Evaluation of the remineralization potential of two non-fluoridated remineralizing pastes using scanning electron microscope with energy dispersive X-ray analysis: A randomized controlled in-vitro trial

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CERTIFICATE

This is to certify that the dissertation titled "Evaluation of the remineralization potential of two non-fluoridated remineralizing pastes using scanning electron microscope with energy dispersive X-ray analysis: A randomized controlled in-vitro trial" is a bonafide workdone by Dr. VIJAYASANKARI.V, Postgraduate student, during the course of the study for the degree of "Master of Dental Surgery" in Department of Pedodontics and Preventive Dentistry, KSR Institute of Dental Science and Research, Tiruchengode during the period of 2015-2018.

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CONTENTS

INTRODUCTION

Dental caries is a localized chemical dissolution of the tooth surface caused by metabolic events taking place in the biofilm covering the affected area. Biofilm is a prerequisite for the carious lesion to occur. Any shift in the ecology and metabolic activity of the biofilm can cause an imbalance in the equilibrium between the tooth minerals and the biofilm in the form of pH fluctuations. The shift in the pH can influence the chemical composition of the tooth structure. The tooth surface apatites are liable to such chemical modifications on countless events from the very moment of eruption.[45] Hydroxyapatite is the main component of enamel (95%) and dentine (75%). The crystals of hydroxyapatite are hexogonal in cross section which are arranged to form enamel rods. The solubility of hydroxyapatite is the primary determinant of dissolution of enamel and it is related to pH.[52]

The enamel surface is in a state of dynamic equanimity with its surrounding environment.[45] When the pH in the surrounding medium drops down, the solubility of the apatite crystals increases[45] resulting in demineralization phase. During this phase, environment of the oral cavity becomes undersaturated with mineral ions compared to the minerals content of the tooth.[50] The reverse takes place when the pH increases and remineralization takes place in the interface between biofilm and the tooth surface.[45] When the demineralization phase prolongs, excessive loss of mineral ions takes place making the enamel surface sufficiently porous to be seen clinically called as white spot lesion (WSL). This appearance has also been described as an early, initial or incipient lesion.[45] This process may progress further that eventually leads to cavitation and total destruction of the tooth. Thus formation of caries is an ubiquitous, natural process. It is impossible to prevent the formation of the biofilm and its metabolic activity, but the progression of the disease can be controlled.[18] As dental caries is a slow process, during early stages non-invasive interventions can convert the initial lesion from an active to inactive state.[26]

Traditionally, the management of dental caries is through surgical-restorative approach. It involves diagnosis of carious lesions followed by surgical intervention to remove and restore the affected part of the tooth. It is known that restoring the carious tooth alone might not stop the disease process. This results in replacement of larger and larger subsequent restorations and shorter associated survival times, resulting in a more invasive procedures over time. It is estimated that 71% of all restorative treatments are performed on previously restored teeth, with recurrent carious lesions as a predominant cause.[31]

The preventive approach of identification, conservative, non-restorative treatment of incipient caries saves both dental manpower for profession as well as expense and suffering for the patient.[33] Thus the concept of minimal intervention dentistry has changed the perspective of caries management, from "extension for prevention," as proposed by GV Black. Today's cavity preparation is designed to preserve the health of the tooth over lifetime. The goal of modern dentistry is to manage non-cavitated carious lesions non-invasively through remineralization in an attempt to prevent disease progression, and to improve strength, esthetics and function of teeth.[22] Diagnosis of carious lesions at earlier stages and its remineralization has led to new era in the modern preventive dentistry.[33]

Remineralization is defined as the process whereby calcium and phosphate ions are supplied from a source external to the tooth to promote ion deposition into crystal voids in demineralized enamel, to produce net mineral gain.[12] For the past 60 years fluoride has been used as a gold standard treatment for remineralizing teeth.[61] The topical application of fluoride agents, including dentifrices, mouthwash solutions, gels, and varnishes can help in remineralization of initial enamel carious lesion.[16] It results in the formation of calcium fluoride like layer on the enamel surface thus preventing the subsequent acid attack and partially reduces

the enamel mineral loss.[16] Intake of fluoride during tooth formation systemically has been found to be effective in prevention of caries.[52]

According to recommended daily allowances, a dose of 0.1mg fluoride/kg body weight /day in children up to 8 years of age are considered to be safe causing no significant form of fluorosis in permanent teeth. However it is crucial to consider the amount of fluoride in water, tooth paste, dietary supplements and topical applications which have been identified as source of enamel fluorosis. Moreover, the probable toxic dose of fluoride is 5 mg/kg of bodyweight which triggers therapeutic intervention and hospitalization.[52] Since most of the dental products contain sufficient amount of fluoride, there is a high chance of chronic consumption of these products by young children which could result in exceeding the toxic dose.[42] Research has been looking for an alternative caries remineralizing agents which should be as effective as fluoride.

Newer tooth remineralizing agents such as calcium phosphate based technologies and biomimetic dental products containing nano sized hydroxyapatite particles are emerging in modern preventive dentistry. Synthetic nano hydroxyapatite (nHAP) has the physical and chemical properties similar to apatite structure in enamel[42] making it most biocompatible and bioactive material. nHAP has strong affinity to tooth and adsorbs strongly on the enamel surfaces. Nano sized crystals smaller than 100nm improves bioactivity of the agent due to the increase in the superficial surface area and wettability of nHAP. It promotes the remineralization by increasing the saturation level of calcium and phosphate in saliva.[16] With advancements in nanotechnology, incorporation of nano sized biomimetic apatite particles in dentrifices and mouthrinses have been increased. Since 1980, nHAP has been used in tooth paste in Japan and it was accepted as anti caries agent in 1993.[42] Various in-vitro and in-situ studies in the literature have proved that nHAP has the potential to remineralize the artificial carious lesion. A study

done by Roveri et al in 2009[52] demonstrated that biomimetic nanosized hydroxyapatite particles produced an apatitic coating deposition on the demineralized enamel surface. Huang et al in 2009[30] proved that nHAP had the potential to remineralize the initial enamel carious lesions in which 10% nHAP has been proved to be most effective. In an in situ study by Najibfard et al in 2011[42], nHAP dentrifice caused remineralization comparable to fluoride dentrifice. They suggested that nHAP dentrifice can be used as an effective alternative to fluoride dentrifice.

Aclaim® (Group pharmaceuticals ltd) is one of the commercially available nHAP toothpaste in India. According to the studies done by Verma P et al 2013[65] and Singh A et al 2017[57] Aclaim® has both densensitizing and remineralizing effect. So, the present study attempts to synthesize nHAP toothpaste at 1% and 10% concentrations and compare its remineralization efficacy with commercially available non-fluoridated remineralizing paste.

AIMS

AIMS

The present study was conducted with following aims:

1. To formulate experimental nHAP tooth pastes with 1% and 10% concentrations.

2. To evaluate the remineralization potential of experimental 1% and 10% nHAP tooth paste.

3. To compare the remineralization potential of experimental 1% nHAP with commercially available nHAP toothpaste using energy dispersive X-ray analysis (EDX).

4. To compare the remineralization potential of experimental 10% nHAP with commercially available casein phosphopeptide amorphous calcium phosphate (CPP ACP) using scanning electron microscope with energy dispersive X-ray analysis (SEM EDX).

HYPOTHESIS

The study hypothesis was that experimental nHAP pastes were equally effective in comparison with commercially available pastes and control.

REVIEW OF LITERATURE

Shen P, Cai F, Nowicki A, Vincent J, Reynolds EC (2001)[54] conducted a randomized controlled cross over in-situ double blind study to evaluate the ability of sugar free CPP ACP chewing gum in remineralizing enamel subsurface lesions. A total of 30 human subjects wore removable palatal appliances with six human-enamel half-slabs inset containing sub-surface demineralized lesions. The appliances were inserted immediately before gum chewing for 20 minutes and then retained for another 20 minutes. This was performed 4 times a day for 14 days. At the completion of each treatment, the enamel half-slabs were paired with their respective demineralized control half-slabs, embedded, sectioned, and subjected to microradiography and densitometric image analysis, for measurement of the level of remineralization. They found that addition of CPP-ACP to either sorbitol or xylitol based gum resulted in an increase in enamel remineralization.

Andersson A, Skold-Larsson K, Hallgren A, Petersson LG, Twetman S (2007)[2] compared the effects of a dental cream containing complexes of CPP ACP and fluoride mouthwashes on the regression of WSLs. A total of 26 healthy adolescents exhibiting 60 teeth with 152 visible WSL sites on incisors and canines were included. Baseline visual scoring and laser fluorescence measurements were carried out. The patients were randomly assigned to two different remineralization protocols A) daily topical applications of a dental cream containing CPP ACP (Topacal) for 3 months followed by a 3-month period of daily tooth brushing with fluoridated dentifrice, or B) daily 0.05% sodium fluoride mouthwash combined with fluoridated dentifrice for 6 months. The laser fluorescence measurements and visual scoring were repeated at 1, 3, 6 and 12 months. A significant improvement (regression of WSL) of the clinical WSL scores was found over time in both groups, but CPP ACP showed a statistically significant difference ($p < 0.01$) in the decrease in number of lesions after 12 months. There was no

statistically significant difference in laser fluorescence measurements. They concluded that both the treatment could promote the regression of white spot lesions, but CPP ACP provided a more favourable aesthetic outcome.

Kumar VL, Itthagarun A, King NM (2008)[35] investigatedthe efficacy of CPP ACP containing Tooth Mousse on the remineralization of enamel lesions and compared its efficacy to that of a fluoride containing toothpaste. A total of 50 specimens were prepared from permanent third molars and were randomly assigned in to 5 groups. Group A, fluoridated toothpaste (positive control), Group B, non-fluoridated toothpaste (negative control), Tooth Mousse containing CPP ACP was tested by three different means: Group C (as a toothpaste); Group D (as a topical coating); and Group E (as a topical coating after treating the sections with Group A paste). All the specimens underwent a pH cycling for a period of 10 days with respective groups. Polarizing light microscopy and microradiography were utilized to record the lesion depth and the mineral content of each lesion before and after the 10 days of pH cycling. They found that the lesion depth decreased by 13.1 per cent in Group E and it was statistically significant. They concluded that CPP ACP containing Tooth Mousse showed a higher remineralizing potential when applied as a topical coating after the use of fluoridated toothpaste.

Pai D, Bhat SS, Taranath A, Sargod S, Pai VM (2008)[46] performed an in vitro study to evaluate the remineralization of incipient enamel lesions by the topical application of CPP ACP using laser fluorescence and scanning electron microscope (SEM). Sixty caries free teeth were randomly divided in to 3 groups; 40 teeth were used as test sample (CPP ACP), 10 as positive control (artificial saliva), 10 as negative control (normal saline). The samples were demineralized and then remineralized by the topical application of CPP ACP for a period of 14 days. The results showed that both laser fluorescence readings and SEM scores of test samples after

remineralization were highly significant (p <0.001). They concluded that CPP ACP can prevent demineralization and also bring about remineralization in enamel lesions.

Yengopal V, Mickenautsch S (2009)[67] did systematic review with meta analysis to assess the caries preventive effect of CPP ACP. Five in situ randomized control trials (RCT) werepooled for meta-analyses. The results of the clinical in situ trials indicated a short-term remineralization effect of CPP ACP. Additionally, in vivo RCT results suggested a caries preventing effect for long-term clinical CPP ACP use. They concluded that further in vivo randomized trials are needed to confirm these initial results.

Bailey DL, Adams GG, Tsao CE, Hyslop A, Escobar K, Manton DJ, Reynolds EC, Morgan MV (2009)[4] compared the effect of CPP ACP on post orthodontic WSL among adolescents of 12-18 years of age for a period of 12 weeks. Four hundred and eight WSLs in 45 participantswere randomly divided in to 2 groups: CPP ACP group and placebo group. Clinical assessments were performed according to International Caries Detection and Assessment System (ICDAS) II criteria. They found that WSLs with severity codes 2 and 3 at baseline had a significantly greater chance of regressing at 12 weeks in the CPP ACP group compared with those in the placebo group.

Srinivasan N, Kavitha M, Loganathan SC (2009)[59] conducted a in-situ study to compare the remineralization potential of pastes containing CPP ACP and CPP ACP with 900 ppm fluoride on human enamel softened by a cola drink. Forty five enamel specimens obtained from human third molar teeth were eroded in a cola drink for 8 minutes and then attached to intra-oral devices worn by five volunteers. The specimens were subjected to three different in situ remineralization protocols using: (1) CPP ACP (Group I), (2) CPP ACP with 900 ppm

fluoride (Group II), and (3) saliva (Group III, control). Vickers microhardness measurements were recorded at baseline, demineralization and remineralization stages. The results revealed statistically significant differences in the mean microhardness values between pastes containing CPP ACP and CPP ACP with 900 ppm fluoride. They concluded that both pastes substantially remineralized the softened enamel, with the CPP ACP and fluoride combination showing higher remineralization potential than CPP ACP.

Altenburger MJ, Gmeiner B, Hellwig E, Wrbas KT, Schirrmeister JF (2010)[1] performed an in-vivo study to evaluate the effect of CPP ACP on initial enamel caries in pits and fissures using DIAGNOdent and visual assessment. A total of 32 volunteers with premolars and molars showing DIAGNOdent scores between 15 and 20 were randomly assigned in to 2 treatment groups. The intervention period consisted of 3 weeks, first 2 weeks of wash out period and third week of treatment period. During a wash-out period of 2 weeks and during the 3-week treatment period all subjects used only standard fluoride toothpaste without any oral hygiene products. During the treatment period, one group additionally applied a CPP ACP containing cream on the respective fissures for 3 minutes, once per day. At days 1, 8, 15, and 22, DIAGNOdent measurements and a visual assessment of the fissures were done. They found that CPP ACP group showed significantly lower fluorescence values at day 15 and day 21 compared to the control group. There was no significant difference in the visual assessment scores in both the groups.

