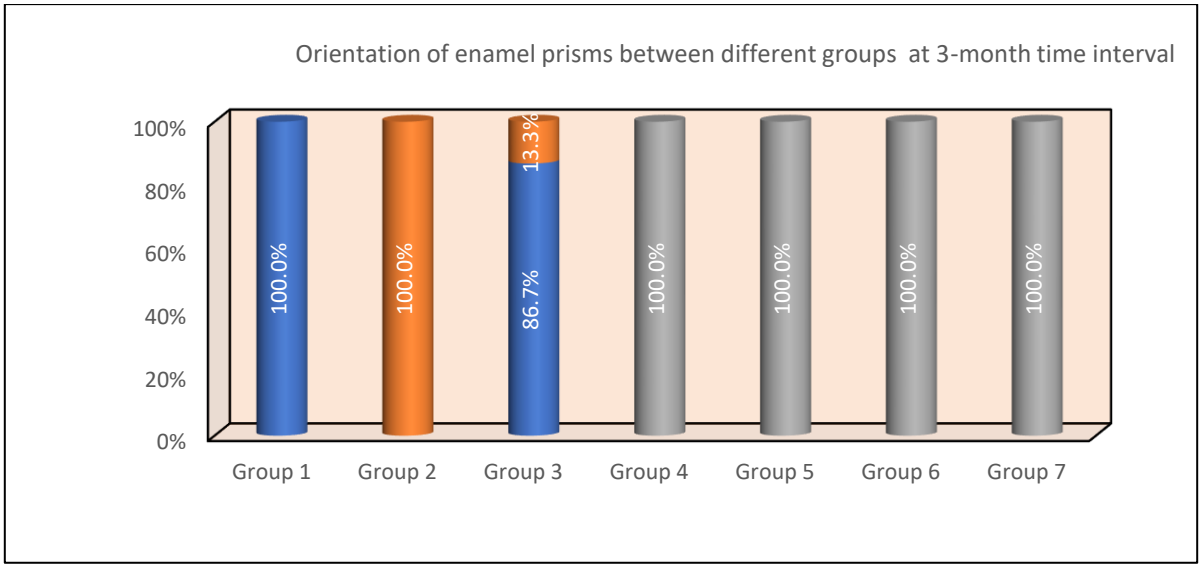


Figure 2(c)

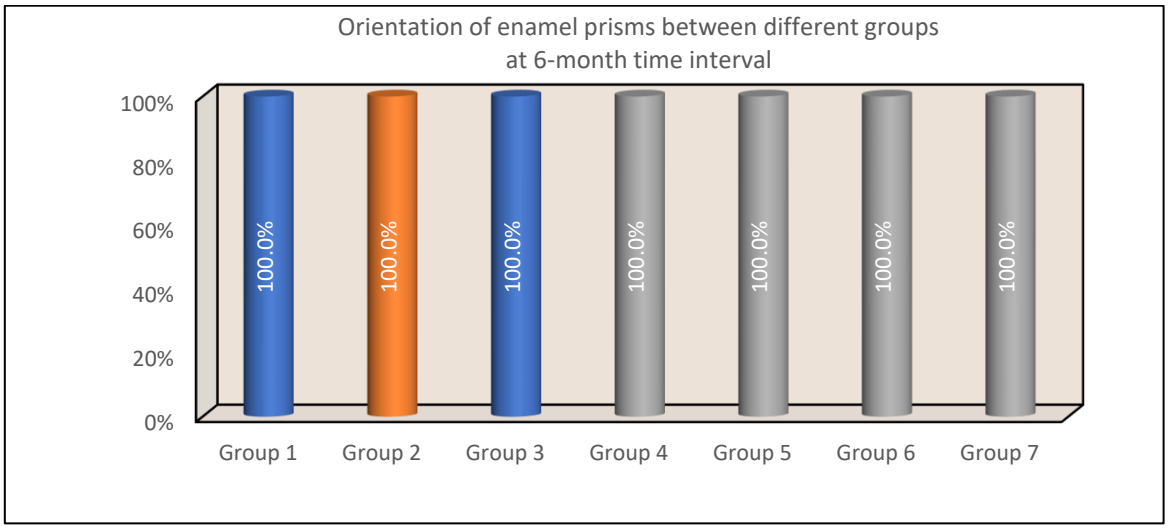


Figure 2(d)

IV. Discussion

An emerging goal of modern dentistry is the non-invasive treatment of early non-cavity white-spotted carious lesions by remineralization to prevent disease progression and improve tooth strength, esthetics and function. to improve [12]. Biomimetic science is a new concept that helps focus dental research on simulating natural tooth enamel. This system is known as a biomimetic regeneration system. and primarily focuses on his two strategies of tissue engineering and nanotechnology.

