COMPARISON OF REMINERALIZATION EFFICACY ON TOOTH WITH VARIOUS REMINERALIZING AGENTS USING POLARIZED LIGHT MICROSCOPY- AN INVITRO STUDY

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CERTIFICATE

This is to certify that this dissertation titled "**COMPARISON OF REMINERALIZATION EFFICACY ON TOOTH WITH VARIOUS REMINERALIZING AGENTS USING POLARIZED LIGHT MICROSCOPY- AN INVITRO STUDY"** is a bonafide record of work done by **Dr. Sudheesh Sudarsan** under my guidance and to my satisfaction during his postgraduate study period between 2010 – 2013. This dissertation is submitted to THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY, in partial fulfillment for the award of the degree of Master of Dental Surgery in Conservative Dentistry and Endodontics, Branch IV. It has not been submitted (partial or full) for the award of any other degree or diploma.

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CONTENTS

INTRODUCTION

Dental caries afflicts quite a large proportion of the population by adulthood and is the most common chronic disease of childhood. The caries process involves an imbalance of acid attack from the metabolic products of oral microbes during carbohydrate consumption[55]. Dental caries is a site specific disease starting at the enamel surface that undergoes many cycles of demineralization and remineralization during lesion development[12].

Demineralization can be seen as a reaction of protons (H^+) derived from disassociation of plaque acids with tooth enamel causing mineral dissolution. When the critical pH of 5.5 is breached, calcium and phosphate exit the enamel, weakening it and eventually causing the chalky white spots of demineralization that may eventually become caries[43]. Because of its developmental characteristics dynamics, the caries lesion can be arrested and even repaired at its early stages without operative intervention by increasing the net mineral gain during the demineralization and remineralization.

Remineralization is the natural repair process for noncavitated lesions, and relies on calcium and phosphate ions assisted by fluoride to rebuild a new surface on existing crystal remnants in subsurface lesions remaining after demineralization[50]. Remineralization can occur naturally when calcium and

phosphorus reenters the enamel with the help of good quality saliva[43]. Through remineralizing therapies we can hasten this remineralization procedure. One of the first systematic clinical studies on carious reversal was reported in 1966, where half of 72 white spot lesions observed on buccal surfaces of first molar in children at age 8 were found to have remineralized at age 15[7]. Since then many in vivo and in vitro reports of remineralization of enamel caries with different remineralizing agents have been published[13,16,19]. Remineralization is increasingly accepted as a viable non-invasive approach for caries reversal, during the earlier stages of the carious process.

The ideal remineralizing agent will provide adequate amounts of calcium and phosphate ions to the body of the carious lesion where they are needed and will not readily precipitate on the tooth surface or increase calculus formation[55]. A variety of compounds are currently available such as Fluoride, NovaMin, Tricalcium phosphate, Sodium monofluorophosphate, CPP-ACP etc. Historically fluoride was best used as a remineralizing agent for a long period of time and till now it is used as an efficient remineralizing agent. Fluorides cause remineralization through various mechanisms, mainly through formation of fluorapatite in place of hydroxy apatite, which is more acid resistant. Fluorides are

available in various forms such as dentifrices, mouth rinses, lozenges etc and in various concentrations.

Currently, casein phosphopeptide and amorphous calcium phosphate are also used as remineralizing agents. A study conducted by Reynolds et al[41] (1997) has shown that CPP stabilized calcium phosphate solutions can remineralize enamel subsurface lesions. Casein phosphopeptides (CPP) binds to amorphous calcium phosphate, forming small clusters of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), thereby stabilizing calcium phosphates in solution[19]. Under acidic conditions, this localized CPP-ACP is claimed to buffer the free calcium and phosphate ions, substantially increasing the level of calcium phosphate in plaque and, therefore, maintaining a state of supersaturation that may inhibit enamel demineralization and enhance remineralization[34].

Another product that promotes remineralization of teeth is calcium sodium phosphosilicate, known as NovaMin. NovaMin is a bioactive glass that delivers silica and ionic calcium, phosphorus, and sodium for bone and tooth mineralization. In the mouth, NovaMin releases sodium, calcium, and phosphate ions, which then interact with oral fluids and result in the formation of a crystalline

hydroxycarbonate apatite (HCA) layer that is structurally and chemically similar to natural tooth mineral[43].

To evaluate demineralization and remineralization, it is important to know how much mineral has been lost or gained, or where the loss or gain occurred. There are different techniques available for direct and indirect mineral quantification including Microhardness, Polarized light microscopy, Micro-computed tomography, scanning electron microscopy, Raman spectroscopy and Microradiography[5,54]. Polarized light microscope analysis is the one of the most commonly used and sensitive techniques for showing mineral changes in hard tissue with respect to demineralization and remineralization. Birefringence experiments can qualitatively show mineral loss and mineral gain[5].

The aim of this study was to utilize polarized light microscope for the assessment of the efficacy of CPP-ACP, NovaMin and Amine Flouride pastes on remineralization of enamel over time.

AIM AND OBJECTIVES

The aim of this study was to utilize polarized light microscope to assess and compare the efficacy of CPP-ACP, NovaMin and Amine Flouride pastes on remineralization of enamel over time.

REVIEW OF LITERATURE

Ogaard et al[33] (1988) investigated the effect of fluoride on carious lesion development and on lesions established during fixed orthodontic therapy with a very low pH fluoride solution. They found out that daily fluoride mouth rinsing with a 0.2% solution sodium fluoride retarded lesion development significantly, whereas the fluoride solution with low pH inhibited lesion formation completely and also fluoride applied as a mouth rinse to plaque-covered lesions underneath orthodontic bands retarded lesion progression.

Reynolds et al[37] **(1995)** investigated the anticariogenicity of Calcium Phosphate Complexes of Tryptic Casein Phosphopeptides in the Rat. Solutions of CPP-ACP were applied to the animal molar teeth twice daily. The anticariogenic effects of CPP-ACP and fluoride were additive, since animals receiving 0.5% CPP-ACP plus 500ppm F had significantly lower caries activity than those animals receiving either CPP-ACP or fluoride alone. The Tryptic digest of casein with the phosphopeptides selectively removed showed no anticariogenic activity. They found that CPP-ACP complex substantially increased the level of calcium phosphate.

Reynolds et al[41] (1997) demonstrated that CPP stabilized calcium phosphate solutions can remineralize enamel subsurface lesions. They did remineralization on molars for a period of ten days and then the lesions were sectioned and subjected to microradiography and the mineral content was determined by microdensitometry. The study concluded that CPP-stabilized calcium phosphate in the solution maintains high concentration gradient of calcium and phosphate ion and ion pairs into subsurface lesion and thus the high rates of enamel remineralization.

Cai et al[11] (2003) determined the effect of CPP-ACP incorporation into a sugar free lozenges on enamel remineralization in a human in situ model. In this study ten subjects wore removable palatal appliances with four, human enamel, half slab insets containing subsurface lesions. Treatment included lozenges containing 56.4mg CPP-ACP, 18.8mg CPP-ACP, lozenge without CPP-ACP and a control group with no lozenge. The enamel slabs were subjected to microradiography and computer assisted densitometric image analysis to determine the level of remineralization. The authors concluded that incorporation of CPP-ACP into the lozenge significantly increased enamel subsurface lesion remineralization relative to the control sugar free lozenge and that lozenges are a suitable vehicle for the delivery of CPP-ACP to promote enamel remineralization.

Ramalingam et al[35] (2005) conducted a study to determine a minimal concentration of casein phosphopeptide stabilized amorphous calcium phosphate (CPP-ACP) which when added to a sports drink would eliminate such erosion in vitro. Enamel specimens were used for the study which were obtained from fifteen molars and were immersed in the sports drink Powerade, Powerade plus 4 concentrations of CPP-ACP and double deionized water. The study concluded that sports drink can erode human enamel and with the addition of 0.125% CPP-ACP the drinks color, clarity and taste were unaffected. With increased CPP-ACP the erosive potential decreases and also addition of 0.09% - 0.25% CPP-ACP eliminated the erosion step.

