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JULY- DEC 2016 • VOL 6 • ISSUE 2

Indian Journal of Comprehensive Dental Care

EVALUATION OF REMINERALIZATION OF ENAMEL WITH TOPICAL APPLICATION OF CALCIUM PHOSPHATE BASED AGENTS: A QUANTITATIVE ENERGY DISPERSIVE X- RAY ANALYSIS USING SCANNING ELECTRON MICROSCOPY : AN IN-SITU STUDY

1. Tawanpreet Kaur
2. Rajesh Khanna
3. Rupam Kaur
4. Aashish Handa

1. PG student, Sri Guru Ram Das Institute of Dental Sciences and Research, Amritsar.
2. Professor & Head Deptt. of Conservative Dentistry and Endodontics, Sri Guru Ram Das Institute of Dental Sciences and Research, Amritsar.
3. MDS, Reader, Sri Guru Ram Das Institute of Dental Sciences and Research, Amritsar.
4. MDS, Reader, Sri Guru Ram Das Institute of Dental Sciences and Research, Amritsar.

Corresponding author:
Name: Dr. Tawanpreet Kaur
Address: Sri Guru Ram Das Institute of Dental Sciences and Research, Amritsar.

Abstract

This study quantitatively evaluated the remineralization potential of Casein phosphopeptide-amorphous calcium phosphate, Casein phosphopeptide-amorphous calcium phosphate containing 900 ppm fluoride and Novamin on enamel subsurface lesions using scanning electron microscopy with energy dispersive X-ray analysis (SEM-EDX). Forty five patients were divided equally into three groups: group A, group B and group C (n=15), based on the remineralizing agent that was used on the experimental demineralized tooth slab. Casein Phosphopeptide-Amorphous Calcium Phosphate containing 900 ppm fluoride (CPP-ACPF) had significantly higher remineralization potential than Novamin. However no statistically significant difference was found between the remineralization potential of Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP) and Casein Phosphopeptide-Amorphous Calcium Phosphate containing 900 ppm fluoride and between the remineralization potential of Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP) and Novamin. It can be concluded that all the three agents significantly remineralized the artificial enamel subsurface lesions.

Keywords: CPP-ACP, Novamin, demineralization, remineralisation, energy dispersive X-ray analysis.

Date of Submission : 28/8/15 Date of Acceptance : 1/10/15

INTRODUCTION

Preservation of a healthy set of natural teeth for each patient should be the objective of every dental surgeon. All work in the health field is aimed at conservation of the human body and its function; similarly, dentistry's goal should be to preserve healthy, natural tooth structure.[1]

In the past, dentistry's approach towards management of a caries lesion was focused primarily on operative treatment. An increased understanding of the caries process and remineralization, and changes in caries prevalence has catalyzed the evolution in caries management from G.V. Black's “extension for prevention” to “minimally invasive”.[1]

The minimal invasive approach focuses on the usage and application of remineralizing agents to tooth structure (enamel and dentin lesions). These agents aim at controlling the demineralization/ remineralization cycle, depending upon the microenvironment around the tooth.[2]

Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), derived from milk protein casein, has been reported to reduce demineralization of the tooth structure and enhance remineralization. The anticariogenic potential and remineralizing effects have been shown in in-vitro and in situ studies.[3] Also Bioactive glass (Novamin) in an aqueous environment releases bioavailable calcium, sodium and

phosphate ions contributing to the remineralization process.[4]

Quantitative measurements of changes in a tooth's mineral content in a single caries lesion are desirable. One of the most recent techniques is Scanning Electron Microscopy with an Energy Dispersive X- ray analysis.[5]

The remineralization potential of CPP-ACP has been evaluated mainly in in-vitro conditions and rarely in the oral environment of the patients. Further, the remineralization potential of bioactive glass has so far not been evaluated and compared with CPP-ACP and CPP-ACP containing fluoride. Therefore the objective of this study was to evaluate the remineralization potential of three different calcium phosphate based remineralizing agents in the oral environment of the patients; so that caries process can be intervened at an early stage thus preserving maximum amount of natural tooth structure.

MATERIALS AND METHOD

The in-situ study was conducted on forty-five freshly extracted caries free premolars obtained from the patients undergoing orthodontic treatment. Teeth with intrinsic stains, teeth with any wasting diseases like attrition, abrasion, erosion, teeth with developmental anomalies and teeth with any restoration were excluded from the study.