In our study, biomimetic regenerative agents are Self-Assembly Peptide(Curodont repair) and Synthetic Nanohydroxyapatite (Apagard) were used were individually combined with Casein Phosphopeptide Amorphous Calcium Phosphate Fluoride (CPP-ACPF) and Silver Diamine Fluoride (SDF) with an aim to assess the orientation and order of enamel prisms formed in primary teeth after their application either alone or in combination under Scanning Electron Microscope.

The result of the present study revealed that the mean Ca/P ratio of the demineralized samples were 1.6235 which was in accordance to N. Sabel et.al[13].

In this study Curodont Repair was used as Self-Assembly Peptide, a novel treatment developed by Credentis. It is a liquid that contains a peptide called P 11-4 which diffuses into the subsurface micro-pores and forms a 3D scaffold made up of small fibres. The scaffold mimics proteins found in teeth development and supports hydroxyapatite (a calcium phosphate ceramic which makes up to 50% of bones) crystallisation around it to regenerate tooth enamel[14].

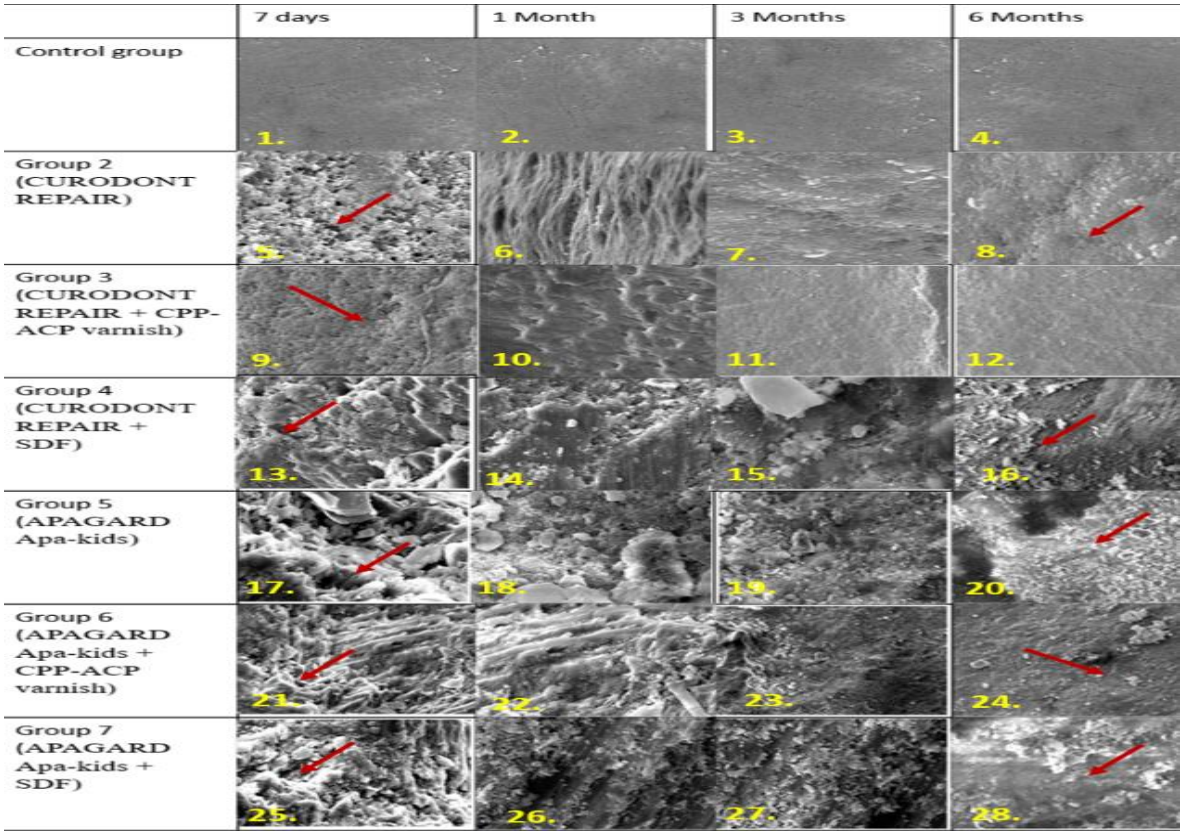
In the present research, (G2) Self-Assembling Peptide (SAP) P_{11}-4 showed a significant increase in Ca: P ratio with evidence of mineral deposition at 7 Days. Similar observations were noticed when the remineralizing efficacy of Self-assembling peptide was evaluated with EDX at 1, 3 and 6 months. Evidence suggested that 93.06% of remineralization was attained over 6 months. The SEM analysis of the surface after remineralization induced by (G2) Self-Assembling Peptide, at 7 days time interval in all the samples(100%), reflected the presence of microporosities (ill-defined irregular, large prisms, having a wide central depression and irregular peripheral borders[15]). This could be attributed to additional acid etching before the application of P_{11}-4 as recommended by the manufacturer which could have caused further loss of minerals from the tooth surface (Vemulapalli Sindhura et.al)[16]. The surface also revealed the newly formed apatite layer which were grown into amorphous clusters were completely covering the prismatic and interprismatic enamel structures. Interestingly, 66.7% samples showed change in prism pattern by the end of 1month from microporosities to shallow prisms pattern (They are smoothly rounded prisms arranged uniformly on the surface with delineated prism outlines, their surface is in the level with the tooth surface). The surface also showed reduction in the amorphous clusters. At 3 months all the samples(100%) showed shallow prisms pattern with uniform mineral deposition that was maintained the same even after 6 months. Our results were in accordance with studies by Vemulapalli Sindhura et.al[16], Kamal D et. al. [17], Nilüfer Üstün and Oya Aktören [18], Kucukyilmaz and Savas[19], Kind et. al.[5], Brunton et.el.[20], Takahashi et.al[21], Schmidlin et.al[22] where they also found P11-4 able to induce biomimetic mineralization of early caries lesions.