Hassanein et al[20] (2006) investigated remineralization potential of bioactive glass on artificial caries enamel and dentin using Raman spectroscopy on human molars. Two remineralizing solution bioactive glass and ITS mineralizing solution with and without the use of zeolite powder were used. The results revealed that bioactive glass had the potential of remineralization and that with the addition of zeolite powder enhances the effect of remineralization.

Lennon et al[25] (2006) conducted a study to determine whether a tooth cream containing casein/calcium phosphate (CasCP) protects enamel against erosion.

Study was conducted on enamel specimens with treatment groups casein calcium phosphate, sodium fluoride and amine fluoride gel. They concluded that highly fluoridated acidic Amine Fluoride gel can protect enamel against erosion while CasCP, 250 ppm fluoride or a combination of CasCP and 250 ppm fluoride provide little protection.

Arnold et al[5] (2006) investigated the effect of four different toothpaste with different fluoride compounds on enamel demineralization on human premolars. Amine fluoride, sodium fluoride and sodium monofluorophosphate was used for the study. The study concluded that amine fluoride compounds in toothpastes result in a clearly marked remineralization of caries like enamel lesions followed by sodium fluoride and sodium monofluorophosphate formulations.

Yamaguchi et al[56] (2006) determined the effect of Casein Phosphopeptide—Amorphous Calcium Phosphate (CPP–ACP) paste on demineralization of bovine enamel by measuring changes in the ultrasound transmission velocity. They found that sonic velocity was found to decrease with time for specimens stored in the demineralization solution and increased for specimens stored in the CPP–ACP solution. The authors concluded by suggesting that the conditions of enamel remineralization of the enamel structure could be measured non-destructively by

using an ultrasonic pulse method. They also concluded that the inorganic components contained in high concentrations in CPP–ACP acted to enhance remineralization of the enamel structure.

Cai et al[10] (2007) investigated the effects of CPP-ACP in a fruit-flavored sugar-free chewing gum containing citric acid on enamel remineralization, and acid resistance of the remineralized enamel, using an in situ remineralization model. In this study ten subjects wore removable palatal appliances, with 4 half-slab inserts of human enamel containing demineralized subsurface lesions. Treatment included sugar-free gum containing 20 mg citric acid and 18.8 mg CPP-ACP, sugar-free gum containing 20 mg citric acid alone, sugar-free gum not containing CPP-ACP or citric acid. The enamel slabs were subjected to microradiography. Authors concluded that remineralization was greater for lesions exposed to gum containing CPP-ACP.

Arnold et al[4] (2007) investigated the influence of different pH levels on enamel remineralization in an in vitro experiment using polarization light microscopy and EDX quantitative element analysis on human premolars. Study included toothpaste with amine fluoride (1400ppm) at pH 4.1, 4.5, 5.1 and 6.9 or control toothpaste without fluoride at pH 4.3, 4.7, 5.3 and 7.0. They found that there was a decreased

porous volume of the body of the lesion after incubation with fluoridated toothpaste at pH 4.53 and 5.16. They concluded that pH also plays an important role in remineralization and slightly acidified fluoridated dentifrices may have a certain positive effect on enamel remineralization.

Azarpazhook et al[6] (2008) reviewed systematically the clinical trials of casein derivatives. They included clinical studies that examined the efficacy of casein derivatives in dentistry and excluded in vitro studies, case series, case reports etc. They found that the quantity and quality of clinical trial evidence are insufficient to make conclusions regarding the long-term effectiveness of casein derivatives, specifically CPP-ACP, in preventing caries in vivo and treating dentin hypersensitivity or dry mouth.

Cochrane et al[14] (2008) determined the effect of ion composition of CPP-ACP and CPP-ACPF solutions on enamel subsurface lesion remineralization on human molars. They found that CPP-ACPF produced greater remineralization than CPP-ACP solutions at pH 5.5 and below. The mineral formed in the subsurface lesions was consistent with hydroxyapatite and fluorapatite for remineralization with CPP-ACP and CPP-ACPF, respectively.

Kumar et al[24] (2008) investigated the efficacy of CPP-ACP containing Tooth Mousse on the remineralization of enamel lesions and comparing its efficacy to that of fluoride-containing toothpaste on molars. After placing in demineralizing solutions, samples were sectioned and grouped into 5 groups as fluoridated tooth paste, non fluoridated tooth paste, CPP-ACP as toothpaste, CPP-ACP as topical coating and topical coating after treating the sections with fluoridated tooth paste. They found that CPP-ACP containing Tooth Mousse remineralized initial enamel lesions and it showed a higher remineralizing potential when applied as a topical coating after the use of a fluoridated toothpaste.

Meurman et al[30] (2008) investigated the effect of amine fluoride–stannous fluoride preparations on oral yeasts in 194 elderly patients. They found that at the end of trial, giving mouth rinse and tooth paste twice daily, mucosal lesions and the total bacterial count had reduced. They found that fluoride combinations were useful as a support therapy for oral candidiasis.

Reynolds et al[40] (2008) determined the ability of CPP-ACP to increase the incorporation of fluoride into plaque and to promote enamel remineralization in situ by using mouth rinses and dentifrices containing CPP-ACP and fluoride. Supragingival plaque was collected and analysed for fluoride. The study showed

increased fluoride incorporation and remineralization with CPP-ACP containing products compared to fluoride alone products. The study showed that CPP-ACP plus fluoride products may be superior in reducing caries risk compared with fluoride alone products.

Ten Cate et al[44] (2008) investigated the dose response between 0 and 5000ppm fluoride of demineralization and remineralization of advanced enamel lesions using fresh bovine incisors. Mineral uptake and loss were assessed from the calcium changes and microradiographs. They found that treatment with 5000ppm fluoride enhanced remineralization and inhibited demineralization than 1500ppm fluoride. Treatment with 5000ppm Vs 1500ppm fluoride resulted in 31% remineralization enhancement and 12% demineralization inhibition.

Toda et al[47] (2008) investigated the effect of three different fluoride compounds on demineralization and remineralization. Sodium fluoride, amine fluoride and sodium monofluorophosphate were used for treating human enamel specimens and the lesions were assessed by cross sectional microhardness. They found that sodium fluoride with 1100ppm fluoride showed better results than amine fluoride with 1250ppm fluoride.

Burwell et al[9] (2009) demonstrated the potential of NovaMin and mechanism of action in areas of remineralization and caries prevention. They conducted a series of three invitro studies and one insitu study. Study I involved investigating the ability of dentifrices to prevent remineralization of dentin surfaces. Study II explored the potential of dentifrices to remineralize existing lesions on root tissue. Study III investigated the ability of dentifrices to heal existing white-spot lesions on enamel. Study IV was an insitu study designed to characterize the morphological changes on tooth surfaces that were subjected to different types of damage and then treated with a dentifrice. They found that in study I NovaMin containing dentifrice creates a tenacious surface that protects dentin from demineralization. In study II NovaMin containing dentifrice rehardened and repaired the lesions. In study III inclusion of NovaMin improved hardening of white spot lesions. In study IV it showed that NovaMin containing dentifrice helped in repair of surface defects by deposition onto the defects.

Willershausen et al[53] (2009) in an invitro study evaluated the effect of a casein phosphopeptide and amorphous calcium phosphate (CPP–ACP) paste on untreated enamel surfaces that had previously been exposed to an erosive challenge. Teeth were incubated with apple juice and later covered with CPP-ACP for fifteen minutes. The results revealed that there was a slight gain in mineral contents after

the application of CPP-ACP mainly in the upper enamel layer. The authors suggested that the application of CPP–ACP paste may enhance the remineralization after an erosive challenge and thus offer some protection for patients who are at risk for erosion.