Huang S, Gao S, Cheng L, Yu H (2010)[28] investigated the combined effects of nanohydroxyapatite and Gallachinensis (GCE) on remineralization of initial enamel lesion. In vitro demineralized bovine enamel blocks were subjected to a pH-cycling regime for 12 days. Each daily cycle included 3-4 minutes application with one of five treatments: NaF (positive

control), deionised water (negative control), crude aqueous extract of GCE, nHAP and GCE with nHAP. The samples were subsequently evaluated using a microhardness tester, polarised light microscopy (PLM), X-ray diffraction (XRD) and SEM. GCE–nHAP combined treatment group showed significant reduction in lesion depth and more mineral deposition occurred in the lesion body. They concluded that there was a significant synergistic effect of combined GCE and nHAP treatment on promoting the remineralization of initial enamel lesion.

Brochner A, Christensen C, Kristensen B, Tranæus S, Karlsson L, Sonnesen L, Twetman S (2011)[6] compared the effect of CPP ACP on post orthodontic WSLs among fifty adolescents. Twenty two belonged to the intervention group (CPP ACP) and 28 in the control group (standard fluoride toothpaste). The outcome was measured by quantitative light induced fluorescence (QLF) and visual scores from digital photographs at baseline and 4 weeks. Both the groups showed a statistically significant regression of WSL from the baseline, but there was no difference between the groups. They concluded that topical application of CPP ACP resulted in significantly reduced fluorescence and reduced area in the lesion size after 4 weeks.

Najibfard K, Ramalingam K, Chedjieu I, Amaechi BT (2011)[42] evaluated the efficacy of nHAP dentifrices on caries remineralization and demineralization inhibition by conducting a double-blind randomized crossover in situ study. A total of 30 adults wore an intra oral appliance containing 3 demineralized enamel blocks and one healthy enamel block cut from each of 30 molars, were exposed respectively to dentifrices of A) 5% nHAP, B) 10% nHAP, C) 1100 ppm fluoride, and D) 10% nHAP in 4 phases lasting 28 days per phase. Baseline and post-test mineral loss and lesion depth were quantified using microradiography. They concluded that nHAP dentifrice caused remineralization comparable to a fluoride dentifrice, and inhibited caries

development, thus suggesting that an HAP dentifrice can be an effective alternative to fluoride toothpaste.

Ferrazzano GF, Amato I, Cantile T, Sangianantoni G, Ingenito A (2011)[21] investigated the remineralizing effect of GC Tooth Mousse on early dental enamel lesions using SEM analysis. 40 volunteers were randomly divided into two groups. In group (CPP ACP group) two demineralized enamel specimens were placed on the buccal surfaces of the first molars and were instructed to apply GC Tooth Mousse only on the right-sided specimen and a placebo mousse on the left, for 1 month. In Group B (control group) two enamel specimens were placed into the mouth without any intervention. The results of SEM analysis revealed a diffuse and homogenous mineral coating reducing the surface alteration in CPP ACP group. They concluded that CPP ACP was able to promote remineralization of early enamel lesions.

Tschoppe P, Zandim DL, Martus P, Kielbassa AM (2011)[62] performed anin-vitro study to evaluate the effects of nHAP toothpastes on the remineralization of bovine enamel and dentine subsurface lesions. A total of 70 enamel specimens were randomly divided in 5 groups and exposed to an aqueous remineralizing solution for two and fiveweeks (37˚ C).Brushingprocedures were performed with the respective toothpaste/storage solution slurry twice daily:1) storage in remineralizing solution only; 2) additional brushing with 20 weight% zinc carbonate nano hydroxyapatite, $ZnCO_3$/nHAP; 3) 24 weight% $ZnCO_3$/n-Hap; 4) 0.14 weight% amine fluoride; 5) 7 weight% pure nHAP. Differences in mineral loss (DDZ) before and after storage/treatment were microradiographically evaluated. They concluded that toothpastes containing nHAP revealed higher remineralizing effects compared to amine fluoride toothpastes.

Zhang Q, Zou J, Yang R, Zhou X (2011)[68] conducted an in-vitro study to evaluate the remineralizing effect of CPP ACP cream on the artificial enamel lesion of the primary teeth andto assess its caries prevention efficiency. A total of 90 enamel specimens were randomly divided in to 3 groups: group A: distilled and deionized water, group B: CPP ACP, group C: sodium fluoride solution. The enamel surface microhardness was measured before, after demineralization and 30 days after remineralization. The enamel specimens were also subjected to SEM analysis. They found that there was a significant increase in microhardness in all the groups. On comparison between the groups, CPP ACP group showed significant increase in microhardness than sodium fluoride group. The results of SEM analysis revealed CPP ACP group exhibited more homogenous arrangement of crystals than sodium fluoride group. They concluded that CPP ACP cream effective in remineralizing enamel lesions in primary teeth.

Hegde MN, Moany A (2012)[27] quantitatively evaluated the remineralization potential of CPP ACP paste on enamel subsurface lesions using SEM EDX analysis. A total of 90 specimens were randomly assigned into two groups: group 1 contained 15 specimens (control group) and group 2 contained 75 specimens (study group). The study group was subdivided into five groups of 15 specimens per group. Each subgroup was treated with remineralizing paste [10% CPP ACP paste seven days (subgroup 2a), 14 days (subgroup 2b), 21 days (subgroup 2c), 28 days (subgroup 2d), and 35 days (subgroup 2e), twice daily for three minutes. SEM EDX was done to measure mineral content before, after demineralization and remineralization procedure. They found that all the study groups showed very highly significant differences between Calcium phosphorus ratios of the demineralized and remineralized samples. There was no significant difference seen in the control group. They concluded that the remineralization achieved was

dose-dependent as the remineralizing rate increased with the timeof exposure of enamel to CPP ACP paste.

Comar LP, Souza BM, Gracindo LF, Buzalaf MA, Magalhães AC (2013)[14] conducted an in-vitro trial to evaluate the preventive potential of experimental pastes containing 10% and 20% nHAP, with or without fluoride, on dental demineralization. According to the surface hardness, bovine enamel (n=15) and root dentin (n=15) specimens were divided into 9 groups : control (without treatment), 20 Nanop paste (20% HAP), 20 Nanop paste plus (20% HAP + 0.2% NaF), 10 Nanop paste (10% HAP), 10 Nanop paste plus (10% HAP + 0.2% NaF), placebo paste (without fluoride and HAP), fluoride paste (0.2% NaF), MI paste (CPP ACP), and MI paste plus (CPP ACP + 0.2% NaF). All the specimens were subjected to pH cycling model for 7 days and the dental subsurface demineralizat   ion was analyzed using cross-sectional hardness. They found that 0.2% NaF significantly reduced the loss of enamel and dentin sub surface hardness. Experimental Nanop pastes, regardless of the addition of fluoride, were unable to reduce demineralization in-vitro

Rallan M, Chaudhary S, Goswami M, Sinha A, Arora R, Kishor A (2013)[49] determined the effect of three remineralizing agents on eroded enamel of human primary anterior teeth. A total of 40 primary anterior teeth were randomly divided in to 4 groups. Group I: CPP ACP, Group II: CPP ACP with fluoride (CPP ACPF) and Group III: fluoridated toothpaste and Group IV: artificial saliva (control). A thin layer of respective paste were applied on the enamel surface and left undisturbed for 3 minutes and then stored in artificial saliva for 8 hours. The Knoop microhardness of the labial surface of enamel was measured at baseline, after erosion and after the remineralization procedures. They found that CPP ACPF showed significant increase in microhardness compared to the other groups.

de Carvalho FG, Vieira BR, Santos RL, Carlo HL, Lopes PQ, de Lima BA (2014)[16]
analyzed the protective effect of remineralizing agents on enamel caries lesions. An in vitro
study conducted using 48 human enamel specimens were randomly divided in to 4 groups: (1)
control (without agent); (2) fluoride varnish (Duraphat); (3) nHAP paste (DesensibilizeNano P);
and (4) CPP ACPF paste (MI Paste Plus). Artificial carious lesion were developed in all the
specimens and subjected to cariogenic challenge (pH cycling) for 7 days. The surface
microhardness was evaluated at baseline, after artificial caries lesion formation and after pH
cycling. The percentage of surface microhardness recovery (%SMHR) was performed, and the
surface morphology was evaluated by atomic force microscopy (AFM). They found after the pH
cycling, the nHAP group showed significantly higher Knoop hardness number (KHN) and
%SMHR values than varnish whereas CPP ACP group showed no increase in KHN. On
evaluation by AFM nano HAP group showed protective layer formation with globular deposits
on the surface and concluded that nHAP paste has protective effect against in-vitro enamel caries
development.

Haghgoo R, Rezvani MB, Salehi Zeinabadi M (2014)[25] compared sodium fluoride
mouthrinse and nHAP at different concentrations (0-2-5-10%) for remineralization of initial
enamel carious lesions. A total of 60 human premolars were randomly divided and subjected to
microhardness evaluation at baseline, post demineralization and remineralization. They
concluded that nHAP and NaF mouthrinses can greatly enhance remineralization and increase
tooth microhardness though the results were not statistically significant.

Vano M, Derchi G, Barone A, Covani U (2014)[63] conducted a double blind randomized
controlled trial among 105 subjects to compare the efficacy in reducing dentin hypersensitivity.
The subjects were divided into 3 groups receiving treatment, 1) nHAP 15% tooth paste fluoride

free; 2) fluoride toothpaste 3) placebo. By using air blast and tactile test dentin hypersensitivity was evaluated at baseline and after 2 weeks and 4 weeks. In addition, subjective evaluation using visual analog scale (VAS) was also used. The results showed statistically significant lower values for sensitivity and VAS scores for group 1. They concluded that fluoride free nano hydroxyapatite is considered as an effective desensitizing agent providing quick relief from symptoms of sensitivity after 2 and 4 weeks.

Mielczarek A, Michalik J (2014)[40] performed an in-vitro study comparing toothpaste containing nHAP and 1,450 ppm F; toothpaste containing 1,450 ppm F; and Placebo group (distilled water). Ninety human enamel specimens were randomly divided into 3 groups and subjected to demineralization and remineralization phases. The surface microhardness of each specimen were measured at each phase. They concluded surface microhardness increased significantly in all the groups but none of the group reached the baseline level.

Memarpour M, Fakhraei E, Dadaein S, Vossoughi M (2015)[39] conducted a 1 year randomized controlled trial among 146 preschool children with WSLs on maxillary anterior teeth. They compared the effect of CPP ACP, fluoride varnish and oral hygiene with dietary counseling. They used dmft index and scored dental probe. There was a significant reduction in the size of the lesion in CPP ACP group. They concluded that preventive interventional methods play an important role in reducing white spot lesions in children. Oral hygiene instruction together with the application of CPP ACP and fluoride varnish was an effective method to reduce WSL size and dmft index values in primary teeth.

Llena C, Leyda AM, Forner L (2015)[37] performed a double blind prospective study to evaluate the effect of CPP ACP and CPP ACPF versus fluoride varnish on the remineralization

of enamel white spot lesions. The participants were divided randomly into three groups. Groups A: GC Tooth Mouse (CPP ACP), Group B: Mi Paste Plus (CPP ACPF) and Group C: Duraphat fluoride varnish. WSLs were categorized according to the ICDAS criteria (ICDAS II; grades 0–3) and assessed by laser fluorescence (DIAGNOdent) at baseline and at 4, 8 and 12 weeks. DIAGNOdent values were significantly reduced in Group B (CPP ACPF) at 4 weeks, and in Groups A (CPP ACP) and group C (Fluoride varnish) at 8 weeks. They concluded that CPP-ACPF have a significant effect on smooth surface caries.

Vyavhare S, Sharma DS, Kulkarni VK (2015)[66] evaluated the effect of nHAP (10%), CPP-ACP (10%), NaF (1000 ppm) and Deionized water (negative control) on remineralization of initial enamel lesion. A total of 26 human permanent incisors were subjected to demineralization and pH cycling. Surface microhardness measurements were performed before, after demineralization and after 3, 6, 9 and 12 days of pH cycling. The specimens were examined by SEM. Percentage surface microhardness of nHAPand fluoride was significantly greater than CPP ACP and negative control. When observed under SEM, nHAP particles were deposited on the demineralized surface whereas CPP-ACP does not show any significant surface remineralization. They concluded that nHAP and fluoride had the potential to remineralize initial enamel lesions.

Souza BM, Comar LP, Vertuan M, Fernandes Neto C, Buzalaf MA, Magalhães AC (2015)[58] conducted a randomized cross over double blind in situ study. They evaluated the effect of an experimental paste containing hydroxyapatite in nanoparticles/ fluoride on dental de-remineralization. Thirteen subjects took part in this study which was performed in 4 phases (14 days each). Four sound and 4 predemineralised specimens were worn intraorally at each phase corresponding to the following treatments: Nanop Plus (10% HA, 0.2% NaF, nano-HA/fluoride),

MI Paste Plus (CPP-ACP, 0.2% NaF), F (0.2% NaF) and placebo. The demineralization and remineralisation was quantified by transversal microradiography. They concluded that Nanop Plus significantly reduced the dentin demineralization and improved enamel remineralization.

Haghgoo R, Ahmadvand M, Moshaverinia S (2016)[24] evaluated the remineralizing effect of topical NovaMin and nHAP on caries-like lesions. Thirty human primary incisors were randomly subjected to demineralization / remineralization cycle and surface microhardness of each tooth was measured at baseline, post demineralization and remineralization. They concluded that no significant differences were detected in their efficacy and both nHAP and NovaMin were effective for remineralization of caries like lesion in primary teeth.