Over the years, studies on Nanohydroxyapatite as a biomimetic material for the reconstruction of tooth enamel suffering from mineral loss have been discussed and have received significant attention[23].

The current study showed that daily application of (G5) Synthetic Nanohydroxyapatite (Apagard Apa-kids, Sangi Co., Ltd., Japan) significantly increased the Ca:P ratio with signs of mineral deposition after 7 days. Similar observations were made when the remineralizing effects of Synthetic Nanohydroxyapatite were assessed by EDX at 1 month, 3 months, and 6 months. There is evidence that 73.84% of remineralization was achieved over 6 months. The SEM analysis in this group reflects this observation where HA nanocrystals were found to adhere to the pores created by demineralization. These adherent nanocrystals were found to aggregate and grow into microclusters on the demineralized enamel. The surface also revealed microporosities and the amorphous structures throughout the study period with only reduction in pore volume was observed. Therefore, we could conclude that remineralized pattern did not show any significant improvement or uniformity when compared to Self-assembled peptide group(G2). The remineralization potential of Synthetic Nanohydroxyapatite was found to be significantly less when compared to other Biomimetic regenerative system i.e Self-Assembled Peptide at different time intervals with EDX and SEM evaluation. This could be attributed to the solubility of nano-HA which plays a significant role in remineralization, when the demineralized specimens were subjected to the treatment solutions continuously for several days (Lu K L, Zhang J X) [24]. Similarly in this study nanohydroxyapatite paste was used for 6 months and due to low solubility of pure hydroxyapatite, not enough Ca^{2+} and PO_4^{3-} were available to increase the stability of hydroxyapatite in enamel and promote remineralization. This might have been the reason for low remineralization efficacy of nanohydroxyapatite when compared to Self-assembled peptide.

In the present research, both Biomimetic Regenerative System i.e Self-Assembled Peptide and Synthetic Nanohydroxyapatite when combined with CPP-ACP varnish showed a significant increased remineralization, 99.25% (G3) and 82.14% (G6) respectively than when used alone. However, when combined with SDF the

remineralizing potential was significantly lower [84.89% (G4) and 69.66% (G7) respectively] than when used alone at different time intervals with EDX.



1-4, 11 and 12 represents Well-Defined Prism Pattern, (5, 13-28) represents Microporosities with Amorphous clusters, 6-10 represents shallow prism pattern.