Bansal et al[8] (2010) evaluated and compared the remineralizing efficacy of a urea-based mineral-enriched mouth rinse and a fluoridated dentifrice using an in vivo intraoral appliance model and polarized light microscopic evaluation technique on premolars and molars. In this study 14 children wore an intra oral appliance in which enamel blocks were prepared and inserted. Specimens were retrieved from the patients six month later. After treatment with non fluoridated dentifrices, fluoridated dentifrices and mineral enriched mouth rinses they found that mineral gain occurred in all the groups but it was complete in mineral mouth rinse group.

Diamanti et al[16] (2010) evaluated the effect of toothpastes containing sodium fluoride in different concentrations or a calcium sodium phosphosilicate system on pre-softened dentin demineralization and remineralization using microhardness tests. Pre-softened bovine root dentin slabs were immersed after the demineralization periods, for 2 min in non-fluoridated, 7.5% calcium sodium phosphosilicate, 1450 ppm F, 2800 ppm F and 5000 ppm F toothpaste slurries and

subsequently subjected to a 15-h acid resistance test. The results revealed that the high fluoride toothpastes promoted remineralization and inhibited demineralization more effectively, than the 1450 ppm F, the non-fluoridated and the calcium sodium phosphosilicate toothpastes.

Carolina et al[13] (2010) investigated the in vitro efficacy of amine fluoride or sodium fluoride or tin chloride mouth rinses on 24 volunteers. They found that NaF mouth rinse reduced substance loss by 19% in enamel and 23% in dentin while the amine fluoride or sodium fluoride or tin chloride mouth rinse reduced this parameter by 67% in enamel and 47% in dentin.

Karlinsey et al[22] (2010) evaluated remineralizing potential of 5000 ppm fluoride dentifrices in a pH cycling model on bovine enamel specimens. Evaluating with Vickers microhardness test, mineral loss, transverse microradiography and polarized microscopy on Colgate PreviDent® Booster 5000 ppm fluoride and 3M Clinpro® 5000 ppm fluoride and fluoride free paste, they found that Clinpro® 5000 conferred superior surface and subsurface remineralization potential relative to both PreviDent® Booster 5000 and Tom's of Maine fluoride-free paste. Due to this superiority, these results suggested the combination of 5,000 ppm fluoride plus

the tricalcium phosphate system may provide significant anticaries benefits relative to fluoride-only and fluoride-free dentifrices.

Manton et al[27] (2010) investigated the in vitro effects on enamel erosion of the addition of 0.2%w⁄v casein phosphopeptide amorphous calcium phosphate (CPP-ACP) to four commercially-available soft drinks. They tested four drinks with and without 0.2% CPP-ACP w/v and distilled deionized water. They concluded that adding CPP-ACP at 0.2% w⁄v significantly decreased the erosivity of all four soft drinks. Also the erosivity of the soft drinks with 0.2%w/v CPP-ACP added did not differ significantly from that of distilled water.

Ambarkova et al[2] (2011) assessed the effect of commercially available dentifrices with different fluoride formulae on remineralization of enamel surface on 28 enamel slabs. Five different fluoride toothpastes at different fluoride level and an amine fluoride were used for the study. They found that high fluoride toothpaste with 1450ppm effectively inhibited demineralization.

Chow et al[15] (2011) evaluated the efficacy of orthodontic adhesives with fluoride or amorphous calcium phosphate in reducing bacterial adhesion and enamel demineralization on premolars with three adhesives; light cure composite with

fluoride, light cure composite without fluoride and light cure composite with ACP. The results revealed that composite with fluoride or ACP reduced bacterial adhesion and lesion formation.

Gibson et al[18] (2011) evaluated the research regarding professional and/or supplemental self-applied fluoride for preventing and remineralizing caries in moderate and high caries risk adults. Multiple databases were utilized and studies included randomized control trials or clinical trials conducted in moderate or high caries risk adult populations, evaluating self- or professionally applied fluoride with the outcomes of caries reduction or remineralization. Studies revealing moderatively effective in higher caries risk adults were low strength sodium fluoride rinses for carious lesions, 1.1% sodium fluoride paste or gel for root lesion remineralization and fluoride varnish for root caries remineralization.

Hamba et al[19] (2011) evaluated the effect of CPP-ACP and CPP-ACPF on inhibition of enamel demineralization over time using polychromatic micro computed tomography. Enamel blocks were prepared from bovine teeth and treated with the agents. Mean mineral loss and lesion depth were measured. They found that application of CPP-ACP and CPP-ACPF pastes to sound enamel surfaces resulted in inhibition of enamel demineralization, more so with CPP-ACPF paste.

They also found that quantitative assessment using polychromatic micro-CT was useful for detecting mineral density changes occurring in enamel demineralization.

Jayarajan et al[21] (2011) conducted an invitro study using scanning electron microscope and Diagnodent to investigate the efficacy of CPP-ACP and CPP-ACPF in remineralizing enamel surface on which artificial caries lesion had been created in maxillary premolars. They found that artificial saliva, CPP-ACP, and CPP-ACPF showed a statistically significant amount of remineralization and CPP-ACPF showed marginally more amount of remineralization than CPP-ACP.

Karlinsey et al[23] (2011) evaluated the *in vitro* remineralization effects of four dentifrice systems using microhardness and fluoride uptake analyses. Tests were conducted using 500 ppm sodium fluoride, 1150 ppm sodium fluoride, and 500ppm sodium fluoride plus functionalized tricalcium phosphate. The authors concluded that significant surface and subsurface strengthening can occur from a 500 ppm F dentifrice containing functionalized tricalcium phosphate, and despite having half the fluoride, the remineralization was comparable to that from an 1150 ppm F dentifrice.

Karlinsey et al[29] (2011) evaluated the remineralization potential of three silica-containing NaF dentifrice systems in an intraoral model. Test groups wore a customized orthodontic appliance attached to a mandibular molar and contained one tooth block with caries-like lesion. Treatments included 500 ppm fluoride, 500 ppm fluoride with functional tricalcium phosphate and 1100 ppm fluoride. The appliances were then subjected to surface microhardness, transverse microradiography, and cross sectional microhardness and the authors concluded that the combination of functional tricalcium phosphate and fluoride in a single compartment, water-based dentifrice can cooperate with fluoride to produce significant remineralization.

Agnihotri et al[1] (2012) investigated the efficacy of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) containing tooth mousse on the remineralization of enamel lesions and to compare its efficacy to fluoride containing tooth paste on premolars. Treatment groups contained fluoridated tooth paste and tooth mousse containing CPP-ACP. Authors concluded that tooth mousse remineralized initial enamel lesions and showed a higher remineralizing potential than fluoridated toothpaste.

Naumova et al[32] (2012) investigated the fluoride bioavailability in saliva and plaque comparing sodium fluoride and amine fluoride. Eight trained volunteers were included in the study and they found that fluoride levels in saliva and plaque are interindividually highly variable and there were no significant differences in bioavailability between sodium fluoride and amine fluoride in saliva or in plaque.

MATERIALS AND METHODS

Armamentarium

- Demineralizing solution
    - Calcium chloride
    - Potassium dihydrogen phosphate
    - Acetic acid
    - Potassium hydroxide
- pH strips
- Test tube
- Test tube stand
- Beaker
- Distilled water
- Remineralizing agents
    - Casein phosphopeptide amorphous calcium phosphate (Tooth Mousse, GC Corp, Tokyo, Japan)
    - NovaMin (Calcium Sodium Phosphosilicate) (Sensodyne repair and protect, Glaxosmithkline, U.K)
    - Amine flouride (Amflor, Group pharmaceuticals, India)
- Hard tissue microtome (Leica, germany)
- Digital camera

- Polarized light microscope (Labomed, Labo america inc, Fremount, California)

Methods

Collection of teeth

40 periodontally compromised maxillary anterior teeth were extracted and were used for the study. Decayed, attrited and restored teeth were excluded from the study. The teeth were thoroughly cleaned and washed under running water to remove all adherent soft tissues.

