ORIGINAL ARTICLE

Comparative Evaluation of Microhardness and Enamel Solubility of Treated Surface Enamel with Resin Infiltrant, Fluoride Varnish, and Casein Phosphopeptide-amorphous Calcium Phosphate: An *In Vitro* Study

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Abstract

Aim and objective: The aim and objective of this study was to do a comparative evaluation of microhardness and enamel solubility (ES) of the treated surface enamel with resin infiltrant, fluoride varnish, and casein phosphopeptide-amorphous calcium phosphate (CPP-ACP).

Materials and methods: An *in vitro* study was conducted on freshly extracted 85 sound permanent teeth of which 5 teeth were subjected to check for microhardness by the Vickers microhardness tester and the remaining teeth were exposed to demineralizing solution to create initial enamel lesions. These 80 teeth were assigned to four groups: group I—negative control ($n = 20$), group II—resin infiltrant ($n = 20$), group III—fluoride varnish ($n = 20$), and group IV—CPP-ACP ($n = 20$), and microhardness was checked after application. These teeth were exposed to caries attack three times a day for three consecutive days. The ES of these four groups was checked by calcium ion loss in the artificial cariogenic solution and whole saliva by an atomic absorption spectrophotometer.

Results: It was found that none of the experimental groups reached the microhardness values of sound intact teeth. At 3rd day, the values of microhardness were: group II = group III > group IV > group I. Maximum ES was found for group I (control) followed with group IV.

Conclusion: All agents used in study remineralized initial carious lesion. Fluoride varnish has the highest microhardness and showed least ES compared to other remineralizing agents.

Clinical significance: Fluoride varnish can be regarded as the choice of material to be used for the treatment of incipient carious lesions because of the low application frequency (once every 3–6 months), requires minimal patient compliance as it is a noninvasive procedure and less time consuming.

Keywords: Bifluoride 12, Remineralizing agents, Resin infiltrant (ICON), Tooth mousse.

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Introduction

The most prevalent oral condition globally is dental caries. The contribution of dental caries to the incidence of oral disease among most prevalent ones is about 10 times higher than that of periodontal disease.[1] The prevalence of caries in school-age children is recorded by the World Health Organization (WHO) at 60–90% and as nearly widespread among adults in most countries.[2] Dental caries is a "pandemic" disease characterized by a high proportion of untreated carious cavities causing pain, irritation, and physical disabilities due to its internationally high prevalence.[3] In addition, untreated carious cavities have a direct effect on children's general well-being and the social and economic well-being of societies.[4] Prevention of dental caries is very essential as it affect persons' self-esteem and quality of life.

Initiation of caries is associated with subsurface tooth enamel demineralization. Calcium and phosphate are lost from subsurface enamel, resulting in subsurface lesion formation. Since the advent of dentistry as an academic discipline at the end of the nineteenth century, it has been influenced by a mechanical age of high-speed rotary cutting equipment, and primarily used to remove caries for surgical approach. This involved the radical removal of the diseased part of the tooth, along with geometrical extensions (material-driven) to areas that we believed to be resistant to caries; however, this idea is clinically based and maybe deceptive.

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Today, in the interests of protection, it can be claimed that no sound tooth structure has to be removed, and new procedures are deemed minimally invasive and nondestructive.[5] At the early stage of the carious process, the initial lesion is reversible via the remineralizing process to restore the lost tooth structure. Various remineralizing agents available in the market that remineralize the initial enamel caries are casein derivatives, fluoride varnish, resin infiltrant, tricalcium phosphate, bioactive glass, and many others.

