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Quantitative evaluation of remineralizing potential of three agents on artificially demineralized human enamel using scanning electron microscopy imaging and energy-dispersive analytical X-ray element analysis: An *in vitro* study

Nitin Khanduri[1], Deepak Kurup[2], Malay Mitra[3]

[1]Department of Pediatric and Preventive Dentistry, UCMS College of Dental Surgery, Bhairahawa, Nepal, [2]Department of Conservative Dentistry and Endodontics, Institute of Dental Education and Advance Studies, Gwalior, Madhya Pradesh, [3]Department of Pediatric and Preventive Dentistry, Guru Nanak Institute of Dental Science and Research, Kolkata, West Bengal, India

ABSTRACT

Background: The aim of this study is to quantitatively evaluate the remineralization potential of three remineralizing systems as follows: fluoride, casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), and CPP-ACP with fluoride, under scanning electron microscope with energy-dispersive X-ray analysis.

Materials and Methods: In this *in vitro* study A total of 40 enamel specimens were prepared from the buccal or lingual surfaces of human premolars extracted for orthodontic reason. Specimens were then placed in demineralizing solution for 96 h, to produce artificial caries-like lesion. Calcium and phosphate weight percentage of demineralized specimens was measured. Specimens were divided into four groups as follows: (a) control, (b) CPP-ACP, (c) CPP-ACP with fluoride, and (d) fluoride varnish. Except for the control group, the entire specimens were subjected to remineralization using respective remineralizing agents of their groups. The prepared specimens were assessed for calcium and phosphate weight percentage using scanning electron microscopy-energy dispersive X-ray spectroscopy. One way analysis of variance (ANOVA), followed by Tukey's test, was performed with the help of critical difference (CD) or least significant difference at 5% and 1% level of significance. $P \leq 0.05$ was taken to be statistically significant and $P < 0.001$ as statistically highly significant.

Results: The mean weight percentage of calcium and phosphorus of specimens treated with CPP-amorphous calcium phosphate nanocomplexes plus fluoride (ACPF) was significantly higher than other groups.

Conclusion: All the groups showed statistically significant remineralization. However, because of added benefit of fluoride, CPP-ACPF showed statistically significant amount of remineralization than CPP-ACP.

Key Words: Casein phosphopeptide-amorphous calcium phosphate, demineralization, remineralization

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Address for correspondence:
Dr. Nitin Khanduri, Department of Pediatric and Preventive Dentistry, UCMS College of Dental Surgery, Bhairahawa, Nepal.
E-mail: nitinkhanduri658@gmail.com

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INTRODUCTION

Dental caries is a pathological state that results from an imbalance in the physiological process of demineralization and remineralization of the hard dental structure.[1] The comprehension of dental caries as a multifactorial oral pathology suggests a complex treatment plan in which remineralization assumes a vital part, preventing the progression of the disease and reversing initial signs of demineralization.[2-4]

More than 70 years since Dean *et al.* distinguished fluoride as being accountable for differences in caries prevalence between communities, fluoride remains the benchmark in the prevention and treatment of the caries disease.[5] Its safety and its potency against dental caries have been widely studied and examined over the years.[6] In trace amounts, fluoride increases the resistance of tooth structure to demineralization and is the main methodology that is applied on a population basis for caries prevention.[2] The complex casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) derived from the casein protein of milk was patented in the U.S. in 1991[7] This complex is presented as an alternative remineralizing agent, remarkably capable of stabilizing calcium and phosphate, and maintaining a state of supersaturation of these ions in the oral environment. As an outcome, the tooth structure would gain from the high levels of calcium phosphate in the biofilm, and remineralization would occur.[8] Although product-containing CPP-ACPs are already available in the market, the absence of accord among scientists in regard to the remineralizing capability of this complex is evident.[9]

CPPs contain the cluster sequence-Ser(P)-Ser(P)-Ser(P)-Glu-Glu-which binds fluoride, as well as calcium and phosphate, stabilizes calcium fluoride phosphate as soluble complexes. These complexes are designated CPP-amorphous calcium phosphate nanocomplexes plus fluoride (CPP-ACPF). Investigations of such nanocomplexes in view of the casein alpha-S1 peptide fragment 59–79 have uncovered particle size of somewhere in the range of 2 nm and stoichiometry of one peptide to 15 calcium, 9 phosphate, and 3 fluoride ions.[10]

With the arrival of various commercially available remineralizing agents, it is important to evaluate which agent efficiently restore carious lesion back to normal and find out which system better restores the original mineral content of tooth. As the goal of modern dentistry is to manage noncavitated caries lesions noninvasively through remineralization in an attempt to prevent disease progression and improve esthetics, strength, and function. Therefore, the aim of this study was to investigate and compare the remineralizing potential of fluoride, CPP-ACP, and CPP-ACP with fluoride on artificial caries-like lesions in an attempt to manage noncavitated caries lesions noninvasively.