Fig 1. Tooth samples

The teeth were then divided into 4 groups with 10 teeth each.

Group A (n=10): Recaldent (Casein phosphopeptide-amorphous calcium phosphate) was used as a remineralizing agent.

Group B (n=10): NovaMin (Calcium Sodium Phosphosilicate) was used as a remineralizing agent.

Group C (n=10): Amine fluoride was used as a remineralizing agent.

Group D (n=10): The teeth served as a control in which only demineralization was done.

Preparation of demineralizing solution

2800ml of demineralizing solution was prepared by mixing 2.2 mM Calcium chloride ($CaCl_2$), 2.2 mM Potassium dihydrogen phosphate (KH_2PO_4) and 50 mM Acetic acid. The pH was adjusted at 4.5 by adding 10M Potassium hydroxide (KOH) to the prepared solution. Each specimen was individually kept in 10 ml of the demineralizing solution in a test tube. The demineralizing solution was refreshed every day and the procedure was done for 1 week.

Fig 2. Prepared demineralizing solution

Fig 3. Teeth kept in demineralizing solution

Remineralizing agent

After 1 week, the process of remineralization was started for the first three groups. In group A remineralizing paste was applied over the labial surface of the tooth and left over for 3-5 minutes while in group B and C remineralizing paste was applied over the labial surface of the tooth and brushed for 2-3 minutes with a tooth brush. After application the teeth were rinsed and again stored in distilled water. This was done twice a day for a period of 3 weeks. Teeth in group D were stored in distilled water.

Fig 4. GC Tooth Mousse (CPP-ACP)

Fig 5. Sensodyne repair and protect (NovaMin)

Fig 6. Amflor (Amine fluoride)

Mounting and sectioning

After 3 weeks, the teeth were mounted on cold cure acrylic and the coronal portions were sectioned labiolingually by means of water cooled hard tissue microtome to obtain vertical slices of 400µ*m* thickness. The centre portions of all the teeth were taken for assessing the depth of demineralization so as to maintain the standardization between samples. These sections were then used to observe areas of enamel demineralization under a polarized microscope.

Fig 7. Mounted teeth in acrylic

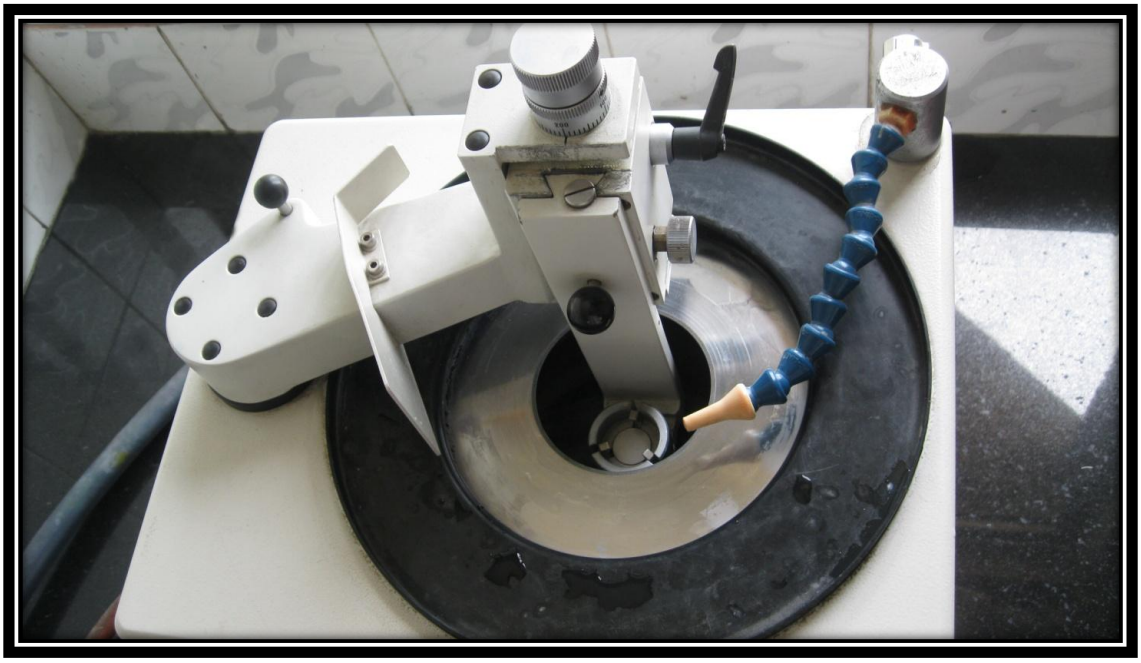


Fig 8. Hard tissue microtome.

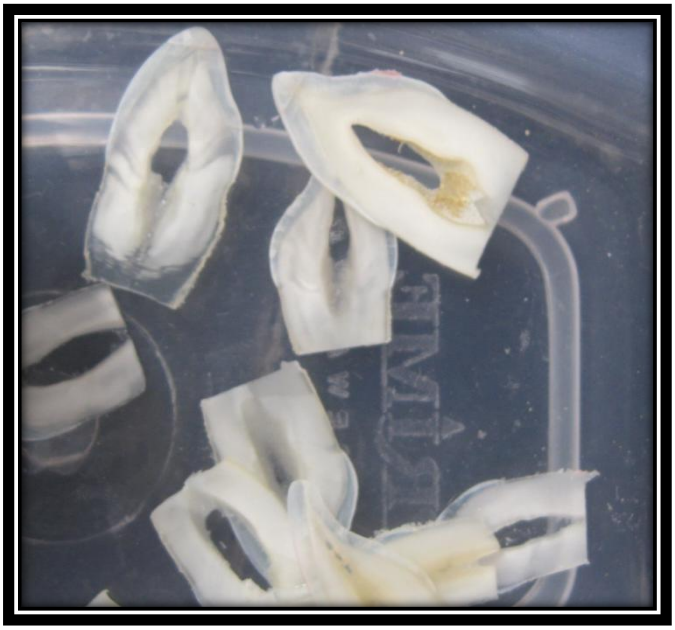


Fig 9. Sections before mounting under a polarized microscope

Viewing under polarized light microscope

The enamel surfaces of the samples were viewed under a Labomed CXR3 microscope with crossed polarizer and analyzer plates for areas of demineralization. Photography was done using a Nikon Coolpix 4500 digital camera at 2x zoom under a 4×10 x magnification. The depth of demineralization was measured using the IMAGETOOL© software. After assessing different areas in the central portion of the tooth, the maximum depth of demineralization was recorded for each sample.

Statistical analysis

The values obtained were statistically analysed using Student's t-test to test the difference between the groups, with the p value set at < 0.05.