Ebadifar A, Nomani M, Fatemi SA (2017)[17] assessed the effect of nHAP on microhardness of artificially created carious lesion. A total of 80 extracted teeth were randomized in two groups, Group A contained nHAP and fluoride and Group B contained fluoride alone. They concluded that toothpaste containing nHAP showed significant increase in microhardness and was more effective than the toothpaste without NHA for the purpose of remineralization.

Esteves-Oliveira M, Santos NM, Meyer-Lueckel H, Wierichs RJ, Rodrigues JA (2017)[20] performed a in vitro study to investigate the caries preventive effect of nHAP containing toothpastes. Two hundred enamel specimens were prepared from bovine incisors and randomized into 5 groups, which received different fluoride treatments: fluoride-free toothpaste (0 ppm F^-), as negative control; AmF (1400 ppm F^-) anti-caries; AmF/NaF/ $SnCl_2$/Chitosan (700 ppm F^-/700 ppm F^-/3500 ppm Sn^{2+}), anti-erosion; NaF/KNO_3 (1400 ppm F^-), anti-erosion; and nHAPcontaining (0 ppm $F-$) toothpastes. Changes in mineral loss and lesion depth were determined using transversal microradiography. All toothpastes caused significantly less

demineralization than negative control except for nHAP. They concluded that both anti-erosive and anti-caries toothpastes reduced mineral loss to a similar extent, whereas the fluoride-free nHAP containing toothpaste did not show inhibition of caries demineralization in vitro.

Vano M, Derchi G, Barone A, Pinna R, Usai P, Covani U (2017)[64] conducted a randomized double-blind clinical trial to compare the efficacy in reducing dentin hypersensitivity of a dentifrice formulation containing nHAP with a fluoride dentifrice. A total of 115 participants were randomly divided into 3 groups: Group 1: Nano-hydroxyapatite 2%gel toothpaste (fluoride free), Group 2: Fluoride gel toothpaste, Group 3: Placebo. The participant's dentin hypersensitivity was evaluated at baseline, after 2 and 4 weeks using airblast, tactile tests. Subjective evaluation was done by visual analogue scale. They concluded that the application of nHAP in gel toothpaste was an effective desensitizing agent providing relief from symptoms after 2 and 4 weeks.

Shaik ZA, Rambabu T, Sajjan G, Varma M, Satish K, Raju VB, Ganguru S, Ventrapati N (2017)[53] quantitatively assessed the remineralization potential of CPP ACP, Vantej and Icon by SEM EDX. A total of 78 maxillary premolars were randomly divided into 3 groups. Group 1:Vantej (Bioactive glass), Group 2: CPP ACP, Group 3: Icon (Resin infiltration). All the samples were subjected to EDX analysis before, after demineralization and after remineralization. Calcium and phosphate ratio increased significantly in CPP ACP group compared to the other groups. They concluded that CPP ACP showed greater potential of remineralization followed by Vantej and Icon.

Kamath P, Nayak R, Kamath SU, Pai D (2017)[32] conducted a in-vitro double blind randomized controlled trial to evaluate the remineralization potential of commercially available

remineralization agents on WSLs in primary teeth. Forty extracted or exfoliated primary teethwere selected and randomized into 4 groups: Group I: FTCP (Tricalcium phosphate with fluoride), Group II: fluoridated dentifrice, Group III: CPP ACPF, and Group IV: nHAP. They evaluated at baseline, post demineralization and post remineralization values by DIAGNOdent and SEM EDX. Evaluation by SEM showed favorable surface changes in all the four study groups. Intragroup comparison of DIAGNOdent and EDX readings showed a highly significant difference between baseline, post demineralization, and post remineralization values. However, the intergroup comparison was statistically non significant. They concluded that all the test agents were comparable in their remineralization potential.

METHODOLOGY

The present randomized controlled invitro study was conducted from the Department of Pediatric and Preventive Dentistry, KSR Institute of Dental Science and Research (KSRIDSR). The study design and protocol was analyzed and approved by the Institutional Review Board and Institutional Ethics Committee of KSRIDSR, Tiruchengode, Tamil Nadu.

Armamentarium

1. 35 human primary molars
2. Contra-angle hand piece
3. Self-cure resin
4. Nail polish
5. Gloves
6. Mask
7. Eye wear
8. Plastic containers
9. Double faced diamond disc
10. 10% formaldehyde
11. 0.1% thymol solution
12. Demineralization solution
13. pH meter (Slope Labtronics, Model LTD 11, Panchkula, Haryana, India)
14. Incubator (Coslab, ISO 9001:2000, Ambala Cant, Haryana, India)
15. Pipette
16. Burette
17. Hot air oven
18. Magnetic stirrer

19. Stirring rod

20. Dry ball mill (PM 100; Retsch Corporation, Germany)

21. Aclaim® Tooth paste (Sorbitol, Glycerine, Silica, Purified water, Hydroxyapatite, Cocomidopropylbetaine, Hydroxyethyl cellulose, Titanium dioxide, Flavour, Sodium saccharin; Group pharmecuticals Ltd, Bangalore, India)

22. CPP ACP paste (GC tooth mousse, Recaldent™, Belgium, Germany)

Inclusion criteria

1. Extracted first and second human primary molars

Exclusion criteria

1. Teeth with developmental defects

2. Teeth with caries and white spot lesion

3. Teeth subjected to previous treatment

Specimen preparation

Thirty five extracted human primary molars were selected for this study. The selected teeth were cleaned using an ultrasonic scalerto remove soft tissue debris. They were disinfected for 2 days using 10% formaldehyde. The teeth were decoronated at the cemento enamel junction and sectioned into buccal and lingual halves using a double faced diamond disc mounted on a contra-angle hand piece. Each specimen was embedded in self-cure acrylic with the enamel surface exposed thus involving 65 enamel specimens from 35 teeth. All the specimens were stored in 0.1% thymol solution till the study was carried out.

Elemental analysis by EDX

The analysis was done in South Indian textile research association (SITRA), Coimbatore. The specimens were kept in moisture free environment and care was taken to avoid contact with air or moisture. The specimens were placed on a metal mounting block and quantification of calcium and phosphorus content was done using EDX analysis.

Baseline EDX analysis

A 3×3 mm square window was prepared on enamel surface of each specimen by applying nail polish varnish in rest of the tooth surface. All the specimens were subjected to EDX analysis for assessing the mineral content at baseline.

Preparation of the demineralization solution

Demineralizing solution was prepared in the Centre for Nano Science and Technology, KSR College of Technology, Tiruchengode. The composition of demineralizing solution used was as follows:

- 2.2 mM calcium chloride, $CaCl_2.2H_2O$ (LobaChemiePvt. Ltd., Mumbai, Maharashtra, India)

- 2.2 mM monosodium phosphate, $NaH_2PO_4.7H_2O$ (LobaChemiePvt. Ltd., Mumbai, Maharashtra, India)

- 0.05 M lactic acid, $C_3H_6O_3$ (LobaChemiePvt.Ltd.,Mumbai, Maharashtra, India).

A digital pH meter was used to check pH during and after preparation of solution.The final pH was adjusted to 4.5 with 50% sodium hydroxide (NaOH).

Induction of artificial carious lesions

Each of the specimens were immersed separately into sterile plastic container containing 12ml of prepared demineralizing solution and incubated for a period of 96 hours at 37°C in universal incubator. After incubation, the teeth were washed with deionized water and dried with the help of an air syringe. All the specimens were assessed for the formation of white opaque areas. The demineralised specimens were subjected to EDX analysis for measuring the loss in mineral content.

Formulation of experimental nano hydroxyapatite tooth paste

Experimental nano hydroxyapatite tooth pastewas prepared in the Centre for Nano Science and Technology, KSR College of Technology, Tiruchengode.

Synthesis of nano hydroxyapatite

nHAP was synthesised according to the method described by Chen et al 2002[10]. HAP was prepared from calcium nitratetetrahydrate $(Ca(NO_3)_2.4H_2O)$ and ammonium dihydrogen phosphate $(NH_4H_2PO_4)$ by sol-gel procedure. The calcium/phosphorus stoichiometric ratio of pure HAP was kept constant as 1.67. Calcium nitratetetrahydrate(3.93 grams) was weighed and mixed with 100 ml distilled water and stirred using a magnetic stirrer. Ammonium dihydrogen phosphate was weighed and mixed with 100 mL distilled water and stirred using a magnetic stirrer. This solution was added drop wise to Calcium nitratetetrahydratesolution using a burette till the colour of the solution changed to milky white. The solution was allowed to stir for 1 hour and the pH of the solution was found to be 6.0. The stable HAP can be obtained when the pH value is above 10. Hence to raise the pH of the solution, ammonia was added drop wise to the solution using pipette, optimizing the pH to 10.4. The solution was allowed to stir for 1 hour and

it was then kept in a hot air oven for 48 hours at 33K. The dried solution was collected, powdered, and then calcined at 773 K to obtain HAP powder. The obtained HAP was ground in a dry ball mill at 400 rpm for 1 hour to obtain fine nHAP.

Characterization of formulated HAP

The Fourier Transform Infrared (FTIR) spectra of theHAP were obtained using an FTIR spectrometer (Spectrum 100; PerkinElmer, USA) in the frequency range from 4000 to 400 cm−1 using a potassium bromide (KBr) pellet. The pellet was obtained by mixing 200:1 ratio of KBr and HAP. The mixture was grinded initially in an agate mortar and then the pellet was obtained using a hydraulic pellet maker. The obtained pellet was used to ascertain the functional groups through measurement of infrared spectra.

The FTIR spectrum shows peaks of HAP. The peak 3572 Cm^{-1} indicates OH^- group[41] and 3431Cm^{-1} stretching peak shows presence of water molecule.[19] 2351 Cm^{-1} peak shows C-H group present material. 1643 Cm^{-1} and 1373 Cm^{-1} peaks indicate CO_3^{2-} functional group present in material.[7] 960 Cm^{-1} and 568Cm^{-1} peaks represent phosphate group present in HAP.[19]

Preparation of experimental nHAP tooth paste

Two experimental nHAP tooth pastes with the concentration of 1% nHAP and 10% nHAP were prepared by mixing with the ingredients of standard tooth paste. The ingredients added to prepare nHAp tooth paste were as follows:

S.no	Ingredients	Role	Quantity/100g
1.	Sorbitol	Humectant	10000mg
2.	Propylene glycol	Humectant	30000mg
3.	Silica	Inorganic thickening agent	1000mg
4.	Sodium lauryl sulphate	Organic thickening agent and emulsifier	1000mg
5.	Nano hydroxyapatite	Active ingredient	1000mg for 1% nHAP paste 10000mg for 10% nHAP paste
6.	Sodium carboxy methyl cellulose	Gum	1000mg
7.	Titanium dioxide	Whitening agent	500mg
8.	Methyl paraben	Antibacterial agent	100mg
9.	Propyl paraben	Antibacterial agent	20mg

10.	Clove oil	Flavor	Quantity required
11.	Sodium saccharin	Sweetening agent	30mg
12.	Distilled water	Bulking agent	Quantity required for 100gm

Remineralization procedure

The demineralised specimens were randomly divided into 5 groups for remineralization procedure.

- GROUP I –Aclaim® (n = 15)

- GROUP II – 1% experimental nHAP tooth paste (n = 15)

- GROUP III – 10% experimental nHAP tooth paste (n = 15)

- GROUP IV – CPP ACP paste (n = 15)

- GROUP V – control (n = 5)

Preparation of artificial saliva

The artificial saliva was prepared according to McKnight Hane and Whitfort formula (1992).[38] The composition used was as follows (grams/litre):

Methyl p- hydroxybenzoate(Merck)	2.00
Sodium carboxy methyl cellulose(Merck)	10.00
Potassium chloride (KCl) (Merck)	0.025
Magnesium chloride dihydrate ($MgCl_2.2H_2O$) (LobaChemiePvt. Ltd.,)	0.059
Calcium chloride dehydrate ($CaCl_2.2H_2O$) (Merck)	0.166
Dipotassium hydrogen phosphate (K_2HPO4) (Rankem)	0.804
Monopotassium hydrogen phosphate (KH_2PO_4)(Merck)	0.326

- The pH of artificial saliva was adjusted to 6.75 with potassium hydroxide (KOH)

Remineralization regimen

The specimens in groups I, II, III and IV were treated with respective tooth paste twice daily for 14 days. Specimens were rubbed with respective tooth paste with the help of cotton applicator for 3 minutes, washed with deionized water, and then placed in artificial saliva maintained at ambient temperature. In the control group, specimens were only washed with deionized water and placed in artificial saliva. Artificial saliva was renewed every 24 hours just before immersion of freshly treated samples.

The specimens in group I and group II were subjected to EDX analysis to measure the change in mineral content after remineralization procedure. Similarly the specimens in group III, IV and V were subjected to SEM with EDX analysis to analyse the surface topographical changes after remineralization.

Structural analysis

The surface characteristics of the demineralized and remineralized enamel specimens were analysed by SEM. The specimens wereplacedonametalmountingblockandthenkept inside the gold sputter coater (Q150R, Quorum technologies, UK). After sputtering the specimens were observed under SEM (GeminiSEM, Zeiss microscopy, Germany) at ×5,000 and ×10,000 magnifications at 15kv.

Statistical analysis

The statistical analysis was done using IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp. The statistical significance was set at p≤0.05. The distribution of normality for the data were done using Kolmogorov-Smirnov and Shapiro-Wilks test. The intragroup comparison was done using Friedman test and Wilcoxan Signed Rank Test.