MATERIALS AND METHODS

In this *in vitro* study, sample size of 40 was estimated using the power calculation α (Type 1 error) = 0.05 and β (Type 2 error) = 0.20 with 80% being the power of the study. Forty enamel specimens were prepared from the buccal and lingual surfaces of the extracted human premolar teeth for orthodontic purpose using a high-speed diamond-tipped disc. Ethical clearance and permission to undertake the study were granted by the Institutional Review Board of Dr. R. Ahmed Dental College and Hospital. The prepared specimens were assessed for calcium and phosphate weight percentage using scanning electron microscopy-energy dispersive X-ray spectroscopy (SEM-EDX). The specimens were then placed in demineralizing solution containing 2.2 mM $CaCl_2$, 2.2 mM NaH_2 PO_4, and 0.05 M lactic acid, 0.2 ppm. Fluoride adjusted with 1M NaOH to a pH 4.5 for 96 h with each specimen submerged in 10 ml of solution incubated at 37°C. The solution was changed after 2 days. All the specimens were assessed for calcium and phosphate weight percentage using SEM-EDX.

The specimens were distributed into four groups. Group I served as a control group where no surface treatment was performed. Group II included specimen treated with fluoride varnish (0.9% silane fluoride [0.1% F]) applied using a microbrush, left to act at the enamel surface for 3 min and then delicately removed using cotton tips immersed in the deionized water. Group III included specimen treated with 10% CPP-ACP-containing paste applied using a mini brush, where the paste was left in contact with tooth for 3 min and was later removed by squirting deionized water to rinse thoroughly. Group IV included specimen treated with CPP-ACPF paste (10% CPP-ACP paste with incorporated fluoride. Fluoride level is 0.2%w/w [900 ppm]) applied using a mini brush where the paste was left in contact with tooth for 3 min and later was removed by squirting deionized water to rinse thoroughly. The

specimens were treated twice daily and then washed and stored in artificial saliva. This remineralizing cycle was continued for 21 days. After completion of the treatment with remineralizing agents, again the calcium and phosphorus weight percentage of all the specimens was assessed.

Statistical analysis was performed with the help of Epi Info (TM) 3.5.3 (Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, US). EPI INFO is a trademark of the Centers for Disease Control and Prevention, Atlanta, the coke city in the USA. The descriptive statistical analysis was performed to calculate the means with the corresponding standard errors. One way analysis of variance (ANOVA), followed by Tukey's test, was performed with the help of critical difference (CD) or least significant difference at 5% and 1% level of significance. $P \leq 0.05$ was taken to be statistically significant and $P < 0.001$ as statistically highly significant.

RESULTS

The baseline mean and standard deviation of the weight percentage of the elements in different groups were calculated and are represented as shown in Table 1. The mean and standard deviation of percentage weights of the elements in different groups after demineralization were calculated and are represented as shown in Table 2. Table 3 shows mean and standard deviation of weight percentage of calcium of Groups I, II, III, and IV after remineralization, respectively, as 23.09 ± 0.60, 24.52 ± 0.38, 26.79 ± 0.41, and 29.20 ± 0.56. It also shows mean and standard deviation of weight percentage of phosphorus of Groups I, II, III, and IV after remineralization, respectively, as 15.46 ± 0.36, 11.78 ± 0.17, 16.97 ± 0.25, and 17.79 ± 0.15. The respective calcium-phosphorus ratio of Groups I, II, III, and IV are 1.49 ± 0.02, 2.08 ± 0.03, 1.58 ± 0.02, and 1.64 ± 0.03.