The same forty-five patients from whom the premolars were extracted were taken for this study. It was ensured that the selected patients were not having any current caries activity, periodontal disease, or any other oral pathology; none of them were using antibiotics or other medications that affect salivary flow rate. Also the medical history of the patients was taken and the patients allergic to milk or milk products were not selected for the study. The treatment plan was explained to the patient and written consent from the patient was taken for following the treatment regime.

The crown of each of these premolar teeth was then sectioned to obtain total of three tooth slabs (4mm×3mm×3mm). Out of the three slabs obtained from each tooth, one tooth slab was kept, in a clean container in a humidified environment, as a positive control. The rest of the two tooth slabs were immersed in 20ml of a demineralizing solution (solution containing 2.2mM KH_2PO_4, 50mM acetic acid and 2.2mM$CaCl_2$ with pH adjusted to 4.4) for a period of 24 hours. Out of the two demineralized tooth slabs, one tooth slab was then kept, in a clean container in a humidified environment, as a negative control. The other demineralized slab was inserted / fixed into a removable appliance.

A removable mid - palatal appliance was fabricated using self-cure acrylic resin extending from the first premolar region to the first/second molar region. A rectangular trough, 5mm×4mm×2mm in size, was cut into acrylic base to house the demineralized tooth slab (4mm×3mm×3mm). Both the appliance and demineralized tooth slab were autoclaved before the tooth slab was subjected to remineralization procedure in the oral cavity of the patient.

Forty five patients were divided equally into three groups: group A, group B and group C (n=15), based on the remineralizing agent that was used on the experimental demineralized tooth slab.

Group A: Casein phosphopeptide-Amorphous calcium phosphate (CPP-ACP) used as remineralizing agent (n=15).

Group B: Casein phosphopeptide-Amorphous calcium phosphate containing 900 ppm fluoride (CPP-ACPF) used as remineralizing agent (n=15).

Group C: Novamin containing dentifrice (Vantej) used as remineralizing agent (n=15).

The remineralising agent was applied three times a day for 3 minutes for 14 days. The appliance was worn for a minimum of 10 hours during daytime. The subjects were instructed to remove their appliances while performing oral hygiene procedures, eating, drinking and sleeping. After removing the appliances, the patient was instructed to store the appliance in a sealed plastic bag in a humidified environment until reinsertion. At the end of the14th day, the tooth slab was removed from the appliance for further evaluation.

The sound, demineralized and remineralized tooth slabs thus obtained from each tooth were then examined under Scanning Electron Microscope and Energy Dispersive X- Ray analysis was used to measure mineral content. The calcium, phosphate and fluoride content, in percentage weight, thus measured in sound, demineralized and remineralized tooth slabs was tabulated and put to statistical analysis.

RESULTS

Tables I, II and III depict the mean calcium, phosphate and fluoride content (in wt %) in sound, demineralised and remineralised enamel slabs in group I, II and III.

ANOVA test showed that the difference in calcium content in remineralized enamel slabs between different groups was statistically significant; p=0.049 (Table I). The difference in phosphorus content in sound, demineralized and remineralized enamel slabs between different groups was not statistically significant; p=0.614, p=0.493 and p=0.104 respectively (Table II). The difference in fluorine content in sound, demineralized and remineralized enamel slabs between different groups was not statistically significant; p=0.476, p=0.857 and p=0.094 respectively (Table III).

DISCUSSION

Casein (derived from the Latin word 'caseus' meaning

Table-I
Statistical Analysis (anova Test) Of Calcium Content In Sound, Demineralised And Remineralised Enamel Slabs Of Different Groups Of Remineralizing Agents

Enamel slabs	Groups	Mean Ca content (Wt %)	Standard deviation	F	P
SOUND	GROUP I	44.29	[illegible]		
	GROUP II	43.68	1.73	0.692	0.506
	GROUP III	43.56	1.74		
DEMINERALISED	GROUP I	40.31	1.03		
	GROUP II	40.37	1.61	0.039	0.962
	GROUP III	40.24	.33		
REMINERALISED	GROUP I	43.26	1.21		
	GROUP II	43.61	.79	1.246	0.049
	GROUP III	42.36	1.50		

Table-II
Statistical Analysis (anova Test) Of Phosphorus Content In Sound, Demineralised And Remineralised Enamel Slabs Of Different Groups Of Remineralizing Agents