KruskallWallis and Mann Whitney U test were employed to analyse the change in the values for intergroup comparison.
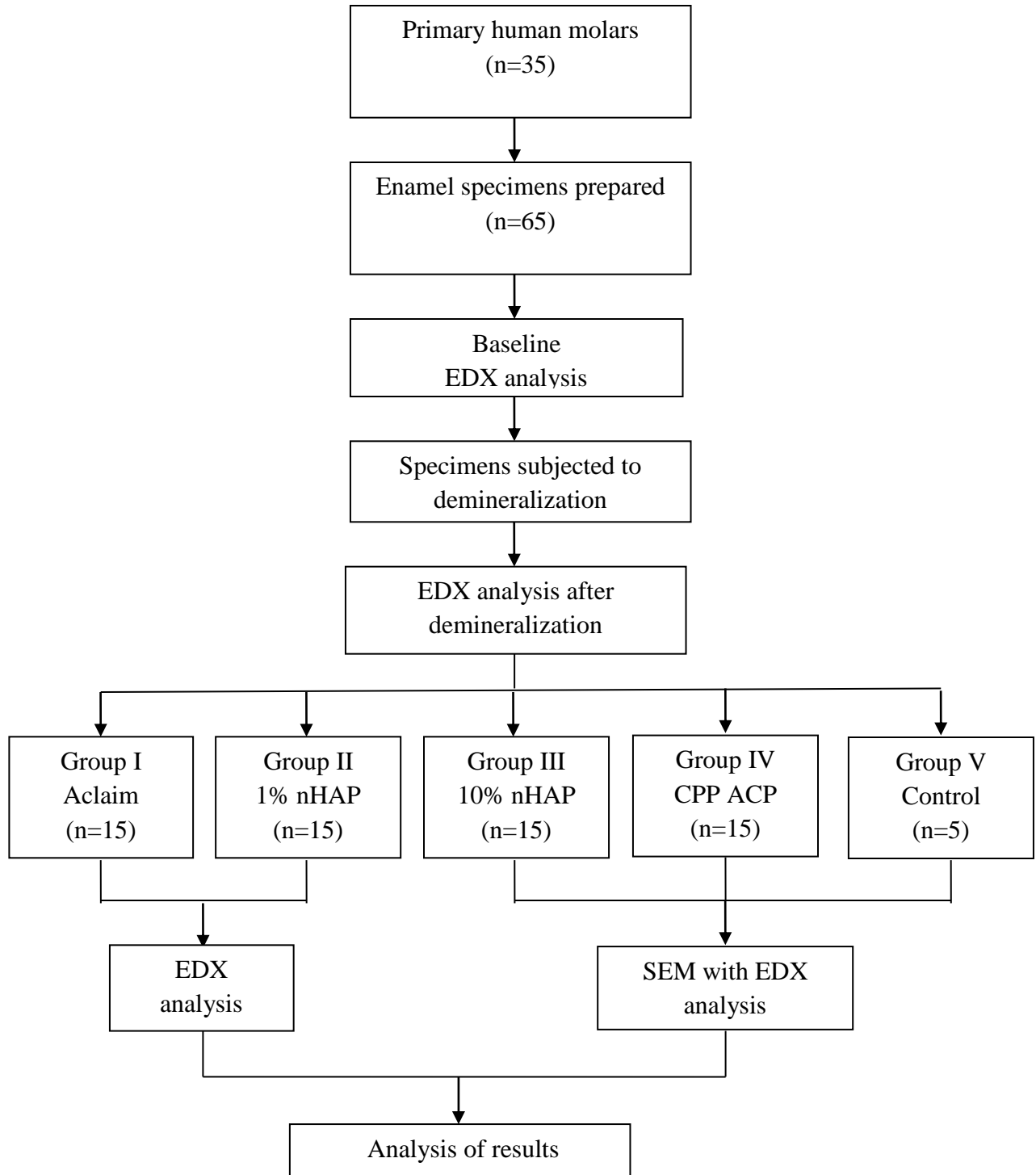
Flow Chart of Methodology

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┌─────────────────────────────┐
│   Primary human molars      │
│        (n=35)               │
└─────────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│  Enamel specimens prepared  │
│        (n=65)               │
└─────────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│         Baseline            │
│       EDX analysis          │
└─────────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│   Specimens subjected to    │
│      demineralization       │
└─────────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│    EDX analysis after       │
│      demineralization       │
└─────────────────────────────┘
```

| Group I Aclaim (n=15) | Group II 1% nHAP (n=15) | Group III 10% nHAP (n=15) | Group IV CPP ACP (n=15) | Group V Control (n=5) |

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        │                                    │
        ▼                                    ▼
┌──────────────┐                    ┌──────────────────┐
│     EDX      │                    │   SEM with EDX   │
│   analysis   │                    │     analysis     │
└──────────────┘                    └──────────────────┘
        │                                    │
        └──────────────┬─────────────────────┘
                       ▼
            ┌──────────────────────┐
            │  Analysis of results │
            └──────────────────────┘
```

Figure 1. Prepared enamel specimens
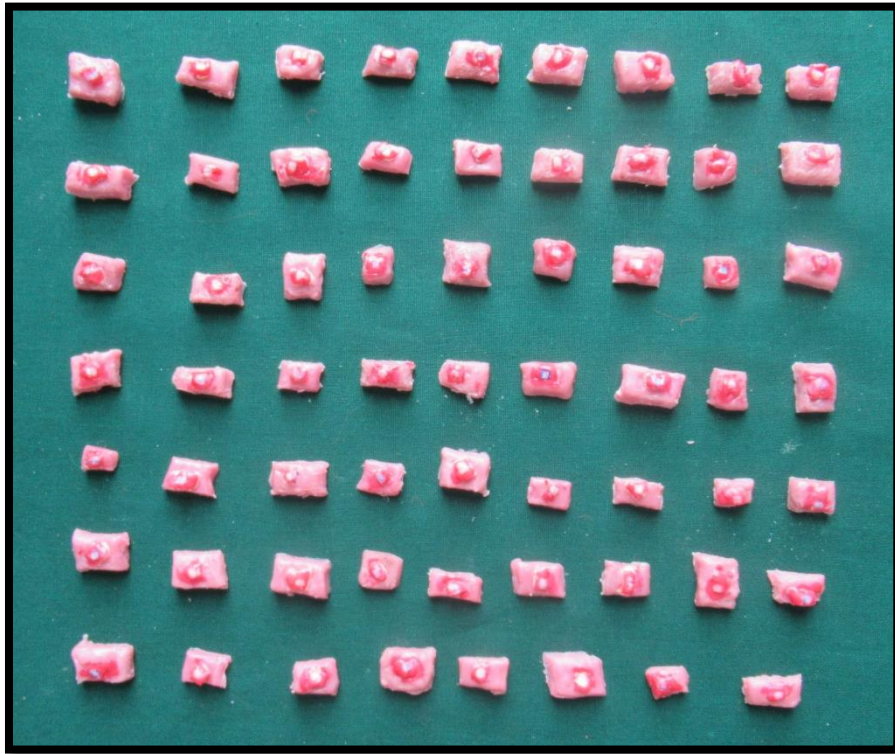

Figure 2. Armamentarium for the preparation of demineralizing solution

Figure 3. Components for demineralizing solution

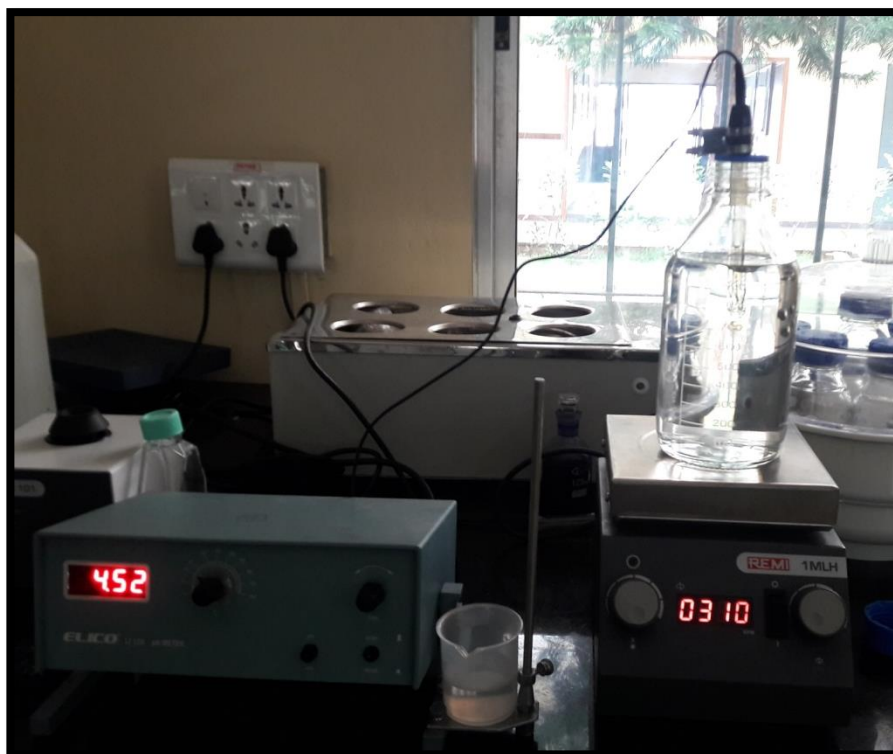**Figure 4. Preparation of demineralizing solution**
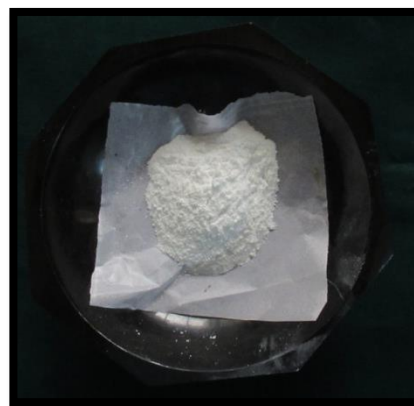
Figure 5. Preparation of nano hydroxyapatite powder
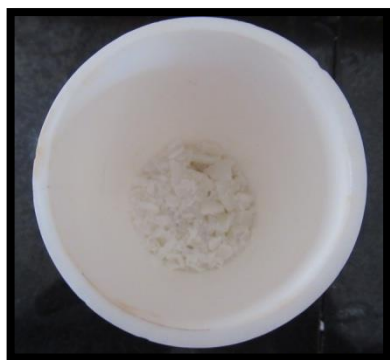
Figure 6. Components for toothpaste preparation

Figure 7. Armamentarium for toothpaste preparation

Figure 8. Preparation of tooth paste

Figure 9. 1% and 10% nHAP paste

Figure 10.Test groups

Figure 11. Colour coded sample distribution

Figure 12. Components for artificial saliva

Figure 13. Preparation of artificial saliva

Figure 15. Ball Milling machine

Figure 16. Fourier Transform Infrared Spectroscopy

Figure 17. Scanning electron microscope with energy dispersive X-ray analysis attachment

RESULTS

Table 1.Comparison of mean distribution of calcium in weight percentage in different stages of demineralization and remineralization cycle within each group

Group	N	Baseline Mean±S.D(wt%)	After demineralization Mean±S.D (wt%)	After remineralization Mean±S.D (wt%)	p value*
Group I (Aclaim®)	15	22.27±7.216	16.53±5.817	23.67±3.792	**0.001**
Group II (1% nHAP)	15	22.87±3.204	17.40±5.591	21.87±4.086	**0.001**
Group III (10% nHAP)	15	22.33±4.237	14.07±4.217	22.33±2.320	**<0.001**
Group IV (CPP ACP)	15	24.00±2.952	18.13±3.357	20.13±3.543	**0.001**
Group V (Control)	5	25.60±4.159	20.20±4.550	24.40±3.050	0.211

*Friedman test

Table 1 shows the comparison of mean values of calcium wt% at baseline, after demineralization and after remineralization in each group. All the groups except Group V showed significant change ($p<0.05$) in calcium wt% after each stage of demineralization and remineralization cycle.

Graph1. Comparison of mean values of calcium weight percentage
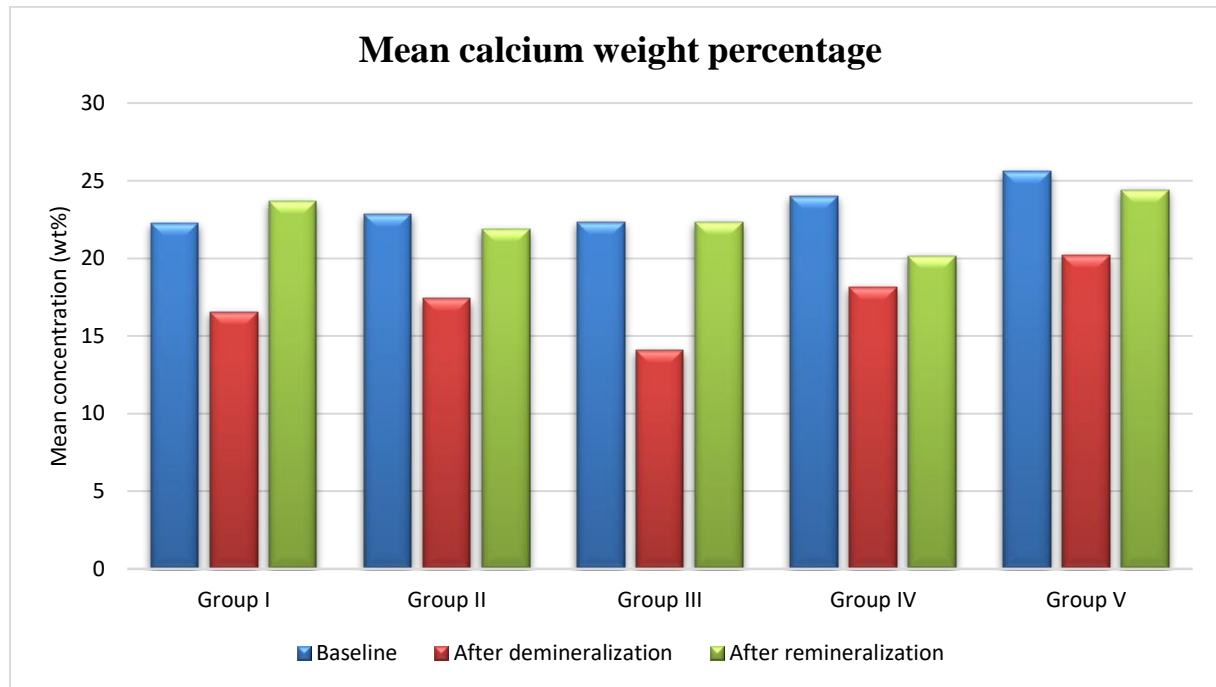
Graph 1 illustrates the comparison of mean values of change in calcium wt% at baseline, after demineralization and after remineralization in each group.