On comparing calcium content of different groups after remineralization, ANOVA showed highly significant differences in calcium content of different groups after remineralization ($F_{3,36} = 28.45$, $CD_5 = 1.42$, and $CD_1 = 1.90$) ($P < 0.01$) [Table 3]. The mean calcium of Group IV was significantly higher than that of other groups ($P < 0.01$). Furthermore, mean calcium of Groups II and III was significantly higher than that of Group I ($P < 0.01$). On comparing phosphorus content of different groups after remineralization, ANOVA showed highly significant differences in phosphorus content of different groups after remineralization ($F_{3,36} = 112.80$, $CD_5 = 0.71$, and $CD_1 = 0.94$) ($P < 0.01$) [Table 3]. The mean phosphorus of Group IV was significantly higher than other groups ($P < 0.01$). Furthermore, mean phosphorus of Group III was significantly higher than that of Group I ($P < 0.01$).

The energy dispersive X-ray analysis [Figure 1] and the SEM pictures [Figure 2] revealed that mineral deposits on the surface when each test group was compared with the control group.

DISCUSSION

Dental caries being one of the most prevalent chronic diseases in adults and children worldwide is a preventable, multifactorial disease that involves bacteria, susceptible teeth, and carbohydrates.[11,12] These factors play a role in the dynamic demineralization-remineralization process that occurs at the surface of each tooth in the oral environment.

Table 1: Distribution of mean and standard deviation weight percentage of calcium and phosphorus in different groups (baseline)

Elements	Group I (n=10)	Group II (n=10)	Group III (n=10)	Group IV (n=10)	F values and critical difference at 5% (CD_5) and at 1% (CD_1)
Ca (mean±SD)	30.70±0.77	31.03±0.64	31.01±0.52	31.07±0.65	$F_{3,36}$=0.06 CD_5=1.86 CD_1=2.48
P (mean±SD)	17.70±0.35	17.48±0.30	17.64±0.24	17.92±0.22	$F_{3,36}$=0.40 CD_5=0.81 CD_1=1.09
Ca/P (mean±SD)	1.73±0.03	1.77±0.02	1.76±0.03	1.73±0.02	$F_{3,36}$=0.42 CD_5=0.08 CD_1=0.11

SD: Standard deviation

Table 2: Distribution of mean weight percentage of calcium and phosphorus in different groups (demineralized)

Elements	Group I (n=10)	Group II (n=10)	Group III (n=10)	Group IV (n=10)	F values and critical difference at 5% (CD_5) and at 1% (CD_1)
Ca (mean±SD)	21.69±0.70	21.63±0.29	21.85±0.40	22.32±0.45	$F_{3,36}$=0.40 CD_5=1.39 CD_1=1.85
P (mean±SD)	14.92±0.38	14.38±0.28	14.93±0.24	15.22±0.32	$F_{3,36}$=1.25 CD_5=0.89 CD_1=1.19
Ca/P (mean±SD)	1.45±0.02	1.50±0.03	1.46±0.02	1.47±0.02	$F_{3,36}$=0.78 CD_5=0.07 CD_1=0.10

SD: Standard deviation

Table 3: Distribution of mean weight percentage of calcium and phosphorus in different groups (remineralized)

Elements	Group I (n=10)	Group II (n=10)	Group III (n=10)	Group IV (n=10)	F values and critical difference at 5% (CD_5) and at 1% (CD_1)
Ca (mean±SD)	23.09±0.60	24.52±0.38	26.79±0.41	29.20±0.56	$F_{3,36}$=28.45 CD_5=1.42 CD_1=1.90
P (mean±SD)	15.46±0.36	11.78±0.17	16.97±0.25	17.79±0.15	$F_{3,36}$=112.80 CD_5=0.71 CD_1=0.94
Ca/P (mean±SD)	1.49±0.02	2.08±0.03	1.58±0.02	1.64±0.03	$F_{3,36}$=78.28 CD_5=0.08 CD_1=0.11