The Surface SEM analysis at 7-day intervals revealed that all samples (100%) of the (G3) Self-Assembling Peptide combined CPP-ACPF, had shallow prisms, in contrast to the self-assembling peptides when used alone. A newly formed homogenous apatite layer on the demineralized enamel was observed and significant pore volume reduction was noticed in prismatic enamel structures. The surface also revealed the uniform mineral deposition without any traces of amorphous structures. Abundant Ca^{2+} and PO_4^{3-} ions of CPP-ACPF might have been the reason behind this pronounced remineralizing pattern. Similar pattern was seen at the end of 1months with further reduction in pore volume. After 3 months, 86.7% of samples showed changed prism pattern from shallow prism pattern to well- defined prisms (The prisms are uniformly arranged having open concave centers with well-defined peripheral prism outlines and their surfaces also in level of the tooth surface). Later at the end of 6 months, well defined pattern was observed in all samples (100%), which closely mimicked the structure of sound enamel (control group).

The SEM analysis for Self-Assembling Peptide+SDF group (G4), Synthetic Nano-hydroxyapatite+CPP-ACPF(G6) group and Synthetic Nano-hydroxyapatite+SDF group(G7) showed formation of crystals which were found to adhere to the pores created by demineralization. These adherent crystals were aggregateed into clusters at 7days time interval. At 1 month, amorphous clusters layer which was completely covering the prismatic and interprismatic enamel structures was observed. At 3 months, similar amorphous cluster layer was noticed along with reduction in pore volume and a similar pattern was maintained after 6 months. Therefore, we concluded that, remineralized pattern did not show any significant improvement and uniformity compared groups G2(Self-assembling peptide), G3(Self-assembling peptide+CPP-ACPF) and G1(control group).

A conclusion that could be drawn from the results of the current study is that, among all the groups tested, the Self-assembled peptide combined with CPP-ACP varnish group(G3) demonstrated the highest remineralization potential and a well defined prism pattern was obtained, had no significant difference when compared to control group(G1)(sound enamel) as the prism pattern were almost similar to sound enamel. This can be due to the composition of CPP-ACP which is a bioactive agent formulated from two parts: casein phosphopeptides (CPP) and amorphous calcium phosphate (ACP). CPPs binds to form clusters of ACP in metastable solution, preventing their growth to the critical size required for nucleation and precipitation. CPP-

ACP nano-complexes bind onto the tooth surfaces, dental plaque and diffuse into the body of lesion releasing the weakly bound, high level of calcium and phosphate ions, which would then deposit into crystal voids. This denotes that the presence of copious amounts of calcium, phosphate and fluoride ions aided in the enhancement of the regenerative potential of self-assembling peptide where these assembled scaffolds supported and initiated an increased hydroxyapatite crystallization utilizing the high level of ions available (Kamal D et.al.) [17]. This was followed by self-assembled peptide group(G2), The results were similar to the study done by Kamal D et.al. [17]. The lowest remineralizing potential was shown by the synthetic nanohydroxyapatite combined with SDF(G7), which reached a Ca/P ratio of 69.66% and had microporosities with amorphous clusters present in the surface morphology.

Although surface remineralization was confirmed in the present study, subsurface enamel remineralization was not assessed which could be a limitation. Also, the remineralization pattern can vary in the dynamically complex biological systems that normally persists in the oral cavity and thus the, clinical status should also be directly estimated.

The synergistic effect of Self-Assembling Peptides and CPP-ACPF used in this study was superior to self-assembling peptides when used alone, supporting the concept of guided enamel regeneration in a single application. Further clinical trials may pave way to achieve this novel treatment outcome and help us curate an effective enamel regenerating regimen.

V. Conclusion

The conclusions that were drawn from the study are:

1 In Biomimetic regenerative systems, Self-Assembling Peptide showed significantly higher remineralizing effect compared to Synthetic Nano-hydroxyapatite.

2 The Single application of Self-Assembling Peptides led to a gradual increase in remineralization, reaching its maximum potential at the end of 6 months, whereas Synthetic Nanohydroxyapatite which was applied daily showed microporosities even at the end of 6 months.

3 The Remineralizing potential /Synergistic effect was significantly higher on combining CPP-ACPF with both Self-Assembling Peptide and Synthetic Nano-hydroxyapatite than when each agent was used alone.

4 For both the systems, the synergistic effect with SDF showed a significantly lower remineralizing potential than when used alone.

5 Self-Assembling Peptide in combination with CPP-ACPF showed the highest remineralizing potential at all intervals i.e 1 week, 1 month, 3 months and 6 months compared to other study groups. A well-defined prism pattern was observed under SEM, which showed no significant difference to sound enamel (G1) of control group. This proved that the prism pattern obtained after application of this potential combination was almost similar to that of sound enamel after 6 months, followed by the prism pattern obtained when Self-Assembling Peptide was used alone.

6 The combined potential of Self-Assembling Peptide and CPP-ACPF should be evaluated with future clinical trials to devise an effective regimen.

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