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Enamel Subsurface Lesion Re-Mineralization Using Casein Phospopeptide-Amorphous Calcium Phosphate: A Sem-Edx Analysis

[1]Dr. Pulkit Vaid, [2]Dr. Sudhir Mittal, [3]Dr. Ankita Gupta

[1,2] Department of Peadiatric and preventive Dentistry

[3]Department of Public Health Dentistry

Corresponding Author: Dr. Pulkit Vaid, Department of Peadiatric and Preventive Dentistry, Jammu, India

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Abstract

Aim: The objective of the study was to evaluate the loss of mineral content from tooth surface in a sub-surface lesion and to assess the ability of casein phosphopeptide amorphous calcium phosphate paste to re-mineralize enamel subsurface lesions using scanning electron microscopy with energy dispersive X-ray analysis (SEM-EDX).

Materials and Methods: 60 enamel specimens were prepared from extracted molar teeth. Mineral content (weight %) of all the specimens were evaluated using SEM-EDX. After the initial scanning, all the specimens were then placed in the demineralizing solution for 4 days in order to produce artificial carious lesions. All the demineralized specimens were then re-evaluated for loss of mineral content (wt. %) using SEM-EDX. After that, the specimens were randomly assigned into 1 experimental group which is further sub-divided into 3 sub-groups and 1 control group containing 15 specimens each. Specimens in experimental group were treated with re-mineralizing paste for a period of 7, 21, and 35 days twice daily for 3 minutes followed by incubation in artificial saliva at 37˚C. Specimens in control group received no treatment with re-mineralizing paste and were incubated in artificial saliva for 35 days at 37˚C. After re-mineralization process, mineral content (% weight) of all the specimens were evaluated using SEM-EDX.

Results: All the experimental groups revealed highly significant difference between calcium/ phosphorous ratios of de-mineralized and re-mineralized enamel samples.

Conclusion: 10% CPP-ACP has high ability to re-mineralize the initial de-calcified pre-cavitated lesion. Moreover re-mineralization potential is dose dependent and maximum re-mineralization has been achieved on the 35th day using CPP-ACP.

Keywords: CPP-ACP, De-mineralization, Re-mineralization, SEM, EDX, Ca/P ratios

Introduction

Oral health is an integral part of the general health of an individual. Dental caries is a complex multifactorial disease caused by the interplay between a susceptible host, fermentable substrate, micro-flora and saliva. Saliva is essential for maintaining the oral equilibrium and the effects of saliva and its constituents on the oral micro-organisms influence the development of dental caries. Salivary components (immunoglobulins, salivary protein, salivary calcium, and inorganic phosphorous and alkaline phosphatase levels), its flow rate, viscosity, buffering capacity, pH etc. plays a major role in initiation, and

progression of dental caries and re-mineralization. Caries is a dynamic process occurring continuously in tooth structure. Disruption in the balance of demineralization and re-mineralization leads to deterioration in tooth structure.[1]

The initiation of caries starts with subsurface enamel demineralization. There is loss of calcium and phosphate ions from the subsurface enamel, resulting in formation of subsurface lesion. Before break in surface integrity, lesion can be re-mineralized by diffusion of calcium and phosphate ions back into subsurface lesions.

Several studies in the past have shown milk and milk products to have anticariogenic properties in human and animal models.[2]This anticariogenic effect has been attributed to the multiphosphoseryl- containing sequences of casein.[3] Casein phosphopeptides (CPP) can stabilize calcium phosphate in solution through binding amorphous calcium phosphate (ACP) with their phosphoserine residues. This allows the formation of small CPP-ACP clusters. CPP-ACP prevents tooth erosion by suppressing demineralization, enhancing re-mineralization or combination of these two processes. [4]

One of the recent technique in measuring tooth's mineral content is scanning electron microscopy with an energy dispersive X-ray analysis attachment. It is a micro analytical technique that is employed to quantitatively estimate the amounts of mineral in a tooth sample.[11] Various studies have been conducted to detect changes in the re-mineralization and de-mineralization status of tooth structure.These studies concluded that the inorganic components such as calcium and phosphate contained in high concentrations in CPP-ACP plays an important role in re-mineralization of initial carious lesions. Therefore the objective of the present study was to evaluate the re-mineralization potential of casein phosphopeptide amorphous calcium phosphate paste (CPP-ACP) on enamel sub surface lesion using scanning electron microscopy with energy dispersive X-ray analysis (SEM-EDX).

Material Method

60 enamel specimens were prepared from the buccal surface of the teeth using a low speed hand-piece with diamond disc. Each slab was shaped into a rectangular form of 4mm*4mm*1mm by using lathe under water cooling. All the specimens were then evaluated for mineral content using SEM-EDX (Fig1). After the initial scanning, all the specimens were then placed in the demineralizing solution containing 20 ml of acid buffer with 2mmol/l $Ca^{2+,}$ 2mmol/l PO_4^{3-} , 0.075mol/L acetate at pH 4.3 for four days at 37^{o}C for producing artificial lesions. On the 5th day all the demineralized specimens were then re-evaluated for loss of mineral content (wt. %) using SEM-EDX (Fig.2).

After evaluating mineral content, all specimens were randomly assigned into 2 groups: group 1 contained 15 specimens (control group) and group 2 contained 45 specimens (experimental group). Specimens in experimental group (Group 2) were randomly assigned into three sub-groups as 2a, 2b and 2c containing 15 specimens each. Specimens in the experimental group were then treated with re-mineralizing paste (GC TOOTH MOUSSE) containing 10% CPP-ACP. The treatment regimen in sub-group 2a is 7 days, sub-group 2b is 21 days and sub- group 2c is 35 days, twice daily for 3 minutes followed by incubation in artificial saliva at 37°C. Specimens in control group (group 1) received no treatment with re-mineralizing paste and were incubated in artificial saliva at 37°C for a period of 35 days. SEM-EDX was then re-evaluated to calculate Ca:P ratio and the amount re-mineralization in each group. The study evaluated the re-mineralization potential of CPP-ACP paste on enamel subsurface lesions using SEM-EDX.

Energy dispersive X-ray analysis was used to determine calcium and phosphorus content in % weight of sound, demineralized, and re-mineralized enamel in each group. The calcium and phosphorus content was then converted into Ca/P ratios for each group from the obtained data.

Results

Figure (1, 2, and 3) demonstrates the elemental analysis of sound, de-mineralized and re-mineralized enamel samples of control group (group 1). Figure (4, 5, and 6) demonstrates the elemental analysis of the experimental group (sub-group 2a-2c). Representative SEM images of the enamel specimens are shown in Figures (7-12). Table 1 demonstrates comparision of mean Ca/P ratios of sound, de-mineralized, re-mineralized enamel samples.

Statistical analysis was done using one-Way Anova, Tukey's HSD, and Student t-test. Comparison between Ca/P ratios of the sound enamel samples and Ca/P ratios of the demineralized enamel samples in all the groups using one-way Anova revealed that there was no statistically significant difference between the groups.