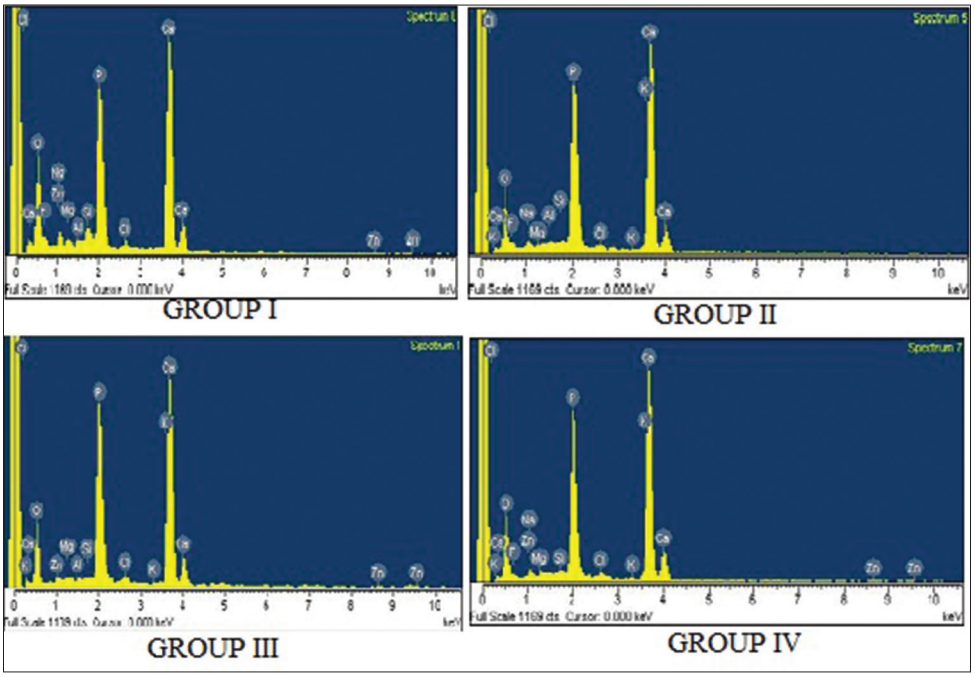

Figure 1: Elemental analysis of enamel sample by energy dispersive X-ray spectroscopy in different group after remineralization.

The primary clinical presentation of dental caries in enamel is a white spot lesion. It is an area of demineralized enamel that usually develops because of prolonged plaque accumulation.[13] The white spot lesion's opaque appearance is a first optically visible evidence of enamel caries. It occurs because of loss of subsurface enamel resulting in the diminution of enamel translucency.[14,15]

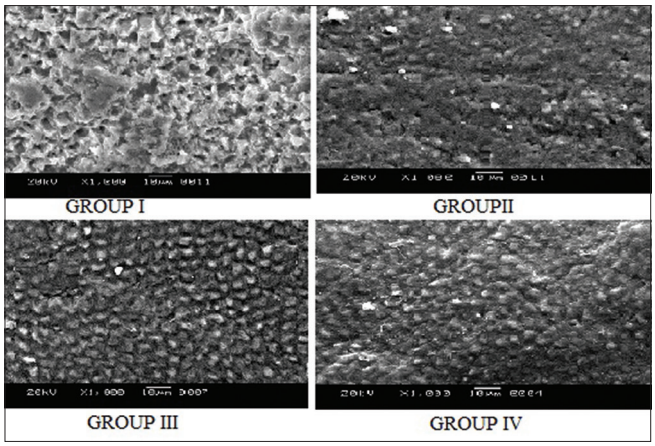

Figure 2: Structural analysis of enamel sample by scanning electron microscopy in different group after remineralization.

In the present study, enamel mineral content was tested using energy dispersive analytical X-ray of the environmental scanning electron microscopy (JOEL JSM 6360 Scanning electron microscope). Energy dispersive spectroscopy is an analytical technique that allows the detection of the elements present in the studied material. It is very versatile and can be used with any type of solid sample, from metals and ceramics to biological tissues. Energy dispersive X-ray analysis was used to determine calcium and phosphorous in weight % of sound, demineralized, and remineralized enamel in each group.