Table 2. Post hoc analysis of change in calcium weight percentage within each group

Group	Baseline to Demineralization p value**	Demineralization to Remineralization p value**	Baseline to Remineralization p value**
Group I (Aclaim®)	**0.002**	**0.001**	0.271
Group II (1% nHAP)	**0.001**	**0.006**	0.977
Group III (10% nHAP)	**0.001**	**0.001**	0.723
Group IV (CPP ACP)	**0.001**	**0.043**	**0.009**
Group V (Control)	0.109	0.588	0.104

**Wilcoxan Signed Rank test

Table 2 shows the comparison of mean values of calcium wt% within the groups. All the 4 groups except Group V showed a statistically significant (p<0.05) change in the calcium wt% from baseline to demineralization. Similarly, there was a statistically significant change in all the 4 groups in calcium wt% from demineralization to remineralization, except in Group V. Only Group IV showed a statistically significant change in calcium wt%, when the baseline values were compared with the post remineralization values.

Table 3. Intragroup and intergroup comparisons for the change in calcium wt% in different stages of demineralization and remineralization cycle

Group	Change in baseline to demineralization Mean±S.D (wt%)	Change in demineralization to remineralization Mean±S.D (wt%)	Change in baseline to remineralization Mean±S.D (wt%)	p value*
Group I (Aclaim®)	5.84±5.90	7.25±6.97	1.40±8.01	**0.002**
Group II (1% nHAP)	5.30±5.47	4.37±4.94	-.92±5.81	**0.001**
Group III (10% nHAP)	8.03±4.43	8.05±3.88	.02±4.11	**0.001**
Group IV (CPP ACP)	5.88±3.02	2.02±3.98	-3.85±4.50	**<0.001**
Group V (Control)	5.38±6.82	4.18±4.46	-1.20±6.42	0.549
p value***	0.227	**0.011**	0.073	

* Friedman test; ***Kruskal Wallis ANOVA

Table 3 shows intergroup and intragroup comparison for change in calcium wt%. In intergroup comparison, there was a statistically significant difference (p=0.011) between the groups when demineralization to remineralization values of calcium wt% was compared. All the groups, except Group V showed statistically significant difference in the change of calcium wt% in the intragroup comparison.

Table 4.Post hoc analysis for change in calcium weight percentage from demineralization to remineralization between the groups

Intergroup comparison		p value[****]
Group I (Aclaim®)	**Group II**	0.494
	Group III	0.330
	Group IV	**0.024**
	Group V	0.827
Group II (1% nHAP)	**Group III**	**0.034**
	Group IV	0.135
	Group V	0.965
Group III (10% nHAP)	**Group IV**	**<0.001**
	Group V	0.190
Group IV (CPP ACP)	**Group V**	0.176

****Mann Whitney U test

Table 4 shows post hoc analysis for intergroup comparison. There was a statistically significant difference between Group I and Group IV (p=0.024). Similarly, on comparing Group II with Group III (p=0.034) and Group III with Group IV (p=<0.001), statistically significant difference was observed.

Table 5. Comparison of mean distribution of phosphorus in weight percentage in different stages of demineralization and remineralization cycle within each group

Group	N	Baseline Mean±S.D (wt%)	After demineralization Mean±S.D (wt%)	After remineralization Mean±S.D (wt%)	p value*
Group I (Aclaim®)	15	12.40±2.501	9.80±2.883	13.67±1.877	**<0.001**
Group II (1% nHAP)	15	13.73±1.792	11.00±3.381	12.73±2.187	**0.034**
Group III (10% nHAP)	15	13.07±2.604	9.20±2.808	13.93±1.831	**<0.001**
Group IV (CPP ACP)	15	14.60±1.957	11.33±1.718	12.73±1.792	**<0.001**
Group V (Control)	5	14.80±2.775	12.60±2.702	13.80±1.924S	0.165

*Friedman test

Table 5 shows the comparison of mean values of phosphorus wt% in each group after each stage of demineralization and remineralization cycle. All the groups except Group V showed significant changes in phosphorus wt% after each stage.

Graph 2. Comparison of mean values of phosphorus weight percentage

Mean phosphorus weight percentage
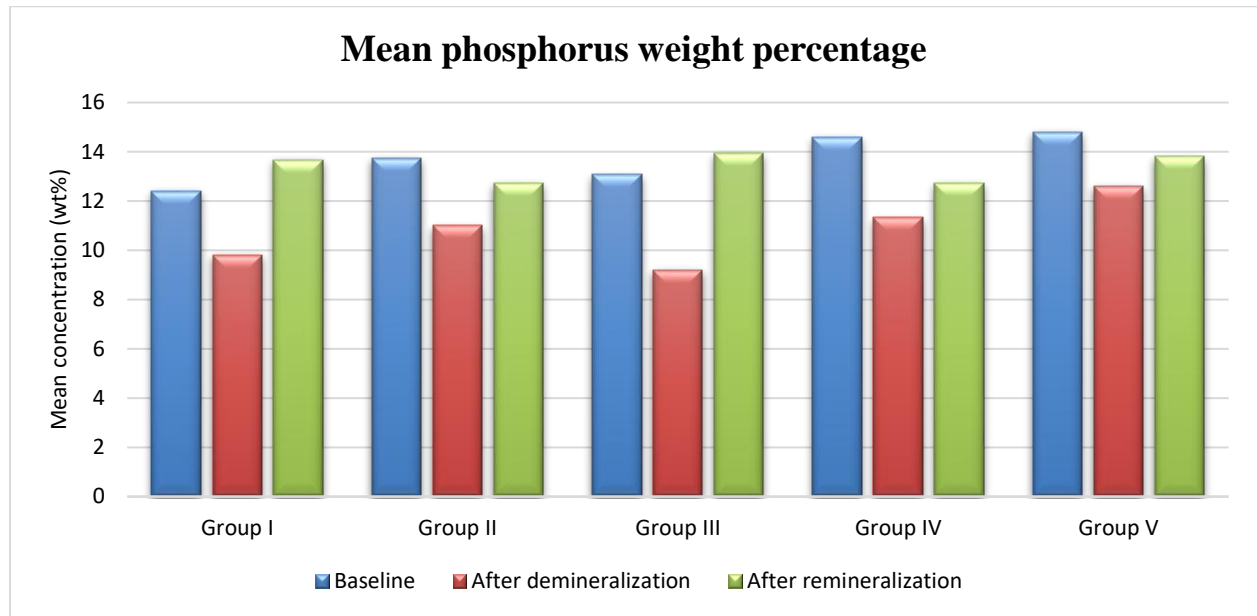
Graph 2 illustrates the comparison of mean values of phosphorus wt% in each group after each stage of demineralization and remineralization cycle.

Table 6. Post hoc analysis of change in phosphorus weight percentage within each group

Group	Baseline to Demineralization p value**	Demineralization to Remineralization p value**	Baseline to Remineralization p value**
Group I (Aclaim®)	**0.001**	**0.001**	0.213
Group II (1% nHAP)	**0.008**	0.092	0.323
Group III (10% nHAP)	**0.001**	**0.001**	0.177
Group IV (CPP ACP)	**0.001**	**0.031**	**0.024**
Group V (Control)	0.279	0.498	0.414

**Wilcoxan Signed Rank test

Table 6 shows the comparison of mean values of phosphorus wt% within the groups. All the groups except Group V showed statistically significant change in the phosphorus wt% from baseline to demineralization. Group I, III and IV showed statistically significant change in phosphorus wt% from demineralization to remineralization. Only Group IV showed a significant change in phosphorus wt%, when the baseline values were compared with the post remineralization values.

Table 7. Intragroup and intergroup comparisons for the change in phosphorus weight percentage in different stages of demineralization and remineralization cycle

Group	Change in baseline to demineralization Mean±S.D (wt%)	Change in demineralization to remineralization Mean±S.D (wt%)	Change in baseline to remineralization Mean±S.D (wt%)	p value*
Group I (Aclaim®)	2.48±2.21	3.60±3.79	1.12±3.50	**0.008**
Group II (1% nHAP)	2.66±4.18	1.69±3.38	-0.97±3.02	**0.002**
Group III (10% nHAP)	3.85±3.30	4.74±2.62	0.89±2.93	**0.011**
Group IV (CPP ACP)	3.18±1.54	1.42±2.30	-1.7±2.93	**<0.001**
Group V (Control)	1.97±4.61	0.91±4.39	-1.06±2.22	0.449
p value***	0.519	**0.021**	**0.035**	

*Friedman test;***Kruskal Wallis ANOVA

Table 7 shows intragroup and intergroup comparison for change in phosphorus wt%. In intergroup comparison there was a statistically significant difference (p=0.021) between the groups, when change in demineralization to remineralization value of phosphorus wt% was compared. Similarly, statistically significant difference (p=0.035) was observed when change in baseline to remineralization values were compared. All the groups, except Group V showed statistically significant difference in the change of phosphorus wt% in the intragroup comparison.

Table 8. Post hoc analysis for change in phosphorus weight percentage from demineralization to remineralization and baseline to remineralization between the groups

Intergroup comparison		Change in demineralization to remineralization p value****	Change in baseline to remineralization p value****
Group I (Aclaim®)	Group II	0.198	0.130
	Group III	0.110	0.852
	Group IV	0.101	**0.019**
	Group V	0.694	0.206
Group II (1% nHAP)	Group III	**0.014**	0.059
	Group IV	0.983	0.468
	Group V	0.965	0.694
Group III (10% nHAP)	Group IV	**0.002**	**0.007**
	Group V	0.81	0.127
Group IV (CPP ACP)	Group V	0.570	0.896

****Mann Whitney U test

Table 8 shows post hoc analysis for intergroup comparison. There was a statistically significant difference, when change in demineralization to remineralization values were compared between Group II and Group III (p=0.014), Group III and Group IV (p=0.002) Similarly, on comparing Group I with Group IV (p=0.019) and Group III with Group IV (p=0.007), statistically significant differences were observed between the baseline values and the post remineralization values.

Table 9.Comparison of mean distribution of calcium phosphorus ratio in weight percentage in different phases of demineralization and remineralization cycle within each group

Group	N	Baseline Mean±S.D	After demineralization Mean±S.D	After remineralization Mean±S.D	p value*
Group I (Aclaim®)	15	2.00±.655	1.67±.488	1.87±.352	0.156
Group II (1% nHAP)	15	1.73±.458	1.80±.414	1.93±.258	0.311
Group III (10% nHAP)	15	1.80±.414	1.47±.516	1.87±.352	**0.045**
Group IV (CPP ACP)	15	1.87±.352	1.80±.414	1.87±.352	0.819
Group V (Control)	5	1.80±.447	1.40±.548	2.00±.000	0.174

*Friedman test

   Table 9 shows the comparison of mean values of calcium phosphorus ratio in each groups after each stage of demineralization and remineralization cycle. Group III showed significant changes in calcium phosphorus ratio after each stage.

Graph 3. Comparison of mean values of calcium phosphorus ratio weight percentage
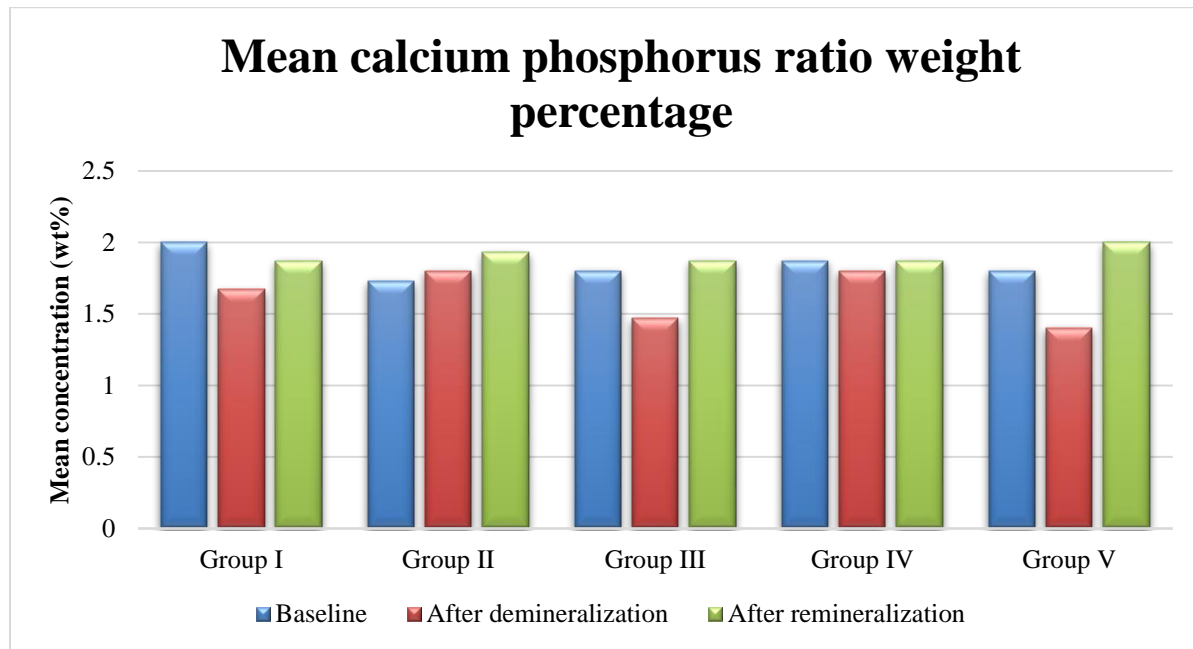
Graph 3 illustrates the comparison of mean values of change in calcium phosphorus ratio wt% in each group after each stage of demineralization and remineralization cycle.