One-way Anova was applied to compare the mean Ca/P ratios of the experimental groups after re-mineralization, which was found to increase to 2.28± 0.06 on the 35th day. This increase in the mean Ca/P ratio from the seventh to the 35th days had p= 0.000, implying a highly statistically significant difference in the re-mineralization potential for this period. Tukey HSD was done for intergroup comparison [Table 2] and $p < 0.05$ for all the comparisons suggested very high significance. Statistical significance differences were seen in re-mineralizing potential at 35 days. In control group when compared to re-mineralization potential at 7 days, no statistical difference was seen between re-mineralization at 21 days and 7 days. When re-mineralization potential at 21 days was compared with other groups, significant difference was seen at 35 days and in control group. No statistical difference was seen between re-mineralization at 7 days and 21 days.

When re-mineralization potential at 35 days was compared with other groups, significant difference was seen at 7 days, 21 days and in control group.

When re-mineralization potential of control group was compared with other groups, significant difference was seen in all experimental groups (7 days, 21days and 35 days).

The t-test was done to statistically analyze the mean Ca/P ratios of demineralized and re-mineralized specimens in each group [Table 3 and Graph 1]. The value of significance was set at $p < 0.05$ and it was seen that all the study groups revealed highly significant results between the Ca/P ratios of the demineralized and re-mineralized samples.

Discussion

For many years caries was considered as a one-way progressive de-mineralization of enamel crystallites followed by degradation of dentin, leading to cavity formation. According to WHO dental caries is defined as the localized post-eruptive pathological process of external origin involving softening of hard tooth tissue proceeding to formation of cavity. Dental hard tissues are constantly undergoing cycles of de-mineralization (when pH is low) and re-mineralization (when conditions favor) leading to variations in mineral status of the teeth throughout the day.[12,13, 14] Mineral loss (de-mineralization) or gain (re-mineralization) by enamel is a dynamic physicochemical process occurring when oral bacteria form a biofilm on the enamel surface and this biofilm is exposed to fermentable dietary carbohydrates, sucrose being the most cariogenic of them.[15, 16] Thus, every time sugar penetrates into a cariogenic biofilm and is converted to acids by bacterial metabolism, the biofilm fluid becomes undersaturated with respect to the enamel

mineral, and de-mineralization occurs.[17, 18, 19, 20] Re-deposition of the mineral lost by enamel can occur by Ca and P found in the biofilm fluid or by direct action of salivary Ca and P soon after the biofilm is removed.[21]

In the present study, the re-mineralization potential of CPP-ACP for enamel subsurface lesions was evaluated using SEM-EDX.[11, 22] The present study consisted of two phases, the first phase represented lesion initiation (de-mineralization) and the second phase represented re-mineralization phase. De-mineralization of tooth specimens was done by placing the specimens in de-mineralizing solution (20 ml of acid buffer containing 2mmol/l Ca^{2+}, 2mmol/l PO_4^{3-} , 0.075mol/L acetate) at pH 4.3 for four days at 37°C in order to produce artificial lesions and re-mineralization process was done using GC Tooth Mousse. GC Tooth Mousse is a water-based, lactose free crème containing 10% w/w Recaldent CPP-ACP.[3, 23] When CPP-ACP is applied in the oral environment, it will bind to biofilms, plaque, bacteria, hydroxyapatite, and soft tissue, localizing bioavailable calcium and phosphate. The proposed mechanism of anti-cariogenicity for the CPP-amorphous calcium phosphate (ACP) is that they localize ACP in dental plaque, which buffers the free calcium and phosphate ions, thereby helping to maintain a state of super-saturation with respect to tooth enamel thus depressing de-mineralization and enhancing re-mineralisation.[2] The presence of CPP–ACP might permit a rapid return to resting calcium concentrations and allows more immediate re-mineralization of enamel substrate. The CPP has a substantial ability to stabilize calcium phosphate in solution. The peptide was found to bind 21 calcium and 14 phosphorus ions per molecule. ACP nuclei spontaneously form in neutral and alkaline supersaturated calcium phosphate solutions. It is proposed that the peptide binds to the forming ACP nano-clusters, producing a metastable solution and preventing ACP growth to the critical size required for nucleation and phase transformation.[11]

Casein is the predominant phosphoprotein in bovine milk present primarily as calcium phosphate stabilized complexes and accounts for almost 80% of its total protein.[5, 6] CPP containing the active sequence – Ser(P)-Ser(P)-Ser(P)-Glu-Glu- has a remarkable ability to stabilize calcium and phosphate as nano-clusters of ions in a metastable solution.[7] In the formation of hydroxyapatite, ACP is a postulated precursor. The ACP's exhibit a very high solubility and are readily converted to hydroxyapatite, which makes them suitable mineralizing agents.[8] The main advantage of ACP is its facile, single solid phase formulation and its biocompatibility with both hard and soft tissues, which is equal to that of hydroxyapatite and various di-, tri- and tetra-calcium phosphates.[9] CPP-ACP is a nano-complex of calcium ions and hydroxide stabilized by casein phosphopeptides. The CPP allow calcium, phosphate and fluoride ions to be stabilized in high concentration, so that it is bioavailable for the promotion of re-mineralization.[10, 5]

According to **Reynolds E.C (1997)[24]** 1.0% w/v CPP solution can stabilize 60 mmol/L CaCl2 and 36 mmol/L sodium phosphate at pH 7.0 to form colloidal amorphous calcium phosphate-CPP nanocomplexes.[3]Also Casein phosphopeptide-amorphous calcium phosphate compounds (CPP-ACP) have been demonstrated to have anticariogenic potential in laboratory, animal, and human in situ experiments. **Rose (2000)[25]** investigated these effects by measuring the affinity and capacity of Streptococcus mutans for CPP-ACP. The study demonstrated that CPP-ACP binds with about twice the affinity of the bacterial cells for calcium. Application of CPP-ACP to plaque may cause a transient rise in plaque fluid free calcium which may assist re-mineralization. Subsequently, CPP-ACP will form a source of readily

available calcium to inhibit de-mineralization.[26, 27] **Bussadori et al.[28]** conducted long-term and short-term cytotoxicity assessment of CPP-ACP paste in rat fibroblasts and concluded that CPP-ACP paste demonstrates low cytotoxicity in rat fibroblast culture. So due to its low toxicity, CPP-ACP could also be used in oral environment as well.[29]

In the present study, CPP-ACP was used in the form of paste. Patients can use this kind of oral hygiene paste just like tooth paste with tooth brushes and also apply the paste with cotton slabs.[2,3] To make it clinically relevant, the re-mineralization treatment regimen of 3 minutes twice daily application was employed as per manufacturer's recommendation.