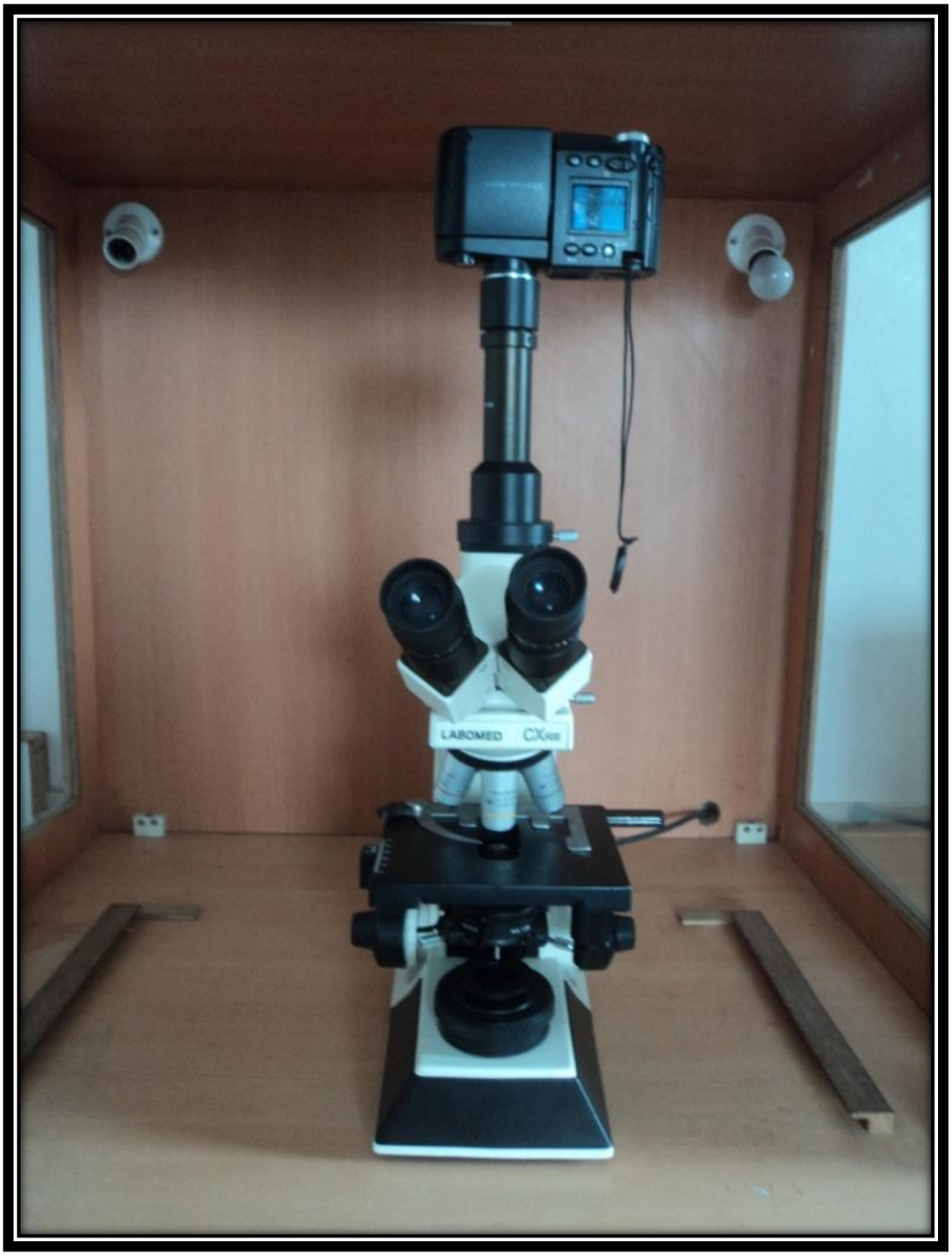


Fig 10. Polarized Light Microscope

RESULTS

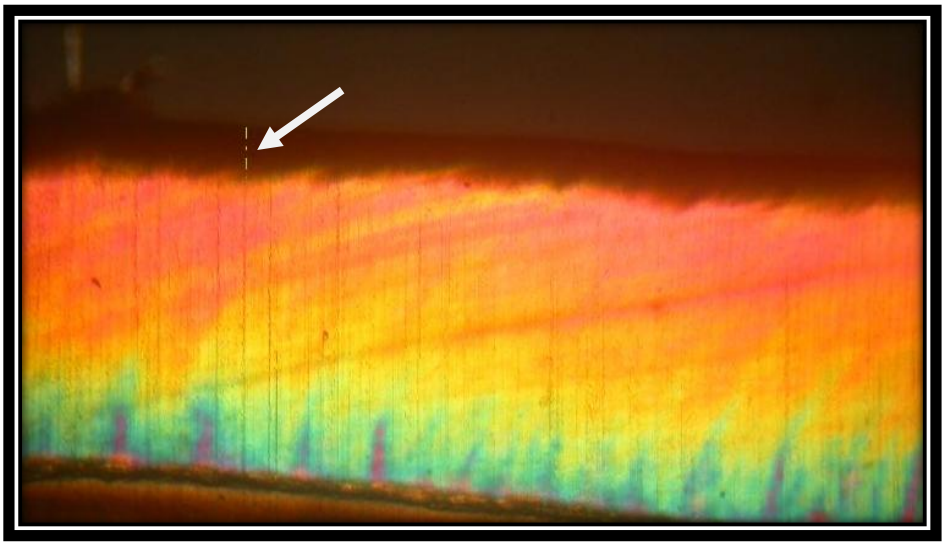


Fig 11. Maximum depth of demineralization noted in CPP-ACP group (Group A) (Arrow shows area of demineralization)

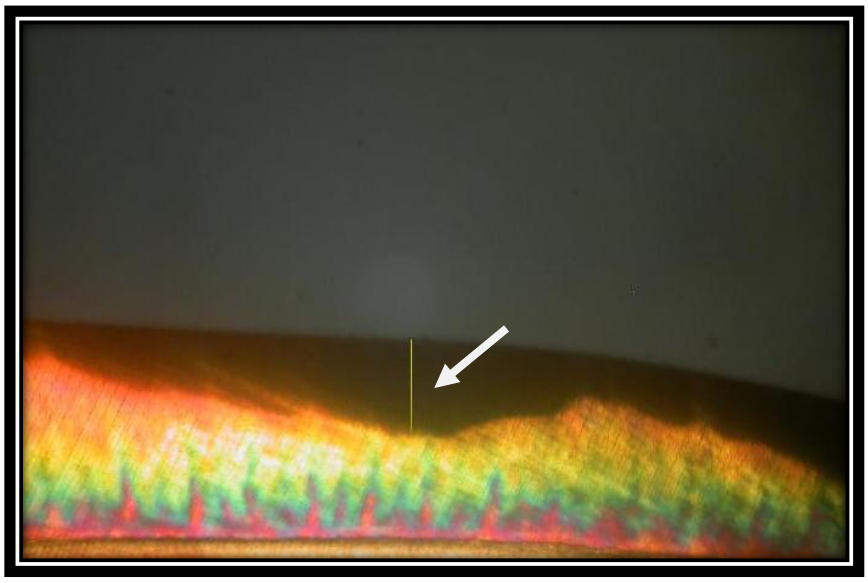


Fig 12. Maximum depth of demineralization noted in NovaMin group (Group B) (Arrow shows area of demineralization)

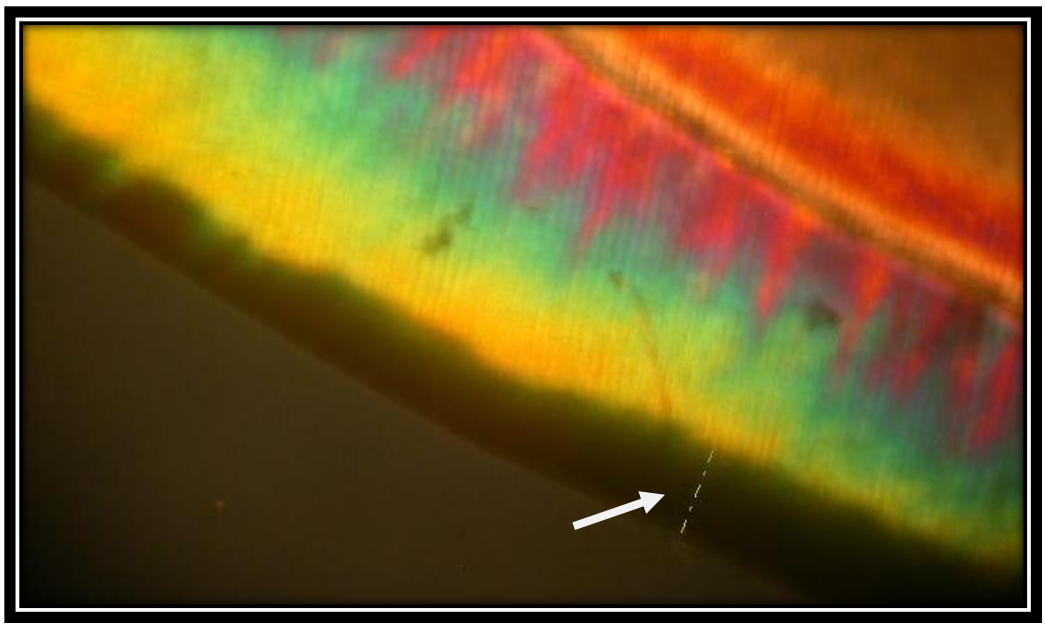


Fig 13. Maximum depth of demineralization noted in Amine fluoride group (Group C) (Arrow shows area of demineralization)