Resin infiltrant is used as a material to treat initial carious lesion by creating the diffusion barrier within the body of lesion and not at the surface of lesion as in case of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP). Caries infiltration utilizes capillary forces to transport resins through enamel porosities with high penetration coefficients (so-called infiltrates). The infiltrant occludes diffusion routes for cariogenic acids and dissolved minerals after polymerization.[6] Such microinvasive therapy of enamel caries has been scientifically proven to be effective in preventing and stabilizing lesions.[7–9]

The various types of topical fluorides used in dentistry for caries prevention and inhibition are sodium fluoride (NaF), calcium fluoride (CaF_2), sodium monofluorophosphate, stannous fluoride, and acidulated phosphate fluoride. Inorganic in nature, all of these fluorides are present in the form of liquids, varnishes, foam, gels, dentifrices, etc.[10] Remineralization of initial caries carious lesions has been seen by fluoride varnish application because of a sustained release ability according to studies done by Castellano and Lin.[11,12]

The CPP-ACP nanocomplexes are derived from bovine milk protein, casein, calcium, and phosphate; amorphous calcium phosphate (ACP), stabilized in the case in phosphate (CPP), is directly absorbed in the enamel or is incorporated into the plaque, serving as a calcium and phosphate reservoir for remineralization of tooth's hard tissues.[13] A number of subsequent studies have shown that CPP-ACP has anticariogenic potential in laboratory, animal and human *in situ* experiments,[14–16] resulting in the incorporation of CPP-ACP as a new tool in the battle against caries[17,18] in dental products and food products.

Given the ability of dental hard tissues to improve resistance to acid attack, it can also be assumed that the remineralizing agent can offer a degree of protection for dental hard tissues against erosive dietary acid insult. Searching for agents that avoid or repair dental degradation from acid attack is important. To measure the degree of protection against erosive insult by dietary acids for dental hard tissue, the physical and chemical analysis checking microhardness and enamel solubility (ES) of the teeth after application of various remineralizing agents can be done. Microhardness is one of the indirect methods and ES testing is one of the direct quantitative methods by which demineralization and remineralization can be checked.

There is a need to carry out a study that provides a true validation regarding the microhardness and ES of treated surface enamel with the various available remineralizing agents in the market. Since no study has been conducted that evaluates and compares the microhardness and enamel solubility of treated surface with resin infiltrant, fluoride varnish, and CPP-ACP (assesses Medline/PubMed, EBSCOhost, ScienceDirect, and Embase database on October 10, 2016), there arises a need to carry out a study that can help us to assess which among these noninvasive, remineralizing agents has a better resistance to acid attack during *in vitro* caries stimulation and can best help in inhibiting the havoc caused by dental caries.

The null hypothesis of the present study was that the microhardness and ES after acidogenic challenge were similar between resin infiltrant, fluoride varnish, and CPP-ACP. The aim of the study is to evaluate and compare microhardness and ES of treated surface enamel with resin infiltrant, fluoride varnish, and CPP-ACP.

Materials and Methods

To test and compare the microhardness and ES of the treated surface enamel with resin infiltrant, fluoride varnish, and CPP-ACP, the present *in vitro* research was undertaken. Ethical clearance was obtained from the institutional ethics committee, and the number being "SVIEC/ON/Dent/BNPG-11/D13192."

Sample Size

The sample size determined for the study was 85 sound extracted single-rooted teeth utilizing the formula: $n = 2 \times (2.802 \times 5/8.8)^2 = 5.069$, where standard deviation (SD) = 5 and $d = 8.8$ with the confidence interval (CI) kept as 95% and power as 80%.

Preparation of Specimens

Freshly extracted single-rooted permanent teeth were obtained from the department of oral surgery and were stored in 0.1% thymol solution till use. All teeth were cleansed of soft tissue debris and inspected for hypoplasia, cracks, and white spot lesions or caries. Extracted permanent single-rooted teeth were included in the study. For teeth that were excluded, the following criteria were used: (1) fractured or cracked permanent single-rooted teeth, (2) carious teeth, teeth with hypoplasia.

The methodology is depicted in Flowchart 1 in a step-wise manner. Eighty-five teeth that met the inclusion criterion (Fig. 1) were coated with an acid-resistant nail varnish (Revlon, New York, USA), leaving an approximately 5 × 5 mm small square "window" on the sound and intact buccal or lingual enamel surface. Eighty-five teeth specimens prepared for this were preserved in 100% humidity until needed for use. This was accomplished by placing the specimens in a sealed plastic container filled with deionized water.