At the ultrastructure level EDX has been used for elemental analysis. It is a microanalytical technique that combines its function with SEM where SEM does the structural analysis and the elemental analysis is done by EDX. The principle is based on the energy emitted in the form of X-ray photons when electrons from external sources collide with the atoms in a material, thus generating characteristic X-rays of that element. When the sample is bombarded by the electron beam of the SEM, electrons are ejected from the atoms on the specimen's surface (secondary electrons). A resulting electron vacancy is filled by an electron from a higher shell, and an X-ray is emitted (characteristic X-rays) to balance the energy difference between the two electrons. The EDX X-ray detector measures the number of emitted X-rays vs. their energy. The energy of the X-ray is characteristic of the element from which the X-ray was emitted. A spectrum of the energy vs. relative counts of the detected X-rays is obtained and evaluated for qualitative and quantitative determinations of the elements present in the specimen using a computer-based program.[3]

The results of this in vitro study showed that 10% CPP-ACP paste re-mineralized enamel subsurface lesions. Re-mineralization was maximum in the specimens kept for 35 days, which explains that the re-mineralization was dose-dependent. The results of our study were consistent with the studies done by **Hegde et al,[11] Reynolds (1997),[24] Kumar et al.[30]** According to **Reynolds EC et al.[31] , Oshiro et al.[4] and Yamaguchi et al.[2]** the inorganic components contained in high concentrations in CPP-ACP acted to enhance re-mineralization of the enamel. This is consistent with the results of the present study.

Enamel specimens treated with CPP-ACP paste revealed slight changes in their morphological features. The surface morphologies of the specimens in the study groups showed no apparent differences among the different storage periods. This is in accordance with the study conducted by **Oshiro et al.[4] and Hegde et al.[3]**

In the present study calcium phosphate stabilized by CPP to produce a metastable solution supersaturated with respect to the amorphous and crystalline calcium phosphate phases has been shown in to re-mineralize enamel subsurface lesions. The CPP-stabilized calcium phosphate solutions re-mineralized subsurface enamel lesions at a rate equal to or greater than those obtained with constant-composition procedures.

Conclusion

The goal of modern dentistry is to manage non-cavitated caries lesions non-invasively involving newer re-mineralization systems to repair the enamel that includes Fluoro-apatite, fluor hydroxyapatite, CPP-ACP. The present study evaluated the enamel subsurface lesion re-mineralization by using casein phosphopeptide-amorphous calcium phosphate.

The following conclusions can be drawn from the present study:-

- That 10% CPP-ACP has high ability to re-mineralize the initial de-calcified pre-cavitated lesion.
- That the re-mineralization potential is dose dependent as in the present study and maximum re-mineralization has been achieved on the 35th day.

Although extreme care has been taken to conduct the study, the study is not free from shortcomings.

The study has been done in ideal in-vitro conditions; the results cannot be completely extrapolated to in-vivo situations, where multiple factors like amount and quantity of saliva, acid attack on tooth site are responsible for modifying the carious lesions. Moreover SEM-EDX measures re-mineralization and de-mineralization potential quantitatively, not qualitatively. So, large sample with qualitative methods of analysis would be required for more precise and accurate results.

Table 1: Comparision of mean Ca/P ratios of sound, de-mineralized, re-mineralized enamel samples using one - way ANOVA

Group	N	Mean, S.D. of sound enamel		Mean, S.D. of demineralized enamel		Mean, S.D. of remineralized enamel	
		Mean	S.D.	Mean	S.D.	Mean	S.D.
1	15	2.416667	.1089998	1.7000	.12006	1.990000	.1668618
2a	15	2.448000	.1199524	1.6913	.13553	1.966000	.0903801
2b	15	2.296667	.0716805	1.6093	.08481	2.125333	.0440562
2c	15	2.465333	.0976046	1.6507	.09691	2.286667	.0699660
		F=8.477, p=.000 (Highly significant)		F=2.108, p=.109 (not significant)		F=30.322, p=.000 (Highly significant)	

Table 2: Intergroup comparison of the re-mineralization potential of study groups done using tukey honestly significant difference post HOC multiple comparisons

Multiple Comparisons			
Tukey HSD			
(I) grouping	(J) grouping	Mean Difference (I-J)	Sig.
2a	2b	.0260000	>0.05
	2c	-.1353333*	<0.05
	1	-.2966667*	<0.05
2b	2a	-.0260000	>0.05
	2c	-.1613333*	<0.05

	1	-.3226667*	<0.05
2c	2a	.1353333*	<0.05
	2b	.1613333*	<0.05
	1	-.1613333*	<0.05
1	2a	.2966667*	<0.05
	2b	.3226667*	<0.05
	2c	.1613333*	<0.05

Table 3: T-test to analyze the mean Ca/P ratios of de-mineralized and re-mineralized specimens in study groups

Group	Mean, S.D. of demineralized enamel		Mean, S.D. of remineralized enamel		Mean difference	T	*P* value
	Mean	S.D.	Mean	S.D.			
2a	1.6913	.13553	1.966000	.0903801	-.274	-11.556	.000(hs)
2b	1.6093	.08481	2.125333	.0440562	-.516	-24.937	.000(hs)
2c	1.6506	.09691	2.286667	.0699660	-.636	-29.931	.000(hs)

KV:15	Mag:3000	Tajeoff: 35.7	Live Time(s):30	Amp Time(µs): 7.68	Resolution: (eV)126.7

EDS spot 1

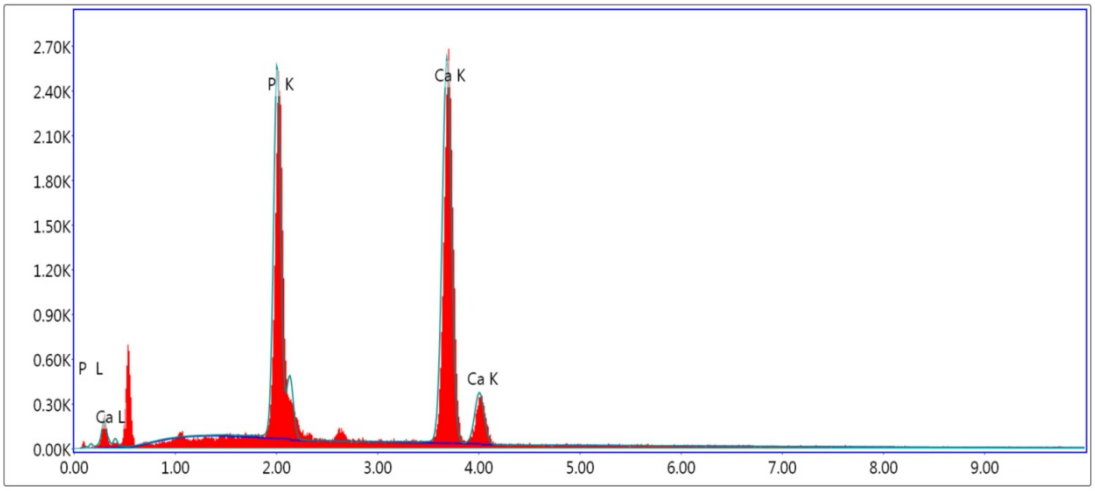


Figure 1: Elemental analysis of sound enamel sample

KV:15	Mag:3000	Tajeoff: 35.7	Live Time(s):30	Amp Time(µs): 7.68	Resolution: (eV)126.7