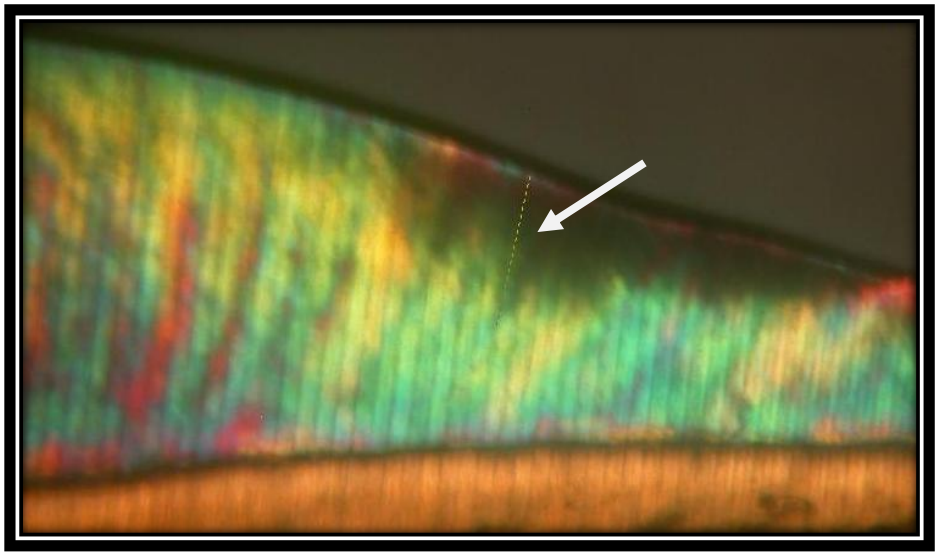


Fig 14. Maximum depth of demineralization noted in Control group (Group D) (Arrow shows area of demineralization)

MASTER CHART

Maximum depth of demineralization noted from enamel surface (in *µm*)

Sl No:	Groups			
	A	B	C	D
1	27.00	195.00	86.33	386.00
2	27.00	99.00	50.99	270.00
3	84.48	183.00	58.14	437.78
4	120.00	213.00	96.83	288.00
5	27.00	153.00	90.00	357.30
6	116.85	91.00	184.40	335.36
7	117.00	132.50	183.22	382.00
8	126.00	162.47	164.15	273.31
9	45.00	149.50	98.52	312.42
10	90.33	189.73	85.50	241.00
Mean value	78.06	156.82	109.808	328.31

Group A – CPP-ACP

Group B – NovaMin

Group C – Amine fluoride

Group D – Control

Mean Value and standard deviation was calculated for the demineralized areas. Descriptive statistics for demineralization are given in the table below

Descriptive statistics for demineralization:

AGENTS	N	MINIMUM VALUE	MAXIMUM VALUE	MEAN	STANDARD DEVIATION
CPP-ACP	10	27.00	126.00	78.0660	42.40584
NovaMin	10	91.00	213.00	156.8200	40.48230
Amine fluoride	10	50.99	184.40	109.8080	49.29027
Control	10	241.00	437.78	328.3170	62.36990

From the above table it can be said that CPP-ACP had the lowest value of demineralization and the highest value of remineralization. Similarly NovaMin had the highest value of demineralization and the lowest value of remineralization.

Further group statistics (Student's t-test) was performed to compare the remineralization efficiency between two groups. Lesser the demineralization more the remineralization efficacy.

t-test between CPP-ACP and Control group :

AGENTS	N	MEAN	STANDARD DEVIATION	t-VALUE	Level of significance/p-VALUE
CPP-ACP	10	78.0660	42.40584	10.493	0.000
Control group	10	328.3170	62.36990		

In the above table the t-value 10.493 for the mean difference between CPP-ACP and Control group were significant (p = 0.000). The mean demineralization value for CPP-ACP and Control group were 78.06µm and 328.31µm respectively. Hence it can be inferred that, since demineralization was lesser in CCP-ACP than the control group, remineralization has occurred in the CPP-ACP group.

t-test between NovaMin and Control group:

AGENTS	N	MEAN	STANDARD DEVIATION	t-VALUE	Level of significance/p-VALUE
NovaMin	10	156.8200	40.48230	7.294	0.000
Control group	10	328.3170	62.36990		

In the above table the t-value 7.294 for the mean difference between NovaMin and Control group were significant (p = 0.000). The mean demineralization value for NovaMin and Control group were 156.82µm and 328.31µm respectively. Hence it can be inferred that, since demineralization was lesser in NovaMin than the control group, remineralization has occurred in the NovaMin group.

t-test between Amine fluoride and control group:

AGENTS	N	MEAN	STANDARD DEVIATION	t-VALUE	Level of significance/p-VALUE
Amine fluoride	10	109.8080	49.29027	8.692	0.000
Control group	10	328.3170	62.36990		

In the above table the t-value 8.692 for the mean difference between Amine fluoride and Control group were significant (p = 0.000). The mean demineralization value for Amine fluoride and Control group were 109.80µm and 328.31µm respectively. Hence it can be inferred that, since demineralization was lesser in Amine fluoride than the control group, remineralization has occurred in the Amine fluoride group.

t-test between CPP-ACP and NovaMin :

AGENTS	N	MEAN(µm)	STANDARD DEVIATION	t-VALUE	Level of significance/p-VALUE
CPP-ACP	10	78.060	42.40584	4.248	0.000
NovaMin	10	156.8200	40.48230		

In the above table the t-value 4.248 for the mean difference between CPP-ACP and NovaMin were significant (p = 0.000). The mean demineralization value for CPP-ACP and NovaMin were 78.06µm and 156.82µm respectively. Hence it can be inferred that, since demineralization was lesser in CCP-ACP than the NovaMin, remineralization has occurred more in the CPP-ACP group.

t-test between CPP-ACP and Amine fluoride :

AGENTS	N	MEAN	STANDARD DEVIATION	t-VALUE	Level of significance/p-VALUE
CPP-ACP	10	78.060	42.40584	1.544	0.140
Amine fluoride	10	109.8080	49.29027		