Table 10. Intragroup and intergroup comparisons for the change in calcium phosphorus ratio weight percentage on different stage of demineralization and remineralization cycle

Group	Change in baseline to demineralization Mean±S.D	Change in demineralization to remineralization Mean±S.D	Change in baseline to remineralization Mean±S.D	p value*
Group I (Aclaim®)	0.16±0.60	0.13±0.22	-0.03±0.66	0.420
Group II (1% nHAP)	0.09±0.28	0.16±0.25	0.06±0.40	0.247
Group III (10% nHAP)	0.17±0.32	0.05±0.26	-0.12±0.25	0.057
Group IV (CPP ACP)	0.72±0.20	-0.006±0.19	-0.07±0.27	0.886
Group V (Control)	0.17±0.47	0.21±0.26	0.03±0.23	0.819
p value**	0.707	0.461	0.250	

* Friedman test; **Kruskal Wallis ANOVA

Table 10 shows intragroup and intergroup comparison for change in calcium phosphorus ratio wt%. There was no statistically significant difference between the groups and within the groups after each stage of demineralization and remineralization cycle.

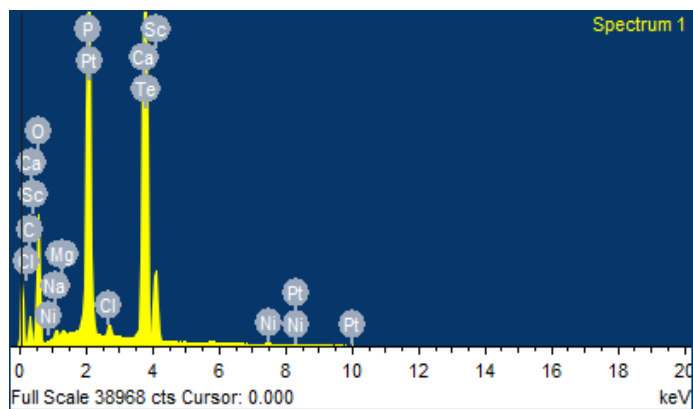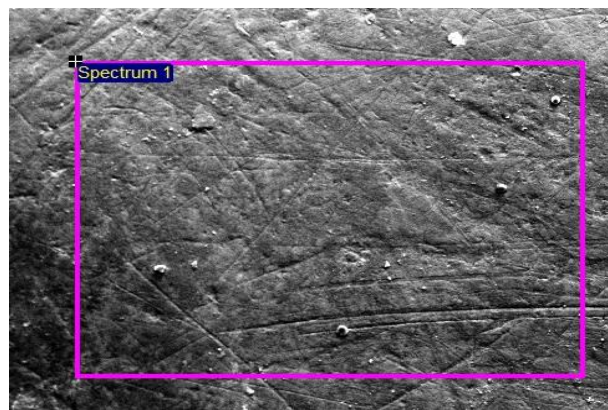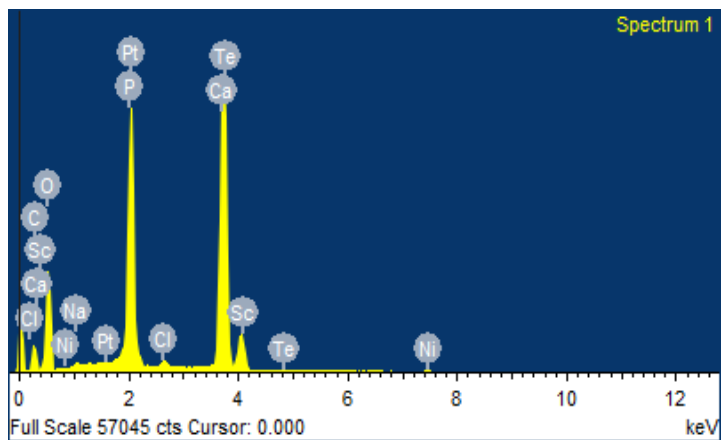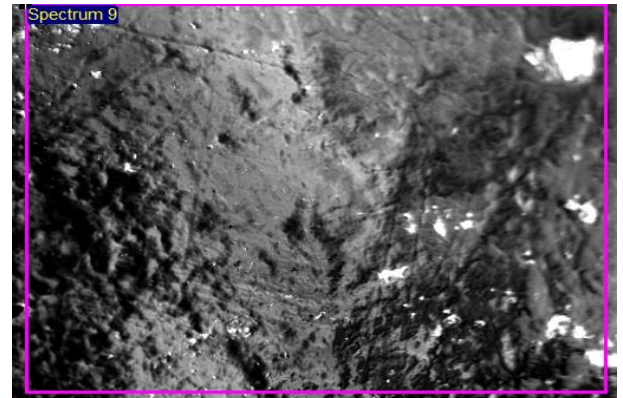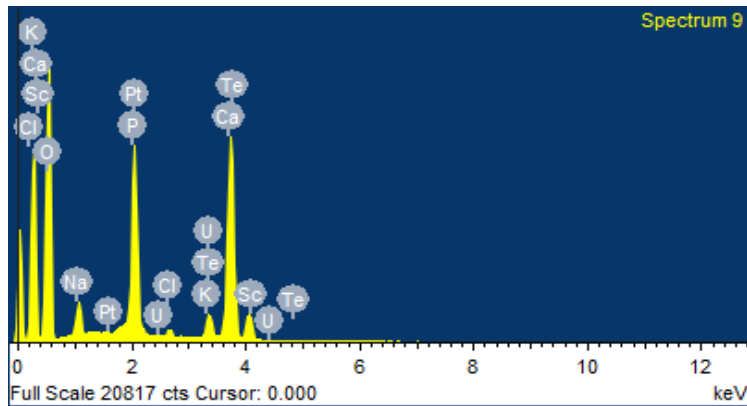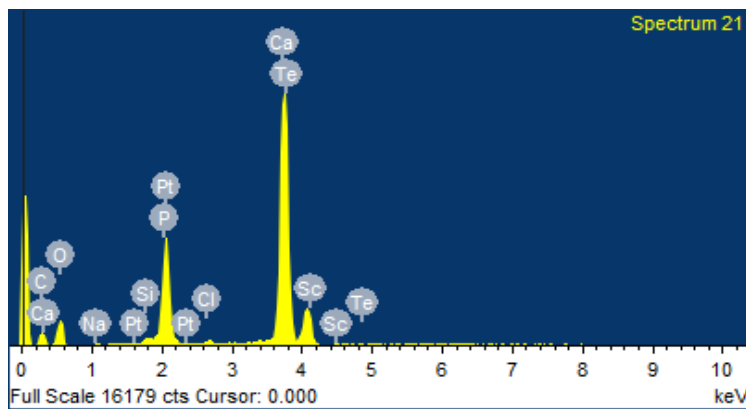# Image 1. Elemental analysis of baseline enamel specimen by EDX analysis

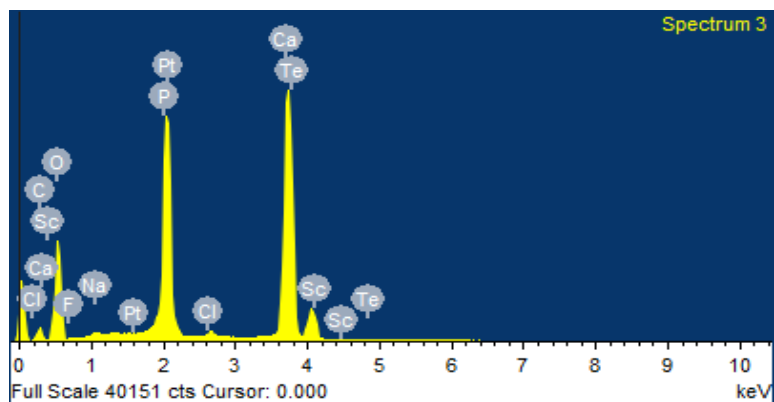# Image 2. Elemental analysis of demineralized enamel specimen by EDX analysis

Image 3. Elemental analysis of remineralized enamel specimen of Group I (Aclaim®) by EDX analysis

Image 4. Elemental analysis of remineralized enamel specimen of Group II (1%nHAP) by EDX analysis
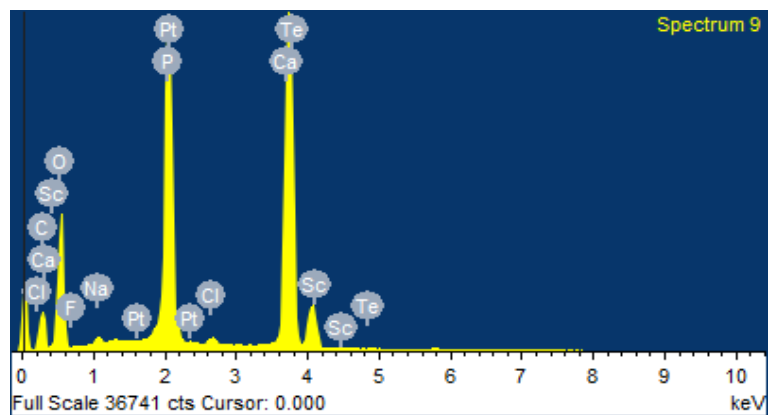
Image 5. Elemental analysis of remineralized enamel specimen of Group III (10% nHAP)

by EDX analysis

Image 6. Elemental analysis of remineralized enamel specimen of Group IV (CPP ACP) by

EDX analysis

Image 7. Elemental analysis of remineralized enamel specimen of Group V (Control) by

EDX analysis
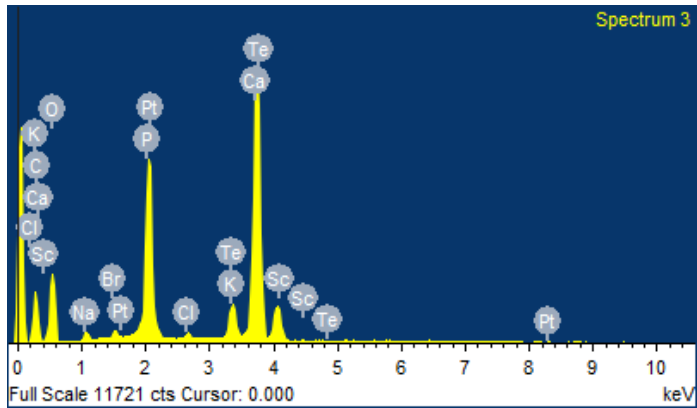
Image 8. Structural analysis of demineralized enamel specimen by SEM analysis

| 2 µm | EHT = 15.00 kV | Photo No. = 4356 | Signal A = SE2 | Date :14 Dec 2017 |
| | WD = 8.9 mm | | Mag = 5.00 K X | Time :11:05:57 |
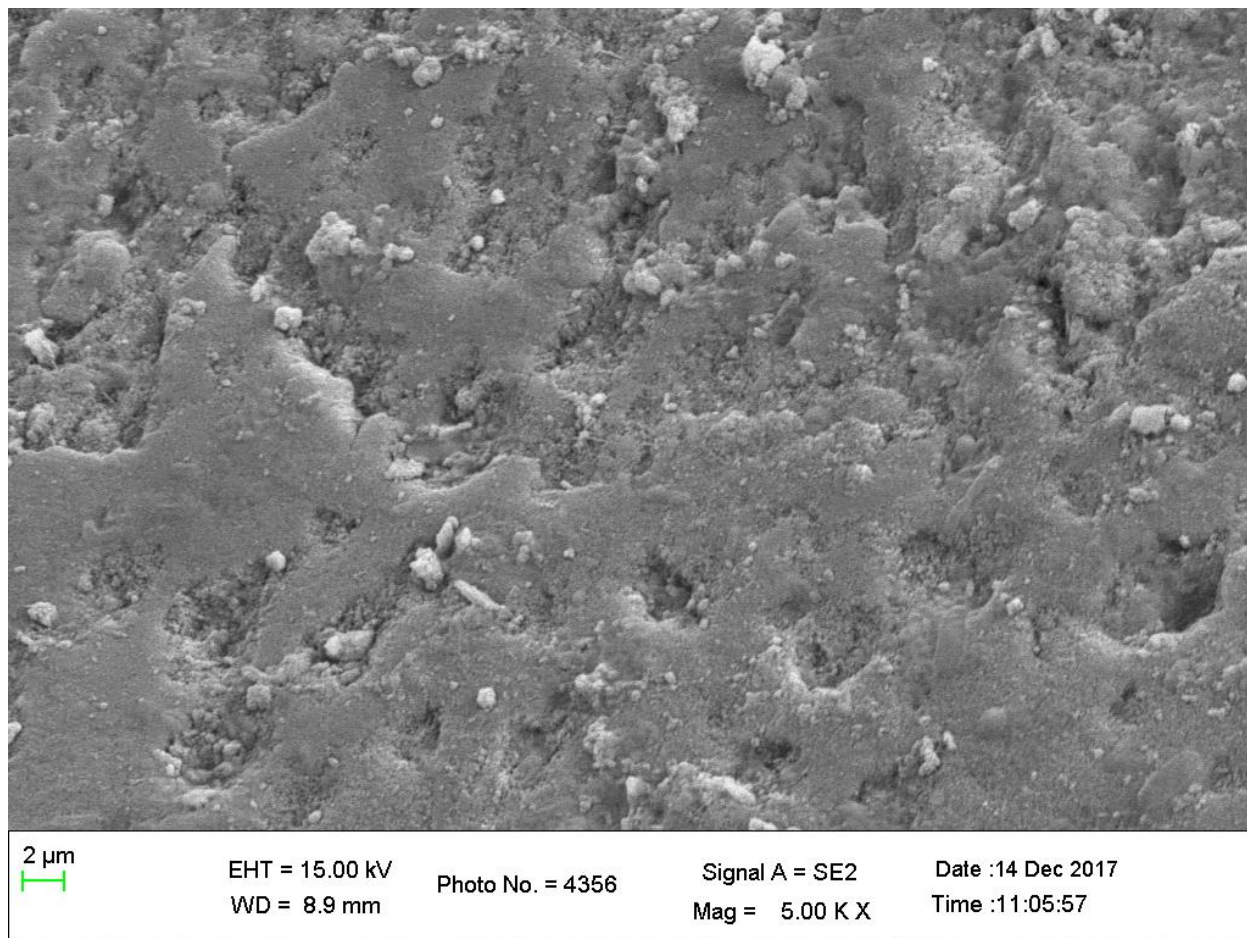
Image 9.Structural analysis of remineralized enamel specimen of Group III (10% nHAP)