EDS Spot 1

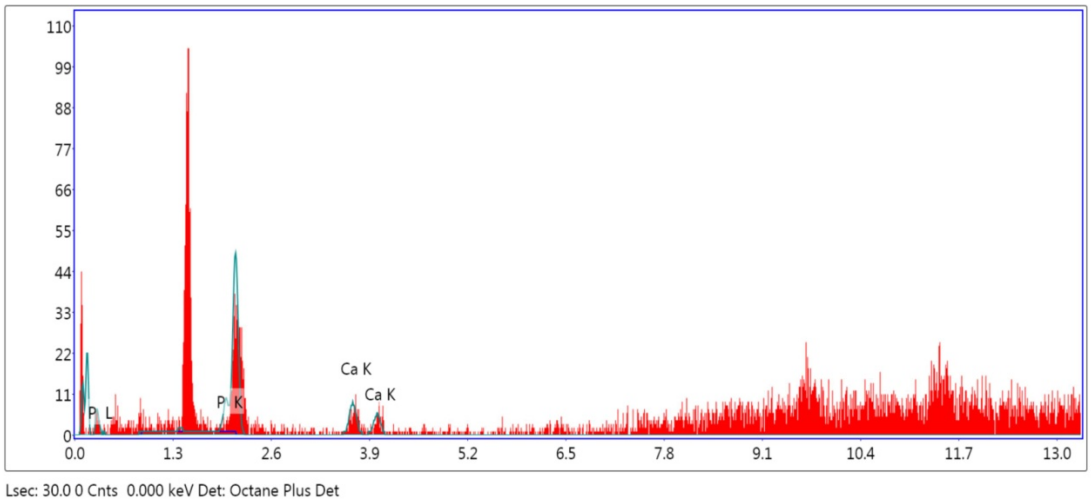


Figure 2: Elemental analysis of demineralized enamel sample

KV:15	Mag:3000	Tajeoff: 35.7	Live Time(s):30	Amp Time(µs): 7.68	Resolution: (eV)126.7

EDS Spot 1

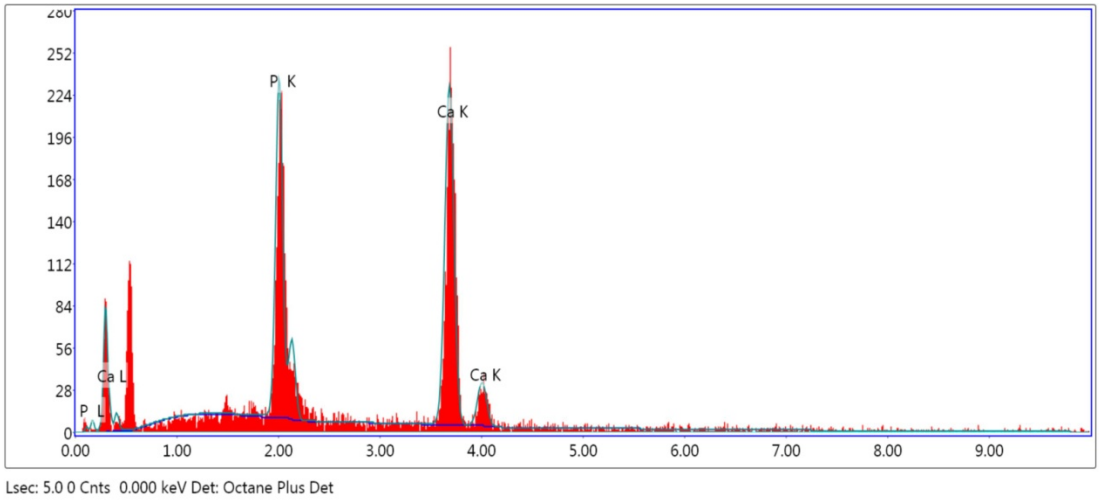


Figure 3: Elemental analysis of re-mineralized enamel sample control group (Group 1)

KV:15	Mag:3000	Tajeoff: 35.7	Live Time(s):30	Amp Time(µs): 7.68	Resolution: (eV)126.7

EDS spot 1

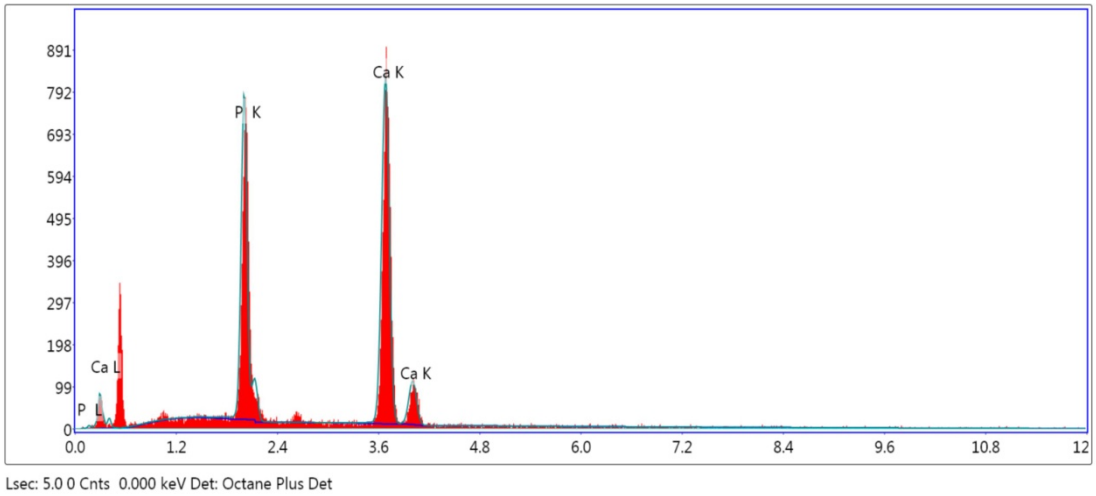


Figure 4: Elemental analysis of re-mineralized enamel sample experimental group (Group 2a)

KV:15	Mag:3000	Tajeoff: 35.7	Live Time(s):30	Amp Time(µs): 7.68	Resolution: (eV)126.7

EDS Spot 1

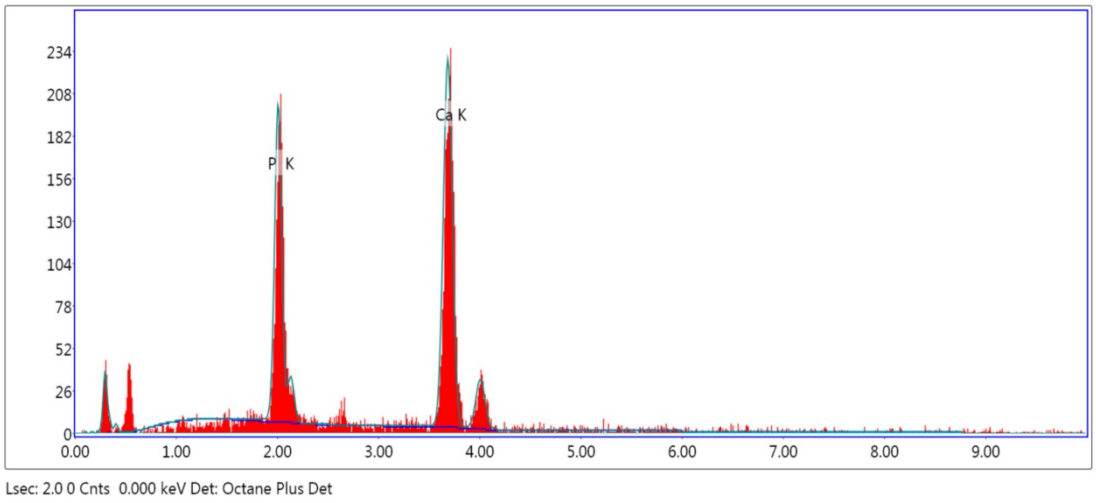


Figure 5: Elemental analysis of re-mineralized enamel sample experimental group (Group 2b)