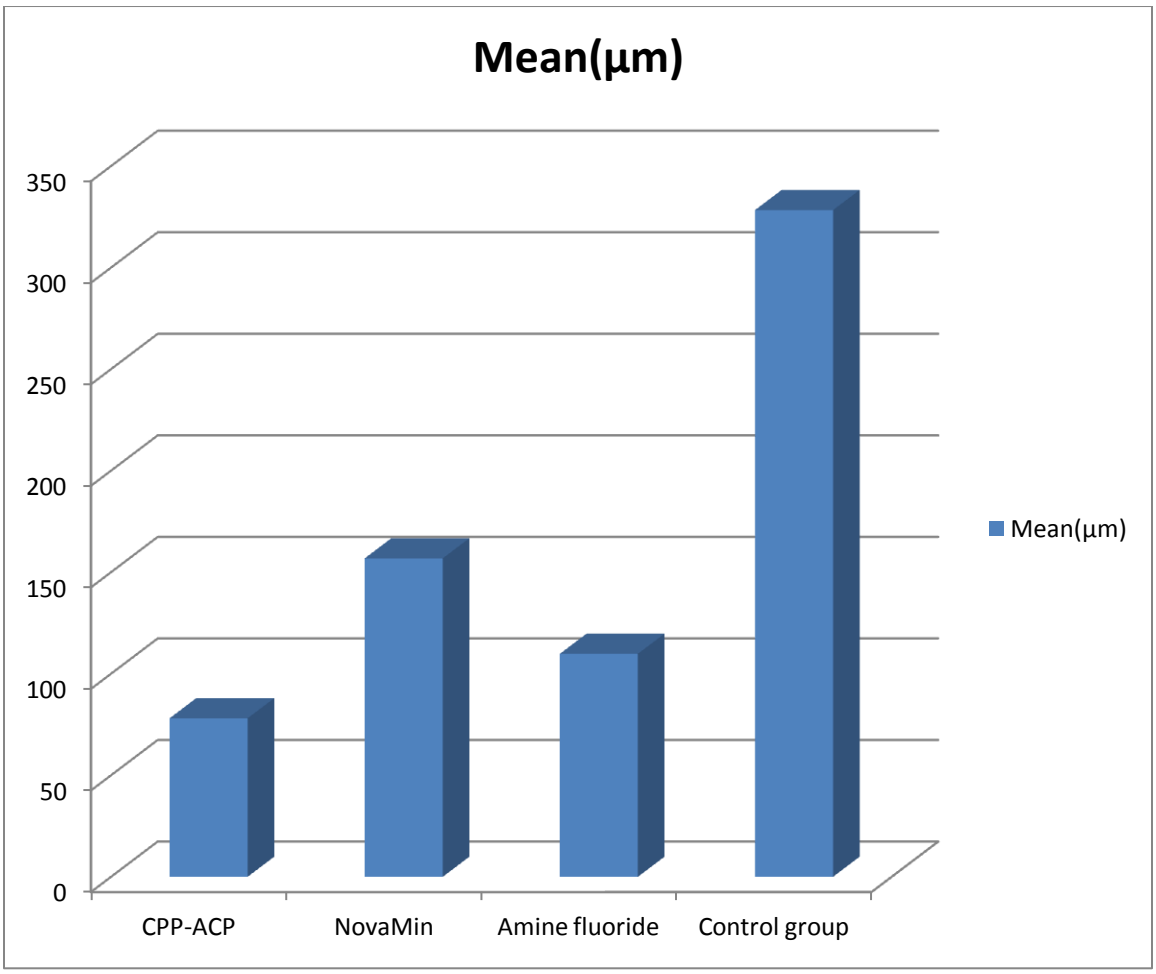
In the above table the t-value 1.544 for the mean difference between CPP-ACP and Amine fluoride were not significant (p = 1.544). The mean demineralization value for CPP-ACP and Amine fluoride were 78.06µm and 109.80µm respectively. Since both the groups were not statistically significant both were equal in remineralizing efficacy.

t-test between NovaMin and Amine fluoride:

AGENTS	N	MEAN	STANDARD DEVIATION	t-VALUE	Level of significance/p-VALUE
NovaMin	10	156.8200	40.48230	2.331	0.032
Amine fluoride	10	109.8080	49.29027		

In the above table the t-value 2.331for the mean difference between NovaMin and Amine fluoride were significant (p = 0.032). The mean demineralization value for NovaMin and Amine fluoride were 156.82µm and 109.80µm respectively. Hence it can be inferred that, since demineralization was lesser in Amine fluoride than the NovaMin, remineralization has occurred more in the Amine fluoride group.

Graphical representation



DISCUSSION

When tooth mineral is exposed to an acidic solution, as during the cariogenic attack, active mineral dissolution occurs by transport of Ca^{2+} and $H_2PO_4^-$ ions from the advancing front of lesion to the plaque. This dissolution can continue until a state of equilibrium with the plaque environment is reached. Hence demineralization can be seen as a process consisting of transport of H^+ ions from plaque to the advancing front and transporting dissolved mineral ions from the advancing front to plaque[49].

Since H^+ ion concentration gradient is the main driving force for the demineralizing process, an increase in pH of the demineralizing medium will reduce the driving force for H^+ transport into the lesion, hence diminishing the rate of demineralization. Similarly, an increase in calcium and phosphate concentrations of the demineralizing medium would increase the concentration gradients against which these ions are transported and should also reduce the rate of demineralization and enhance remineralization[49].

Featherstone in 2006 described that the process of caries lesion formation depends on pathological factors outweighing the preventive factors present in the oral environment[17]. The main clinical sign of demineralization has been found to be the white-spot lesion, which has been identified as having an intact surface zone

and subsurface demineralization (**Silverstone** in 1968)[17]. This formation of a white-spot lesion makes it easier to identify areas of demineralization clinically, although remineralization is not as easily measured or observed clinically[52].

Enamel and dentin undergo unlimited cycles of demineralization and remineralization. A tip in the balance one way or the other will either lead to stronger healthier teeth or greater susceptible teeth. So the advantage of remineralization is that it could counteract the effect of demineralization rather than leading to caries, thereby loss of tooth structure followed by cavity preparation and restoration of the teeth[26].

The cariostatic efficacy of fluorides has been convincingly demonstrated and the recent decline in caries prevalence is primarily attributed to the increased use of fluoride agents. The mechanism by which fluoride increases caries resistance may arise from both systemic and topical applications of fluoride and can be broadly be grouped as follows: Increased enamel resistance, increased rate of maturation, remineralization of incipient lesions, interference with microorganisms and improved tooth morphology[34]. The effectiveness of topical sodium fluoride (NaF) as a cariostatic agent has been established well, and

professional topical NaF applications are commonly used to arrest the progression of active caries. As the ‘‘Minimal Intervention’’ concept becomes widely spread, various products with tooth surface protection, anticariogenic or remineralizing effects have gained an increasing attention[19]. Concentrating mainly on prevention and early intervention of caries, minimal intervention dentistry’s first basic principle is the remineralization of early carious lesions, advocating a biological or therapeutic approach for early surface lesions[36].

Anticariogenic properties of milk and milk products such as cheese have been shown in human and animal models[38,42]. The mechanism of action suggested, is a direct chemical effect from phosphoprotein casein and calcium phosphate components of the cheese. Casein phosphopeptides (CPP), derived from milk, have the ability to stabilize calcium phosphate in solution through binding amorphous calcium phosphate (ACP) with their multiple phosphoserine residues allowing the formation of small CPP–ACP clusters and have an anti-caries protective effect, by suppressing demineralization, enhancing remineralization, or possibly a combination of both[56]. They are reported to promote remineralization through release of free calcium and phosphate ions[19].

Bioactive glass compounds have been available since the late 1960s as materials designed to help repair damaged bone and have also been effective in bone-grafting procedures used in ridge augmentation and repair of periodontal defects[57]. The bioglass products have been said to bind to existing bone and act as scaffolds for new bone growth. NovaMin is a newer class of bioactive glass-ceramic materials that provide calcium and phosphate upon reaction. In the products with NovaMin, the active ingredient is a calcium sodium phosphosilicate that reacts when exposed to aqueous media and provides calcium and phosphate ions that form a Hydroxy-carbonate apatite (HCA) with time[52].When particles come in contact with saliva or water, they rapidly release sodium, calcium and phosphorous ions into the saliva which are available for remineralization of the tooth surface. Unlike other calcium phosphate technologies, the ions that bioactive glass release form hydroxy carbonate apatite (HCA) directly, without the intermediate amorphous calcium phosphate phase[9]. These particles also attach to the tooth surface and continue to release ions and remineralize the tooth surface after the initial application. Ultimately these particles will completely transform into HCA and result in 80% tubular occludance and desensitization. When bioactive glass is incorporated into toothpaste formulations, the ions released from the amorphous calcium phosphate layer are believed to contribute to the remineralization process of the tooth surface[31,28,26].