by SEM analysis

| 2 µm | EHT = 15.00 kV | Photo No. = 4367 | Signal A = SE2 | Date :14 Dec 2017 |
| | WD = 8.9 mm | | Mag = 5.00 K X | Time :11:25:33 |
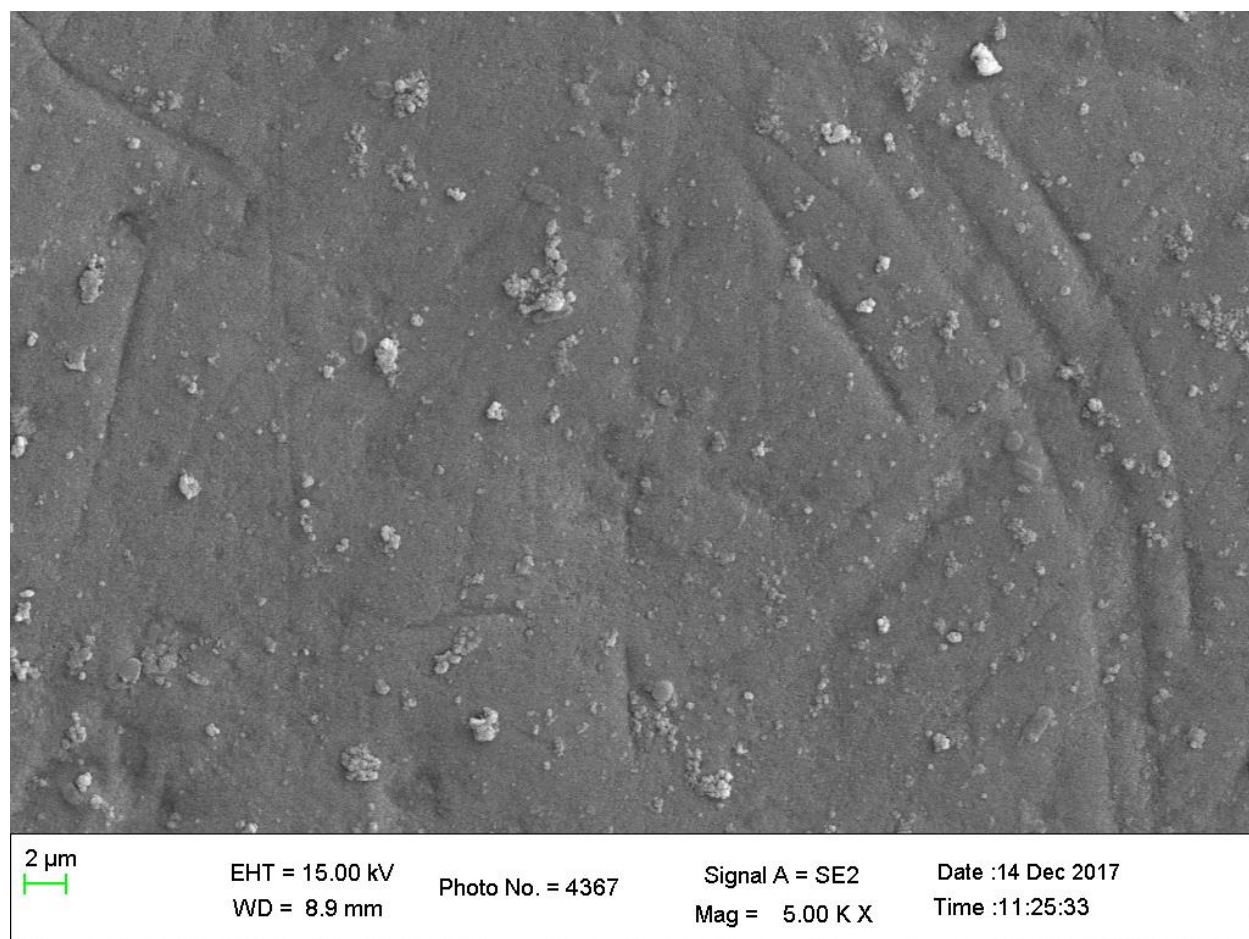
Image 10. Structural analysis of remineralized enamel specimen of Group IV (CPP ACP)

by SEM analysis

| 2 µm | EHT = 15.00 kV | Photo No. = 4378 | Signal A = SE2 | Date :14 Dec 2017 |
| | WD = 9.9 mm | | Mag = 5.00 K X | Time :11:33:36 |
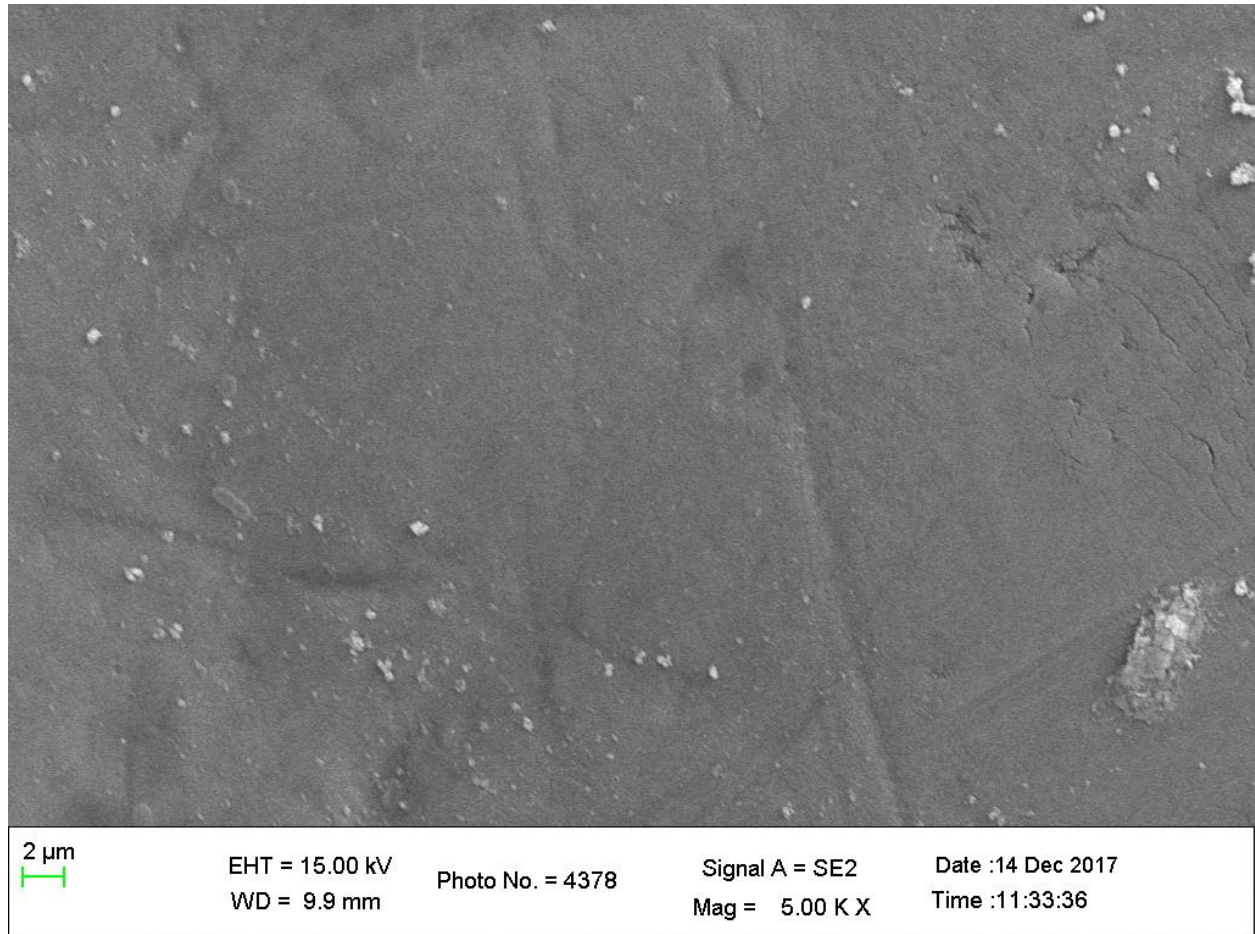
DISCUSSION

Dental caries is the most common reason for tooth loss in children and young adults. It is often described as a chronic disease which causes localized destruction of the tooth.[45] Dental caries is a multifactorial disease occurring as a result of complex interactions among tooth structure, dental biofilm, dietary, salivary, and genetic influences.[23] Any disturbance in the physiological equilibrium of the biofilm (dental plaque) covering the affected site results in demineralization.[45] Demineralization of enamel leads to dissolution of hydroxyapatite and diffusion of calcium and phosphate ions toward the enamel surface. Reprecipitation of hydroxyapatite occurs, when the enamel surface is supersaturated with calcium and phosphate ions forming an intact superficial layer.[32]

Caries process is said to be active when the demineralization period exceeds remineralization.[43] The disease will continue to progress, unless the dental plaque covering the site is removed. The localized destruction of the hard tissues is often referred to as the lesion. The amount of destruction of lesion can range from initial loss of mineral at the ultra structural or nanoscale level to total tooth destruction.[45]

The concept of demineralization and remineralization cycle has led to the scope for remineralizing incipient carious lesion.[43] Thus focus on caries prevention shifted to the development of methods for detecting the caries at early stages. The use of non-invasive treatment for incipient carious lesions by remineralization has been considered as a promising advancement in the clinical management of the disease.[44] It bridges the traditional gap between prevention and restorative procedures.[32] Remineralization of initial carious lesions may be possible with a variety of currently available agents containing fluoride, bioavailable calcium and phosphate, CPP ACP, self-assembling peptide and nano hydroxyapatite.[44]

Presently there is a lack of knowledge about the remineralizing efficacy of biomaterials based on nanotechnology which may be an important preventive approach to be applied in high caries risk patients.[14] The Japanese company Sangi Co. Ltd was the first one to take an interest in hydroxyapatite, after purchasing the rights from NASA (U.S. National Aeronautics and Space Authority) in 1970. NASA introduced a synthetic hydroxyapatite as a repairing material for the astronauts who lost minerals from the teeth and bones in the space due to the absence of gravity. Later in 1978, Sangi Co. Ltd had the idea to create a toothpaste containing nHAP (Apadent) that could repair the tooth enamel.[48] The function of biomimetic hydroxyapatite is to protect the teeth with the creation of a new layer of synthetic enamel around the tooth, rather than hardening the existing layer with fluoride.

Toothpaste with nHAP had the strong ability to bond with the proteins present in plaque and bacteria. The size of the nano particles markedly increases the surface area to which proteins can bind.[48] Accordingly, materials containing nHAP are able to provide calcium and phosphate ions for reducing tooth demineralization and/or improving tooth remineralization. Hydroxyapatite nano particles may penetrate tooth porosities (Mechanical imbrications) and produce a protective layer on the tooth surface.[12,29] nHAP helps in providing more mineral deposits on the outer layer than the body of the lesion.[14] The efficacy of nHAP increases in the presence of biofilm.[58]

Toothpastes containing nHAP are now commercially produced worldwide. This indicates the need for more precise evaluation of their efficacy. Considering the lack of adequate studies, the present study was aimed at formulation of nHAP toothpaste at 2 different concentrations (1% and 10%) and evaluating their remineralization potential with comparison to commercially available nHAP paste (Aclaim®) and CPP ACP (GC Tooth mousse).

Products containing CPP ACP have also proved to have some potential to prevent enamel demineralization and increase remineralization in-vitro.[35,9,69] Based on in-situ studies[8,13] and randomized clinical trials.[50,5] CPP ACP paste was able to increase the remineralization of initial enamel caries lesions. However it does not seem to have a significant difference in its remineralization effect from fluorides.[4,67,36] According to the systematic reviews by Chen et al 2013[11] and Li et al 2014[36], there were a lack of reliable scientific evidence to support the remineralization effect of CPP ACP.