KV:15	Mag:3000	Tajeoff: 35.7	Live Time(s):30	Amp Time(µs): 7.68	Resolution: (eV)126.7

EDS Spot 1

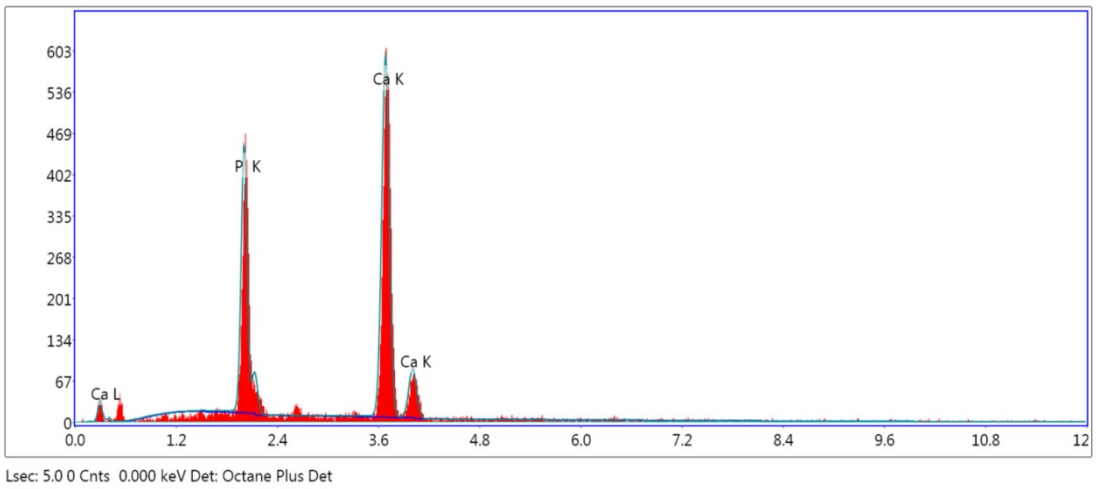


Figure 6: Elemental analysis of re-mineralized enamel sample experimental group (Group 2c)