Enamel slabs	Groups	Mean P content (Wt %)	Standard deviation	F	P
SOUND	GROUP I	21.55	1.09		
	GROUP II	21.89	1.27	0.484	0.6 [illegible]
	GROUP III	21.92	1.04		
DEMINERALISED	GROUP I	18.79	0.88		
	GROUP II	19.15	1.03	0.720	0.493
	GROUP III	19.22	0.76		
REMINERALISED	GROUP I	20.88	1.20		
	GROUP II	21.68	1.14	2.392	0.104
	GROUP III	21.00	1.27		

Table-III
Statistical Analysis (anova Test) Of Fluorine Content In Sound, Demineralised And Remineralised Enamel Slabs Of Different Groups Of Remineralizing Agents

Enamel slabs	Groups	Mean F content (Wt %)	Standard deviation	F	P
SOUND	GROUP I	3.38	0.68		
	GROUP II	3.56	0.77	0.756	0.476
	GROUP III	3.50	0.69		
DEMINERALISED	GROUP I	1.88	0.63		
	GROUP II	2.02	0.61	0.155	0.857
	GROUP III	1.88	0.63		
REMINERALISED	GROUP I	3.09	0.62		
	GROUP II	3.59	0.79	2.497	0.097
	GROUP III	3.11	0.74		

cheese) is the predominant phosphoprotein in bovine milk and accounts for almost 80 percent of its total protein, primarily as calcium phosphate stabilized micellular complexes.[6]

The concept of casein phosphopeptide amorphous calcium phosphate as a remineralizing agent was first postulated in 1998. The remineralization process involves diffusion of calcium and phosphate ions through the protein/water-filled pores of the carious surface enamel into the body of the enamel lesion. Once in the body of the enamel lesion, these calcium and phosphate species increase the activities of Ca^{2+} and PO_4^{3-}, thereby increasing the degree of saturation with respect to hydroxyapatite.[5]

Novamin is the trade name for a calcium sodium phosphosilicate bioactive glass. In aqueous environments, such as saliva, sodium ions (Na^+) in calcium sodium phosphosilicate particles immediately begin to exchange with hydrogen cations (H+ or H_3O+). This rapid exchange of ions allows calcium (Ca^{2+}) and phosphate (PO_4^{3-}) species to be released from the particle structure. A modest localized, transient increase in pH occurs that facilitates the precipitation of calcium and phosphate from the particles and from saliva to form a calcium phosphate (Ca-P) layer on tooth surfaces. As the reactions and the deposition of Ca-P complexes continue, this layer crystallizes into hydroxycarbonate apatite, which is chemically and structurally similar to biological apatite.[7]

Therefore the objective of this in situ study was to evaluate the remineralisation potential of three different calcium-phosphate based remineralising agents i.e. Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP), Casein Phosphopeptide-Amorphous Calcium Phosphate containing 900 ppm fluoride (CPP-ACPF) and Novamin.

In Group I, after remineralisation the calcium content increased to 43.26 wt% from 40.31 wt %; phosphorus content increased to 20.88 wt% from 18.79 wt %; whereas fluorine content increased to 3.09 wt% from 1.88 wt %. In Group II, after remineralisation the calcium content increased to 43.61 wt% from 40.37 wt %; phosphorus content increased to 21.68 wt% from 19.15 wt %; whereas fluorine content increased to 3.59 wt% from 2.02 wt %. In Group III, after remineralisation the calcium content increased to 42.36 wt% from 40.24 wt %; phosphorus content increased to 21.00 wt% from 19.22 wt %; whereas fluorine content increased to 3.11 wt% from 1.88 wt %. These findings are in concurrence with the study by Narayana SS, et al.[4] (2014) who found that calcium, phosphorus and fluorine content in enamel slabs remineralized by CPP-ACP, CPP-ACPF and Novamin was higher as compared to their respective demineralized controls. These results are also in concurrence with the study by Hegde MN and Moany A.[5] (2012) who found that 10% CPP-ACP paste significantly remineralized the artificial enamel subsurface lesions in vitro and the remineralization rates increased with the time for which the enamel was exposed to the CPP-ACP paste. The calcium/phosphate ratio increased from 1.6227 at the end of

7th day to 1.6947 at the end of 14th day; and was 1.9373 at the end of 35th day. These findings are further supported by Grewal N, et al.[8] (2013) who found that application of CPP-ACP for 14 days remineralized erosive enamel lesions.

These findings are also in concurrence with those of Kumar VL, et al.[9](2008) who found that CPP-ACP showed higher remineralization potential when used in combination with fluoride (13% reduction in lesion depth) as compared to CPP alone (10% reduction) or fluoride alone (7% reduction). Moreover in an animal study, Reynolds EC, et al.[10] (1995) showed that rats receiving 0.5% CPP-ACP plus 500 ppm fluoride had significantly lower caries scores than those receiving either 0.5% CPP-ACP or 500 ppm fluoride alone.