Fluorides are important adjunct in the prevention of dental caries, but it takes time for depositioning as it requires about 3 (ppm) shift the balance from net demineralization to net remineralization.[16] Several mechanisms have been suggested to achieve the anticaries effects of fluoride, including the formation of fluoroapatite which is more acid resistant than hydroxyapatite (HA), increase enhancement of remineralization, and interference of ionic bonding and also its effect extend when used *in vivo* where it inhibits the microbial growth and metabolism.[17] The protective effect of fluoride is mainly attributed to the formation of a CaF_2-like layer on the tooth surface, which acts as a fluoride reservoir. These results come in agreement with ours where the fluoride group (fluoride varnish) showed the least findings.[18]

The protective effect of CPP-ACP lies in the fact that it provides a reservoir of neutral ion pair that inhibits enamel demineralization and promotes remineralization.[19] Calcium and phosphate ions are building blocks for the remineralization process and are found in saliva. CPP–ACP complex has been introduced as a supplemental source of calcium and phosphate ions in the oral environment. The ACP is biologically active and can release calcium and phosphate ions to maintain saturation levels of calcium and phosphate at the tooth surface. It is hypothesized that, in addition to the prevention of erosive demineralization, CPP-ACP also remineralizes eroded enamel and dentine crystals.

The greatest amount of increase in mean weight percentage of calcium and phosphorus was found in Group IV that was treated with CPP-ACPF. The mean weight percentage of calcium increased from 22.32 ± 0.45 to 29.21 ± 0.56 and mean weight percentage of phosphorus increased from 15.22 ± 0.32 to 17.79 ± 0.15. The present study results can be justified on the basis that this recently introduced material combines both fluoride and CPP-ACP in one product, thus offering the protective effect of both of them. This protein nanotechnology combines specific phosphoproteins from bovine milk with forming nanoparticles of ACP. Under alkaline conditions, the calcium phosphate is present as an alkaline amorphous phase complexed by the CPP. The nanocomplexes form over a pH range from 5.0 to 9.0, while CPP-ACPF paste pH is 7.8, under neutral and alkaline conditions, the CPPs stabilize calcium and phosphate ions, forming metastable solutions that are supersaturated with respect to the basic calcium phosphate phases. The amount of calcium and phosphate bound by CPP increases as pH rises, reaching the point where the CPPs have bound their equivalent weights of calcium and phosphate.[20]

It has been reported that the CPP-ACP nanocomplexes interact with fluoride ions to produce a novel ACFP phase.[10] The identification of this novel ACFP phase is consistent with the observed additive anticariogenic effect of the CPP-ACP nanocomplexes and F.[21] The anticariogenic mechanism of fluoride is the localization of the fluoride ion at the tooth surface, particularly in plaque in the presence of calcium and phosphate ions. This localization increases the degree of saturation with respect to fluorapatite (FA), thus promoting remineralization of enamel with FA during an acid challenge. It is clear that for the formation of FA ($Ca_{10}(PO_4)_6F_2$), calcium and phosphate ions must also be present with the fluoride ions. The reported additive anticariogenic effect of the CPP-ACP nanocomplexes and F, therefore, may be attributable to the localization of ACFP at the tooth surface by the CPP, which colocalizes calcium, phosphate, and

fluoride as bioavailable ions in the correct molar ratio to form FA.

CONCLUSION

The current study concludes:

1. CPP-ACP and CPP-ACPF are effective in remineralizing the early enamel caries at the surface level.
2. CPP-ACP showed higher remineralizing potential when used in combination with fluoride than when used alone. Since additive effects were obtained when CPP-ACP is used in conjunction with fluoride, it can be recommended that CPP-ACP may be used as a self-applied topical coating after the teeth have been brushed with fluoridated toothpaste by children who have a high caries risk.
3. The presence of calcium and phosphate ions in low concentrations may also represent an effective approach to treat white spot lesion overtime, which means that whole human saliva – in favorable oral conditions – may also act as a slow but powerful remineralizing agent since these two ions are known to be part of it.
4. CPP-ACP can be used in parallel to good oral hygiene practices to help prevent the development of dental caries and erosion. This may be potentially relevant for those segments of the population, such as toddlers, infants, and the elderly, where effective brushing of teeth have poor compliance.

The present study has the obvious limitations of an *in vitro* study, namely that application of the CPP-ACP does not exactly mimic the action of the GC Tooth Mousse in the mouth. While the study supports the existing evidence of the remineralization effect of CPP-ACP on demineralized enamel surfaces, further *in situ* studies are required to establish clinical evidence for its actual capability in enhancing enamel lesion remineralization in the oral cavity. EDX does not assess the absolute concentration of calcium and phosphate. Further studies are needed that will assess the absolute concentration of calcium and phosphate and the creation of new HA crystals under experimental conditions.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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