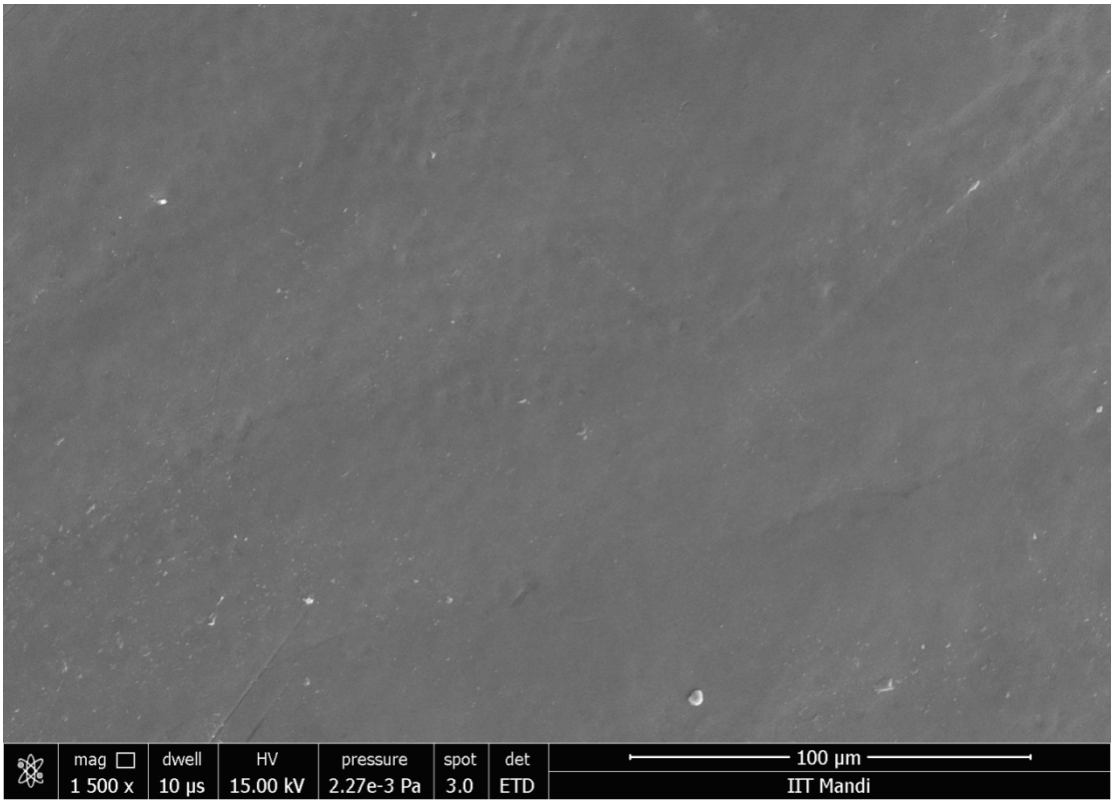


Figure 7: Sturctural analysis of sound enamel sample by SEM

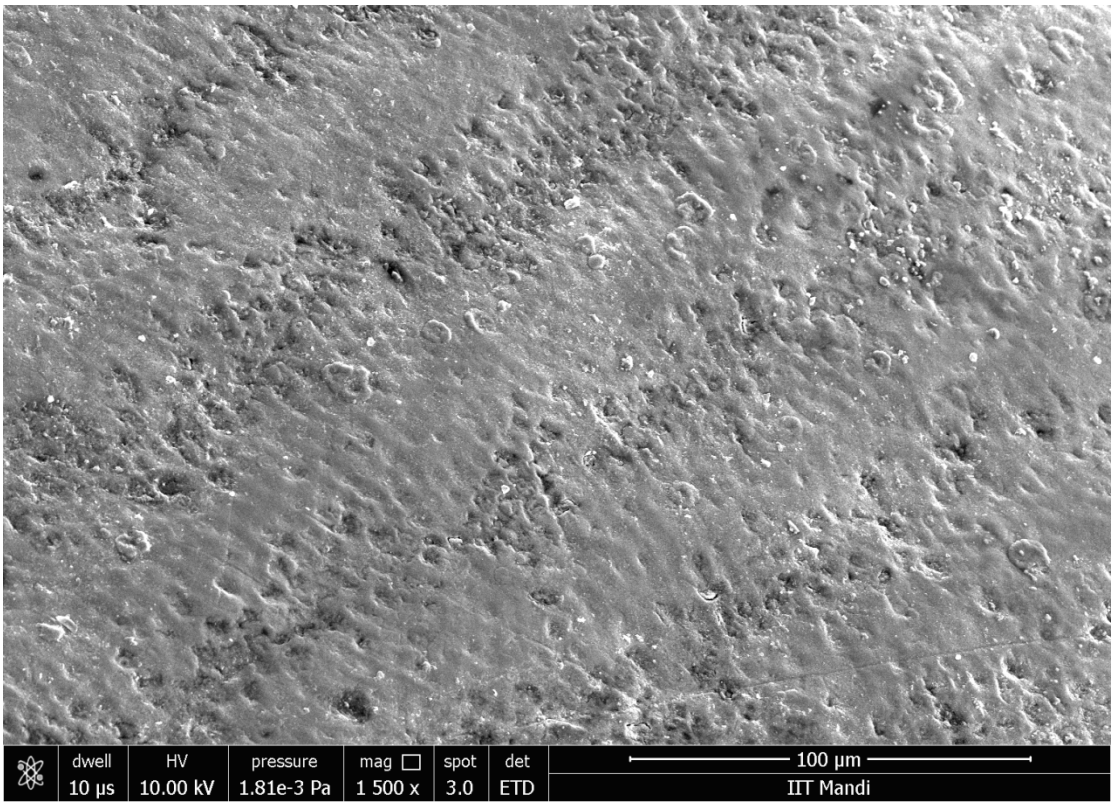


Figure 8: Structural analysis of de-mineralized enamel sample by SEM

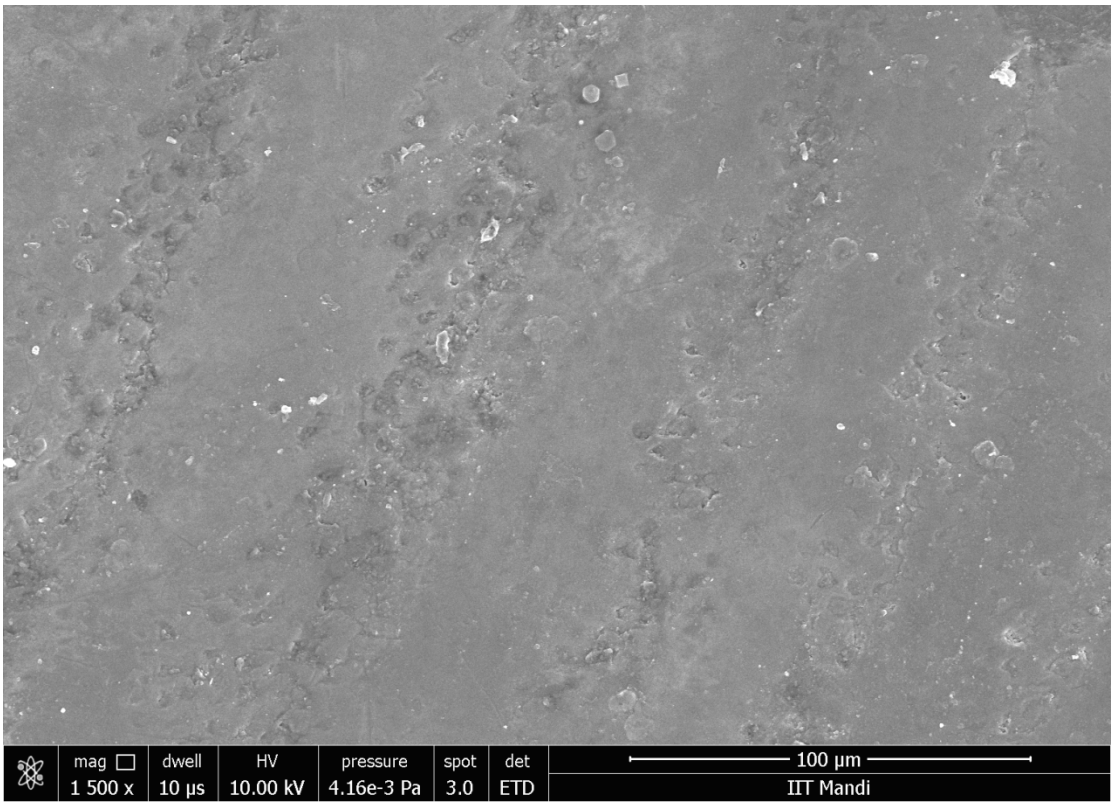


Figure 9: Structural analysis of re-mineralized enamel sample control group (Group I) by SEM

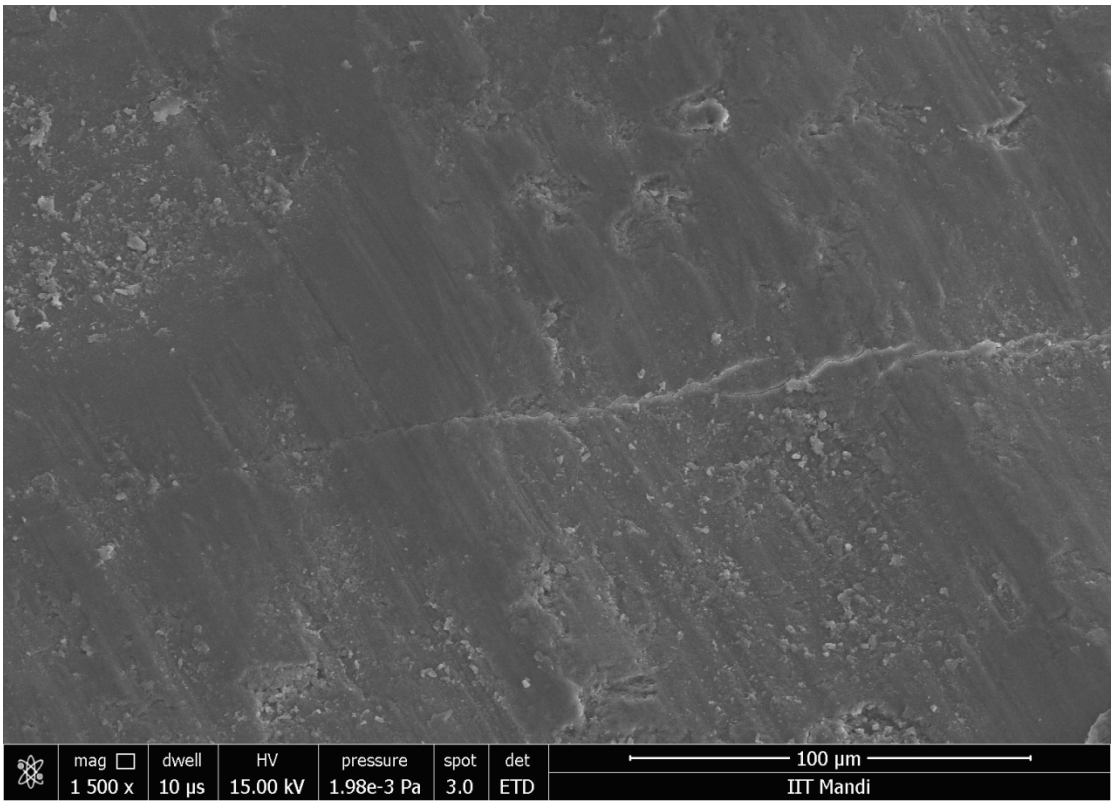


Figure 10: Structural analysis of re-mineralized enamel sample Group II (a) by SEM