ANOVA test of statistical analysis shows that the difference in calcium content in sound and demineralized enamel slabs between different groups was not statistically significant; p=0.506 and p=0.962 respectively. However the difference in calcium content in remineralized enamel slabs between different groups was statistically significant; p=0.049. Also the difference in net gain in calcium content between different groups was found to be statistically significant; p=0.013. The difference in phosphorus content in sound, demineralized and remineralized enamel slabs between different groups was not statistically significant; p=0.614, p=0.493 and p=0.104 respectively. However the difference in net gain in phosphorus content between different groups was found to be statistically significant; p=0.037. The difference in fluorine content in sound, demineralized and remineralized enamel slabs between different groups was not statistically significant; p=0.476, p=0.857 and p=0.094 respectively. Also the difference in net gain in fluorine content between different groups was found to be not statistically significant; p=0.152.

These findings are in concurrence with the study by Krithikadatta J, et al.[11] (2013) who found that fluoride incorporated in 10% CPP-ACP complex did not improve the degree of remineralization when compared to 10% CPP-ACP used alone. The plausible hypothesis for this observation in 10% CPP-ACP was attributed either to the presence of fluoride ions in saliva or subjacent plaque that could have played a role in the formation of fluorapatite or to the pH range in the subjects being closed to neutral as at pH range from 7.0-6.0 CPP-ACPF and CPP-ACP solutions are found to produce similar levels of remineralization. This hypothesis can explain the observations of the present study.

These findings are also in accordance with the study by Mehta R, et al.[12](2013) who found no significant difference between the remineralization potential of CPP-ACP and CPP-ACPF after 14 days (p=0.21). Patil N, et al.[13] (2013) also found no significant difference between the remineralization potential of CPP-ACP and CPP-ACPF (p=0.502). Also Shetty S, et al.[14] (2014) found statistically insignificant difference between the remineralization potential of CPP-ACP and CPP-ACPF (p=1.000).

Further, in a study Lata S, et al.[15] (2010) found that the combination of fluoride and CPP-ACP does not provide any additive remineralization potential when compared to fluoride varnish alone at the surface level. These results are further supported by Jayarajan J, et al.[16] (2011) who found that though CPP-ACPF showed marginally more amount of remineralization than CPP-ACP but the difference between remineralization potential of CPP-ACPF and CPP-ACP was not statistically significant ($p<0.05$).

However these findings are not in concurrence with the study by Srinivasan N, et al.[17] (2010) who found remineralization effect of CPP-ACP with 900 ppm fluoride to be superior to that of CPP-ACP alone. It was observed that post-erosion, CPP-ACP increased surface microhardness by 46.24% whereas CPP-ACPF increased surface microhardness by 64.25%. These findings are also not in concurrence with Reynolds EC, et al.[18] (2008) who found dentifrice with 2% CPP-ACP plus 1100-ppm fluoride to be superior to CPP-ACP alone or fluoride alone in promoting in-situ remineralization ($P < 0.01$). Further the results of the present study are also not in concurrence with Mehta AB, et al.[19] (2014) who compared the remineralization potential of Novamin and CPP-ACP and found Novamin to have higher (statistically significant) remineralization potential compared to CPP-ACP (p=0.013).

Therefore, it can be concluded that three different calcium phosphate based remineralizing agents i.e. Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP), Casein Phosphopeptide-Amorphous Calcium Phosphate containing 900 ppm fluoride (CPP-ACPF) and Novamin do have a remineralizing potential when applied for 14 days. But there is insufficient clinical trial evidence (in quantity, quality or both) to make a recommendation regarding the long-term effectiveness of Casein derivatives and Novamin in preventing caries in vivo.

CONCLUSION

The following conclusions were drawn from the present study:

1. Casein Phosphopeptide-Amorphous Calcium Phosphate containing 900 ppm fluoride (CPP-ACPF) had significantly higher remineralization potential than Novamin.
2. However no statistically significant difference was found between the remineralization potential of Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-

ACP) and Casein Phosphopeptide-Amorphous Calcium Phosphate containing 900 ppm fluoride and between the remineralization potential of Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP) and Novamin.

3. Scanning Electron Microscope images revealed mineral deposits on the surface of remineralized enamel slabs in all the three groups, thus indicating the remineralization potential of these agents.

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