The effect of amine fluoride on enamel remineralization has been studied in clinical investigations and showed similar effects of amine fluoride and NaF and is said to be more effective than sodium monofluorophosphate[51]. A lot of attention has been paid to the amount of fluoride in dentifrices[47]. Most studies showed that even low concentrations of salivary fluoride affect enamel demineralization and remineralization. Salivary fluoride levels decrease with the time after topical application with a fluoride dentifrice. For the effectiveness of fluoride over periods longer than the brushing and the following salivary clearance, fluoride needs to be deposited and slowly released. For Amine fluoride the surface tension has also been responsible for the caries protective effect[44,3].

This study focused on comparing the remineralizing efficacy of Casein phosphopeptide amorphous calcium phosphate (Recaldent), Calcium sodium phosphosilicate (NovaMin) and Amine fluoride as there are no studies comparing these three agents. The demineralization solution used here was 2.2 mM Calcium chloride ($CaCl_2$), 2.2 mM Potassium dihydrogen phosphate (KH_2PO_4) and 50 mM Acetic acid adjusting the pH at 4.5 by adding 10M Potassium hydroxide (KOH) as the previous studies by **Ten Cate et al (1982)[46], Agnihotri et al (2012)[1], Hamba et al (2011)[19], Kumar et al (2008)[24]** have shown demineralization of tooth with

this solution. It has been suggested that the use of a continuous exposure in the acidic environment, as in this study, would simulate a severe case scenario of demineralization, magnifying the effects of each treatment on enamel[19].

While using the polarized light microscope the samples need to be sectioned labiolingually to a 400μm thickness so as to observe under the microscope. So a control group was kept to assess the depth of demineralization with the demineralizing solution alone. Enamel is uniform with adequate thickness in the central portion of the tooth. So depth measurement was standardized to be measured in this specific area and also to minimize sampling errors due to inadequate thickness of enamel. Since remineralization cannot be directly measured it was measured indirectly by the decrease in the depth of demineralized areas.

Polarized light microscopic analysis is a very sensitive technique for showing changes in hard tissues. With respect to demineralization and remineralization, birefringence experiments can qualitatively show mineral loss and mineral gain[3]. Readings of the total path difference are recorded at various intervals along a transverse axis running from the outer surface through the lesion into sound enamel. The polarized light microscopy is a sensitive technique for

assessing demineralization and remineralization in *in vivo* studies. It requires only a polarized light microscope to study the enamel samples quantitatively. Polarized light measurements can provide quantitative information on the pore volume in demineralized and remineralized enamel, and on lesion characteristics[3]. All other techniques either need expensive equipment like microradiography or would need a flat surface of enamel sample, which is unsuitable for *in vivo* evaluation[8]. Keeping in mind all these factors, polarized light microscopy was used.

Maximum depth of demineralization was assessed in each sample. Mean remineralization was assessed by subtracting mean demineralization depth of experimental groups from mean demineralization depth of control group. Students t-tests were performed to analyse the remineralizing efficacy of all the three agents. The mean demineralization depth of experimental groups was much lesser in comparison to the control group, being statistically highly significant, showing that remineralization occurred in all these agents. CPP-ACP showed maximum efficacy having the minimum demineralization depth (78.06 µm) followed by amine fluoride (109.80 µm). However, these values did not have any statistically significant difference. NovaMin showed minimum efficacy (demineralization depth 156.82 µm). This value showed statistically highly significant difference from CPP-ACP and significant difference from fluoride.

Flouride ions get adsorbed onto the surface of enamel crystals, inhibiting dissolution and enhancing remineralization. Softened surface lesions get remineralized faster and more completely than the subsurface lesions. **Ogaard and co-workers** discouraged the treatment for white spot lesions with concentrated fluoride agents as they would arrest the lesions and prevent complete repair by surface hypermineralization[33]. CPP-ACP can stabilize over 100 times more calcium phosphate than is normally possible in aqueous solution at neutral or alkaline ph before spontaneous precipitation occurs. For mineral deposition to occur within the body of the lesion, calcium and phosphate ions must first penetrate the surface layer of enamel[21]. **Jayarajan et al (2011)** stated that CPP-ACP are able to consume the acid generated during enamel lesion remineralization by generating more calcium and phosphate ions, including $CaHPO_4$, thus maintaining the high concentration gradient into the lesion[21].

It has been observed that silicates in bioglass compounds are a stronger inducer of remineralization of the tooth than fluoride. However the hydroxy carbonate apatite precipitate formed by bioglass application is more soluble than fluor hydroxyl apatite formed by fluoride application. Hence the acid resistance of bioglass treated substrate is lesser than that treated by fluoride[16]. This could be a

reason why depth of demineralization was more in NovaMin than in fluoride in our study.

A study conducted by **Kumar et al (2010)**[24] compared the remineralization efficacy of CPP-ACP and fluoride and showed both can inhibit demineralization and enhance remineralization with CPP-ACP having more remineralizing potential. Another study conducted by **Diamanti et al (2010)**[16] compared the remineralization efficacy of Fluoride and NovaMin and showed both can inhibit demineralization and enhance remineralization with fluoride having more remineralizing efficacy. These study results are in accordance with the present study results.

Studies conducted by **Hamba et al (2011)**[19] and **Jayarajan et al (2011)**[21] compared CPP-ACP and CPP-ACPF and showed that fluoride along with CPP-ACP showed more remineralizing potential than CPP-ACP or Fluoride used alone.

Reynolds et al (2008)[40] suggested that products containing CPP-ACP with NaF (CPP-ACPF) might be superior in reducing caries risk, when compared to CPP-ACP. With the use of low fluoride concentration, as in CPP-ACPF, there is a complex localization of free calcium phosphate and fluoride and ion activities

which helps in maintaining a state of supersaturation by suppressing demineralization. Further research using CPP-ACP and CPP-ACPF pastes with different approaches are needed.

This study was done under invitro conditions which may not simulate the complex oral conditions where demineralization and remineralization is a constant cyclic process. Further studies should be conducted in in vivo or ex vivo models for a closer simulation of oral environment to prove the efficacy of these agents in natural oral conditions.

SUMMARY AND CONCLUSION

The aim of this study was to utilize polarized light microscope for the assessment of the efficacy of CPP-ACP, NovaMin and Amine Fluoride pastes on remineralization of enamel over time.

40 teeth were used for the study which was divided into 4 groups:

Group A (n=10): Recaldent (casein phosphopeptide-amorphous calcium phosphate) was used as a remineralizing agent.

Group B (n=10): NovaMin (Calcium Sodium Phosphosilicate) was used as a remineralizing agent.

Group C (n=10): Amine fluoride was used as a remineralizing agent.

Group D (n=10): The teeth served as a control in which only demineralization was done.

Following one week of demineralization and three weeks of remineralization, the teeth were mounted on cold cure acrylic and the coronal portions were sectioned labiolingually by means of water cooled hard tissue microtome to obtain vertical slices of 400μm thickness. These sections were then used to observe areas of enamel demineralization under a Labomed CXR3 microscope with crossed polarizer and analyzer plates for areas of demineralization. Photography was done using a Nikon Coolpix 4500 digital camera at 2x zoom under a 4X10 x

magnification. The depth of demineralization was measured using the IMAGETOOL© software. After assessing different areas, the maximum depth of demineralization was recorded

The values obtained were statistically analysed using Student's t-test to test the difference between the groups, with the p value set at < 0.5 and found that CCP-ACP and Amine fluoride was better in remineralization followed by NovaMin.

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