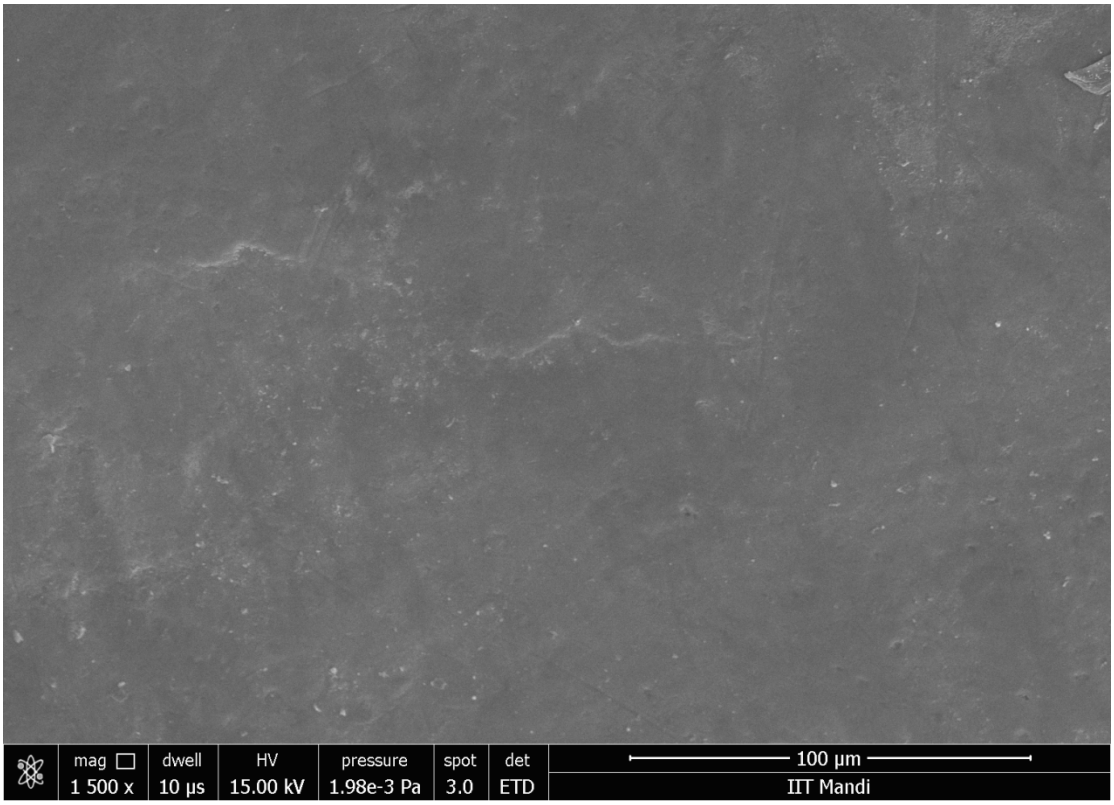


Figure 11: Structural analysis of re-mineralized enamel sample Group II (b) by SEM

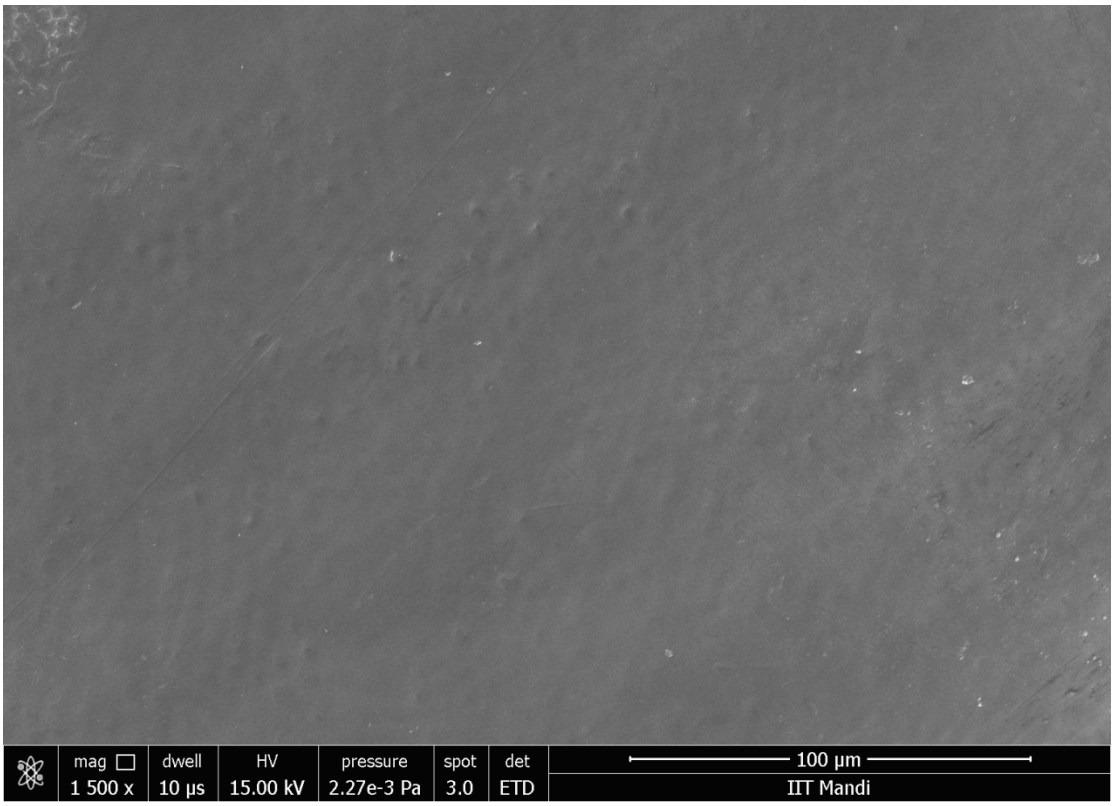
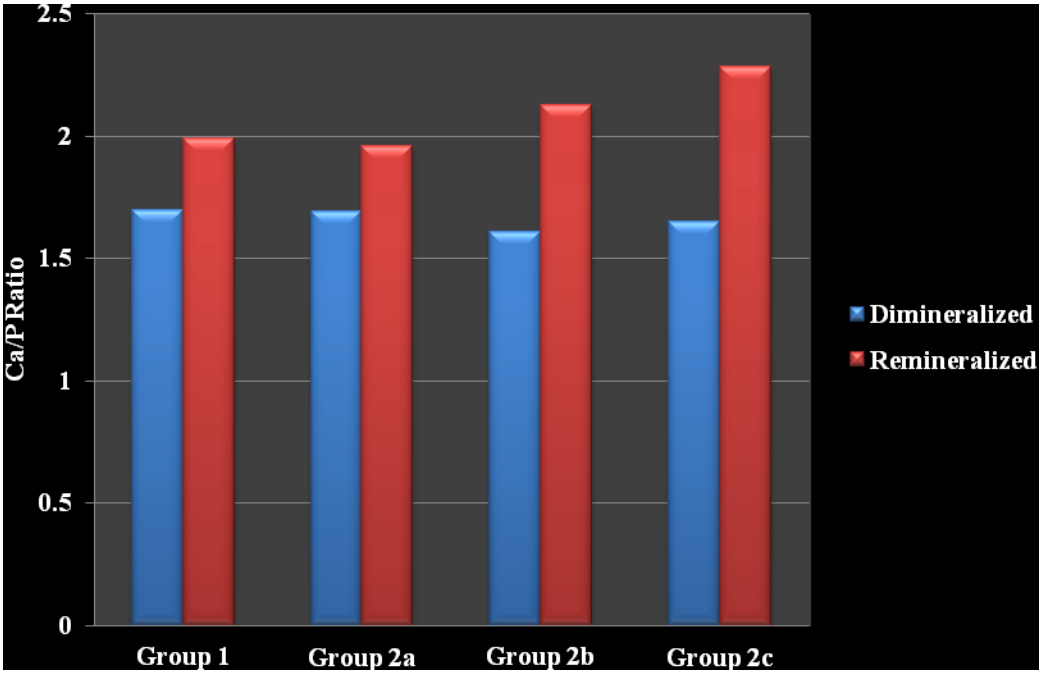