Evaluation method

In-vitro demineralization and remineralization can be assessed by using various methods. The commonly used methods are SEM / SEM-EDX, DIAGNOdent, surface micro hardness, and polarized light microscopy. It is wise to measure the changes in the mineral content of the carious lesions quantitatively in order to provide more promising results in remineralization process. One of the most common techniques was SEM with EDX attachment. It is a micro analytical technique that is employed to estimate quantitatively the amounts of minerals in a given tooth sample. SEM gives the topographical pictures and is used to assess the surface changes seen on enamel. EDX gives quantification of various elements like calcium, phosphorus, fluoride, magnesium and sodium in both atomic and weight percentage.[43] In the present study, quantitative elemental analysis for each specimen was measured at three levels; baseline, after induction of carious lesions (demineralization) and after remineralization.

Demineralization regimen

Artificial caries like lesions can be induced in enamel in in-vitro conditions. These carious lesions were considered to be more homogenously reproducible than natural carious lesions and thus provide more reliable experimental model. The in-vitro model provides an area

of enamel having a defined lesion of constant depth beneath the surface. They facilitate the testing of multiple areas in any enamel lesion at different time intervals, in order to assess the remineralizing phenomena.[3,15,66] In the present study, artificial carious lesions were induced using the demineralization protocol described by Patil N et al[47] which is in accordance to Ten Cate and Duijsters[60]. The results of the present study revealed that calcium and phosphorus wt% of all the enamel specimens were significantly reduced from baseline after demineralization regimen.

Remineralization regimen

The present study used paste type formulation of the test agents, applied with disposable cotton tip applicators. This was done in view of replication of patient convenience in using the tooth cream like a tooth paste with toothbrushes or along with cotton tip applicators. The remineralization regimen comprised 3 minutes twice daily application of 14 days similar to Shriahatti et al.[56] In contrast, study done by Pai et al[46] treatment pastes were applied 3 minutes once daily for 14 days. Hegde and Moany[27] in their study performed twice daily application of CPP ACP for 3 minutes for 7, 14, 21, 28, and 35 days. They mentioned that remineralization efficacy of paste is dose dependant, i.e. remineralization rate increases with the duration of time the paste is in contact with the enamel surface.

In the present study, artificial saliva was changed every 24 hours during the remineralization regimen to ensure ionic balance and maintenance of pH. This is in accordance with Patil N et al[47] and Pujan Kamath et al[32]. In contrast, Shirahatti et al[56] changed the artificial saliva solution every 72 hours in their study.

In the present study quantitative elemental analysis for each specimen was done at three stages: baseline, after demineralization and after remineralization. The results of 4 test groups

and a control group were summarized as calcium wt%, phosphorus wt% and calcium phosphorus ratio wt%.

Experimental nHAP paste

In the present study experimental 1% nHAP paste showed significant increase in calcium wt% after remineralization whereas phosphorus wt% did not increase significantly. Experimental 10% nHAP paste showed a significant higher mean change in both calcium and phosphorus wt% after remineralization compared to 1% nHAP. This was in accordance with the results of Huang et al[30] who compared the remineralization effect of different concentrations (1%, 5%, 10% and 15%) of nHAP on artificially demineralized bovine enamel. They found that 1% nHAP did not show significant effect on remineralization of initial enamel caries whereas 10% nHAP was most effective. They also concluded that 10% nHAP was considered as the optimum concentration for the remineralization of early enamel lesions. Similarly Kim et al[34] reported that higher concentration (10%) of nHAP with longer treatment time showed greater remineralization effect. As the concentration of nHAP increased, the rate and amount of its precipitation also increased along with the deposition of excessive amount of calcium and phosphate ions. Contrary to our results, Haghoo et al[25] concluded that there was no significant difference between the effect of various concentration of nHAP (2%, 5% and 10%). The difference in the result of their study might be because the formulated nHAP was used as mouth rinse. Another reason could be the shorter treatment time of 12 hours, in which the specimens were immersed in the mouth rinse.

The results of the present study revealed that, there was a significant change in calcium phosphorus ratio wt% after remineralization in 10% nHAP paste. This is in contrast with the results obtained by Pujan Kamath et al[32] where there was no significant difference between the

groups. The variation in the result might be due to the concentration of the nHAP used in the present study.

Commercially available nHAP pate (Aclaim®)

The present study revealed that Aclaim® showed a significant increase in calcium and phosphorus wt% after remineralization. This suggests a positive effect on remineralization of early enamel caries. Similar results were obtained in a study done by Singh A et al[57] where Aclaim® demonstrated a significant effect in preventing demineralization under polarized light microscopy. Shetty P et al[55] mentioned that the particle size of nHAP in Aclaim® was <100nm. The nano sized hydroxyapatite remained relatively stable under acidic conditions and thus nHAP layer formed on the enamel surface became resistant to dissolution.

CPP ACP

In the present study CPP ACP demonstrated a significant change in calcium and phosphorus wt% after remineralization, however the mean value was lesser than the values at baseline. When compared with CPP ACP, both Aclaim® and 10% nHAP showed higher mean change of calcium and phosphorus wt% after remineralization. But there was no significant difference between 1% nHAP and CPP ACP. The results were in accordance to the study done by deCarvalho et al.[16] This might be because CPP ACP needs an acid challenge for activation and for the separation of ACP from casein. This activation increases the degree of saturation of calcium and phosphate ions in saliva with respect to hydroxyapatite. In a study done by Comar et al[14] both CPP ACP and nHAP were unable to reduce demineralization which is contrary to our results. The difference in results might be due to the pH cycling method used which focused on the prevention of demineralization rather than the remineralization of preformed lesions.

Structural Analysis

SEM analysis of the demineralized samples at 5,000 magnification revealed rough and irregular surface with marked irregular porosity. Minor honey comb pattern of demineralized enamel was evident on the surface. Distinct surface coating was evident in remineralized specimens of both 10% nHAP and CPP ACP. SEM image of 10% nHAP revealed numerous small deposits covering the irregularities of the enamel surface. CPP ACP also showed similar deposits on the enamel surface but in fewer numbers. Roveri et al[52] demonstrated an apatitic coating deposition on the enamel surface when biomimetic nHAP was used. Pujan Kamath et al[32] noticed superior texture of enamel treated by nHAP paste under SEM. This could be attributed to the nHAP particles that have similar morphology, crystal structure, and crystallinity like the apatite crystals of tooth enamel.

In the present study, null hypothesis was rejected because 1% and 10% nHAP paste showed significant increase in calcium wt% and phosphorus wt% compared to CPP ACP and control group.

Limitations

1) Selection of the enamel specimen was done based on visual inspection. This might be the reason for varied baseline values of calcium and phosphorus wt%.

2) The present study did not make any attempt to compare the remineralization of nHAP with fluoride.

SUMMARY AND CONCLUSION

The present randomized controlled trial was conducted in Department of Pediatric and Preventive dentistry, KSR Institute of Dental Science and Research, Tiruchengode, Tamil Nadu. The in-vitro trial study was conducted with following aims: 1. To formulate two experimental nHAP tooth pastes with two concentrations 1% and 10% concentrations; 2. To evaluate the remineralization potential of experimental 1% and 10% nHAP tooth paste; 3. To compare the remineralization potential of experimental 1% nHAP with commercially available nHAP toothpaste using EDX analysis and 4. To compare the remineralization potential of 10% nHAP with commercially available CPP ACP using SEM with EDX analysis. A total of 65 enamel specimens were randomly divided into 4 test groups and one control group; Group I: Aclaim®, Group II: Custom made 1% nHAP, Group III: Custom made 10% nHAP, Group IV: CPP ACP, Group V: Control. Baseline quantitative measurement of mineral contents (calcium and phosphorus wt%) in enamel specimens were done by EDX analysis. Artificial carious lesion was induced in all the enamel specimens, according to the demineralization regimen by Tencate and Duijsters. All the enamel specimens were evaluated for loss in mineral content using EDX analysis. The specimens in Groups I, II, III and IV were treated with respective paste twice daily for 14 days. Specimens were rubbed with respective paste with the help of a cotton applicator for 3 minutes, washed with deionized water and then placed in artificial saliva and maintained at ambient temperature. In the control group, specimens were only washed once with deionized water and placed in artificial saliva. After the remineralization cycle, all the enamel specimens were again subjected to EDX analysis to evaluate the change in mineral content. The data were collected and subjected to statistical analysis. SEM analysis was done for Group III, IV and control group to analyze the surface topographical changes after remineralization.

The following conclusions can be inferred from this study

1) All the test groups showed significant change in calcium and phosphorus wt% after remineralization.

2) Among the 5 groups, post reminerlization calcium wt% was greatest in the 10% nHAP followed by Aclaim®, 1% nHAP, control and CPP ACP

3) Among the 5 groups, post reminerlization phosphorus wt% was greatest in the 10% nHAP followed by Aclaim®, 1% nHAP, CPP ACP and control

4) Among the test groups, post reminerlization calcium phosphorus ratio wt% was greatest in the 10% nHAP followed by Aclaim®, 1% nHAP and CPP ACP

5) Significant change in calcium phosphorus ratio wt% after remineralization was seen only in 10% nHAP group.

6) The mean change in calcium and phosphorus wt% of 10% nHAP was significantly higher than 1% nHAP and CPP ACP after remineralization.

7) Aclaim® showed significantly higher mean change in calcium wt%, than CPP ACP after remineralization.

8) Both 10% nHAP and CPP ACP showed favorable surface changes in enamel after remineralization in SEM analysis.

9) SEM image of 10% nHAP showed more deposits on the enamel surface than CPP ACP

Conclusion

Within the limitations of this in-vitro trial, nHAP had the potential to remineralize artificially induced carious lesions. A concentration of 10% nHAP followed by Aclaim® was found to be most effective in increasing calcium and phosphorus wt%. CPP ACP and 1% nHAP were found to have less remineralizing potential.

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APPENDIX

APPENDIX I

INSTITUTIONAL ETHICAL COMMITTEE
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Dr. P. PONMURUGAN, Ph.D.,
Prof. & Head Dept. of Biotechnology
KSR College of Technology,
KSR Kalvi Nagar, Tiruchengode.

Member Secretary
Dr. G.S. KUMAR, MDS.,
Principal,
KSR Institute of Dental Science & Research,
KSR Kalvi Nagar, Tiruchengode.

Members

Dr.G.Ayppadasan,Ph.D.,
Biotechnologist

Mr.A.Thirumoorthi,M.A.B.L.,
Human Activist

Dr.R.Renuka,M.D.S.,(Perio),M.Sc.,
Family Counsellor

Dr.K.Sivakumar,MDS.,(Cons.Dent.)

Dr.Suman, M.D.S.,(OMDR)

Dr.Sharath Ashokan,MDS.,(Pedo)

Dr.G.Rajeswari,Ph.D.,(Biochemistry)

Dr.K.Karthick,MDS.,(Cons.Dent.)

Mr.V.Mohan,M.Sc.,M.Phil.,(Physicist)

Mr.A.P.S.Raja,B.A.,
(Layperson)

Ref.: 116/KSRIDSR/EC/2015   Date : 19.12.2015

To

Dr.V.Vijayasankari,
Postgraduate Student,
Dept. of Peadodontics,
KSR Institute of Dental Science & Research,

*****

Your dissertational study titled "Evaluation of the remineralization potential of Two Non fluoridated remineralizing pastes using Scanning Electron Microscope with Energy Dispersive X-ray analysis – A Randomized controlled in vitro trial" presented before the ethical committee on 15th Dec. 2015 has been discussed by the committee members and has been approved.

You are requested to adhere to the ICMR guidelines on Biomedical Research and follow good clinical practice. You are requested to inform the progress of work from time to time and submit a final report on the completion of study.

Signature of Member Secretary
   (Dr.G.S.Kumar)

Urkund Analysis Result

Sources included in the report:

EFFECTIVENESS OF NANOHYDROXYAPATITE DENTRIFICE AND FLUORIDATED DENTRIFICE ON
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hydroxyapatite-dentifrice
https://www.reddit.com/r/conspiracy/comments/5p6k8v/
if_toothpaste_ads_were_honest_honest_ads/

Instances where selected sources appear:

25

CERTIFICATE - II

This is to certify that this dissertation work titled **"Evaluation of the remineralization potential of two non-fluoridated remineralizing pastes using scanning electron microscope with energy dispersive X-ray analysis: A randomized controlled in-vitro trial"** of the candidate **Dr.Vijayasankari V** with registration number _____ for the award of **"Master of Dental Surgery"** in the branch **of Pedodontics and Preventive Dentistry**. I personally verified the urkund.com website for the purpose of plagiarism Check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows **7%** percentage of plagiarism in the dissertation.

Guide & Supervisor sign with seal

APPENDIX IV

ABBREVIATIONS

S no	Abbreviation	Expansion
1.	WSL	White spot lesion
2.	Nhap	Nano hydroxyapatite
3.	CPP ACP	Casein phophopeptide amorphous calcium phosphate
4.	$CaCl_2.2H_2O$	Calcium chloride
5.	$NaH_2PO_4.7H_2O$	Monosodium phosphate
6.	$C_3H_6O_3$	Lactic acid
7.	$NaOH$	Sodium hydroxide
8.	SEM	Scanning Electron Microscope
9.	EDX	Energy Dispersive X-Ray
10.	$Ca(NO_3)_2.4H_2O$	Calcium nitratetetrahydrate
11.	KCl	Potassium chloride
12.	$MgCl_2.2H_2O$	Magnesium chloride dehydrate
13.	K_2HPO_4	Potassium dihydrogen phosphate

14.	KH_2PO_4	Monopotassium hydrogen phosphate
15.	KOH	Potassium hydroxide
16.	NaOH	Sodium hydroxide
17.	kg	Kilogram
18.	mg	milligram