Figure 12: Structural analysis of re-mineralized enamel sample Group II (c) by SEM



Graph 1:Relationship between demineralization and the re-mineralization potential of various groups

References

1. Damle SG et al. Quantitative determination of inorganic constituents in saliva and their relationship with dental caries experience in children. *Dentistry 2012; 2:3.*
2. Yamaguchi K et al. Effect of CPP-ACP paste on mechanical properties of bovine enamel as determined by an ultrasonic device. *J Dent 2006; 34:230-6.*
3. Hegde MN, Shetty S, Pardal D. Re-mineralization of enamel sub surface lesion using casein phosphopeptide-amorphous calcium phosphate. *J Conserv Dent 2007; 10:19-25.*
4. Oshiro M et al. Effect of CPP-ACP paste on tooth mineralization: An FE-SEM study. *J Oral Sci 2007; 49:115-20.*
5. Gurunathan D, Somasundaram S, Kumar SA. Casein phosphopeptide-amorphous calcium phosphate: A re-mineralizing agent of enamel. *Australian Dental Journal 2012; 57: 404–408*
6. Azarpazhooh A, Limeback H. Clinical efficacy of casein derivatives: A systematic review of the literature. *J Am Dent Assoc* 2008; *139:915–924.*
7. Shen P, Cai F, Nowicki A, Vincent J, Reynolds EC. Remineralization of enamel subsurface lesions by sugar-free chewing gum containing casein phosphopeptide-amorphous calcium phosphate. *J Dent Res 2001; 80:2066-2070*
8. Tung MS, Eichmiller FC. Dental applications of amorphous calcium phosphates. *J Clin Dent 1999; 10:1–6.*
9. Brown WE, Chow LC. A new calcium phosphate, water-setting cement. In: Brown PW, ed. Cements Research Progress 1986. Westerville, Ohio: *American Ceramic Society, 1987:352–379.*

10. Reynolds EC. Casein phosphopeptide-amorphous calcium phosphate the scientific evidence. *Adv Dent Res 2009; 21:25–29.*
11. Hegde MN, Shetty S. Re-mineralization of enamel subsurface lesions with casein phosphopeptide-amorphous calcium phosphate: A quantitative energy dispersive X-ray analysis using scanning electron microscopy: An in vitro study. *J Conserv Dent 2012; 15:61-7.*
12. Sathe N, Chakradhar Raju RVS, Chandrasekhar V. Effect of Three Different re-mineralizing Agents on Enamel Caries Formation – An in vitro Study. *Kathmandu Univ Med J 2014; 45(1):16-20.*
13. Hicks J, Flaitz C. Role of re-mineralizing fluid in in vitro enamel caries formation and progression. *Quintessence Int 2007; 38:313-319.*
14. Arends J and Ten Bosch JJ. De-mineralization and re-mineralization Evaluation Techniques. *J Dent Res 1992; 71:924-928.*
15. Dawes C. What is the critical pH and why does a tooth dissolve in acid? *J Can Dent Assoc. 2003; 69:722-4.*
16. Pereira RF, Leal SC. Efficacy of casein derivate CPP-ACP. *Rev Gaúch Odontol, Porto Alegre.2014; 62(3):243-52.*
17. Paes Leme AF et al. In situ effect of frequent sucrose exposure on enamel demineralization and on plaque composition after APF application and F dentifrice use. *J Dent Res. 2004; 83:71-5.*
18. Greenby TH, Andrews AT, Mistry M, William RJ. Dental caries protective agents in milk and milk products: investigation in vitro. *J Dent 2001; 29:83-92*
19. Gordon Nikiforuk. Understanding of dental caries etiology, mechanism& prevention basic clinical aspect *vol. 1&2.*
20. Hicks J, Flaitz C. Role of re-mineralizing fluid in in vitro enamel caries formation and progression. *Quintessence Int 2007; 38:313-319.*
21. Curry JA, Tenuta LMA. Enamel re-mineralization: Controlling the caries disease or treating early caries lesions. *Braz Oral Res 2009; 23:23-30.*
22. Pai D et al. Use of laser fluorescence and scanning electron microscope to evaluate re-mineralization of incipient enamel lesions re-mineralized by topical application of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) containing cream. *J Clin Pediatr Dent 2008; 32(3):201-206.*
23. Pradeep K, Rao PK. Re-mineralizing agents in the non-invasive treatment of early carious lesions. *Int J Dent Case Reports 2011; 1(2):73-84.*
24. Reynolds EC. Re-mineralization of enamel subsurface lesions by casein phosphopeptide-stabilized calcium phosphate solutions. *J Dent Res 1997; 76:1587-95.*
25. Rose RK. Effects of an anti-cariogenic casein phosphopeptide on calcium diffusion in streptococcal model dental plaques. *Arch Oral Biol.2000 Jul; 45(7):569-75.*
26. Al Batayneh OB. The clinical applications of Tooth Mousse and other CPP-ACP products in caries prevention: Evidence-based recommendations. *Smile Dent J 2009; 4:8-12.*
27. Marsh PD. Microbiological aspect of dental plaque & dental caries. *Dent Clin North Am 1999; 43:599-614.*

28. Bussadori KS et al. Cytotoxicity assessment of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) paste. *Conscientiae Saúde 2010; 9:354-9.*

29. Kalra DD, Kalra RD, Kini PV, Allama Prabhu CR. Non-fluoride re-mineralization: An evidence-based review of contemporary technologies *J Dent Allied Sci 2014; 3(1):24-33.*

30. Kumar VLN, Itthagarun A, King NM The effect of casein phosphopeptide-amorphous calcium phosphate on re-mineralization of artificial caries-like lesions: An in vitro study. *Aust Dent Journal 2008; 53:34-40.*

31. Reynolds EC et al. Retention in plaque and re-mineralization of enamel lesions by various forms of calcium in a mouth-rinse or sugar free chewing gum. *J Dent Res 2003; 82:206-11.*