Preparation of Experimental Demineralizing Solution (Artificial Cariogenic Solution)

The buffered demineralizing solution (artificial cariogenic solution) was prepared by analytical grade chemicals and deionized water. It was prepared according to a formulation used by ten Cate and Duijsters[19] consisting of 2.2 mM calcium chloride, 2.2 mM potassium hydrogen phosphate, 0.005 M acetic acid, and 1 M potassium hydroxide with the pH of the solution adjusted to 4.4. In order to determine the weight of the chemical used to create the solution, it is important to know the molecular weight of the chemical along with the volume and concentration of the solution required. The molar solution concentration calculator was used to make the solution with the concentration of the above chemicals that is expressed in molarity. Hence, to calculate the weight of the chemicals the following formula was used:

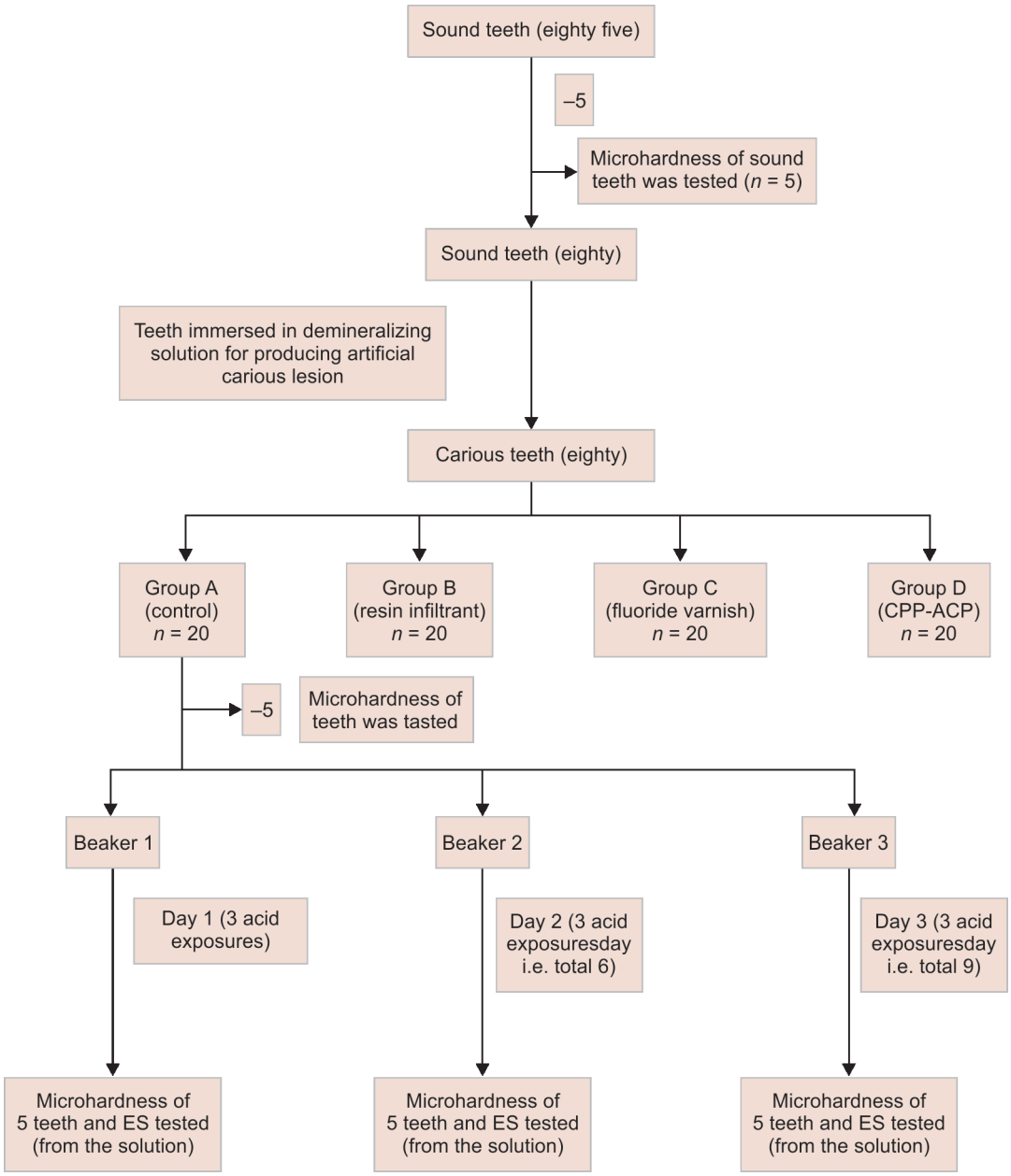
Molar solution concentration equation:

$$C = m / v \times 1 / \text{MW}; \text{hence}, m / v = C \times \text{MV}$$

where, *C* is the molar concentration in mol/L (molarity), *m* is the mass (i.e., weight) of the solute in grams (g), *v* is volume of solution in liters (*L*) in which the indicated mass (*m*) of solute must be dissolved to make the desired molar concentration, and MV is the molecular weight in g/mol.

The values determined were 0.2442 g/L calcium chloride (anhydrous), 0.2993 g/L potassium hydrogen phosphate, 0.3 g/L of acetic acid, and 56 g/L potassium hydroxide. Acetic acid is available as liquid form; hence, weight in grams (g) was converted to milliliters (mL) accounting for 2.9 mL/L. For the preparation of

Flowchart 1: Entire methodology in a flow diagram for the control group, which would be the same for all the other groups



solutions in deionized water, the chemicals were measured by digital weighing scale (Shayona scale, Vadodara, India) (Fig. 2) in the concentration mentioned above. These prepared solutions were than mixed to make 1 L of experimental demineralizing solution (artificial cariogenic solution) (Fig. 3) by adjusting the pH to 4.4 with the help of a digital pH meter (Systronics pH system 361; Systronics India Ltd, Ahmedabad, India).

Preparation of Experimental Remineralizing Solution (Artificial Whole Saliva Solution)

The buffered remineralizing solution (artificial whole saliva solution) was prepared by analytical-grade chemicals and deionized water. It contained 1.5 mM of calcium chloride, 0.9 mM of sodium dihydrogen phosphate, 0.15 M of potassium chloride, and a pH of 7.0. This solution corresponds to the supersaturation of apatite minerals found in saliva and was comparable to that designed and implemented by ten Cate and Duijsters.[19]

The values of these chemicals were calculated with the help of above-mentioned molar solution concentration equation and were 0.1171 g/L calcium chloride, 0.108 g/L sodium dihydrogen phosphate, and 0.87 g/L potassium chloride.

For the preparation of solutions in deionized water, the chemicals were measured by a digital weighing scale (Shayona scale, Vadodara, India). These prepared solutions were than mixed to make 1 L of experimental remineralizing solution (artificial whole saliva solution) by adjusting the pH to 7 with the help of a digital pH meter (Chemi Line Digital pH Meter CI 110: Chemiline Technologies, Ahmadabad, India).

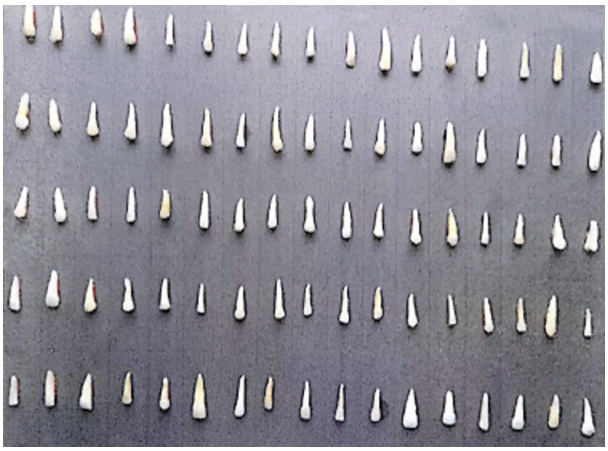


Fig. 1: Eighty-five teeth those were included in the study

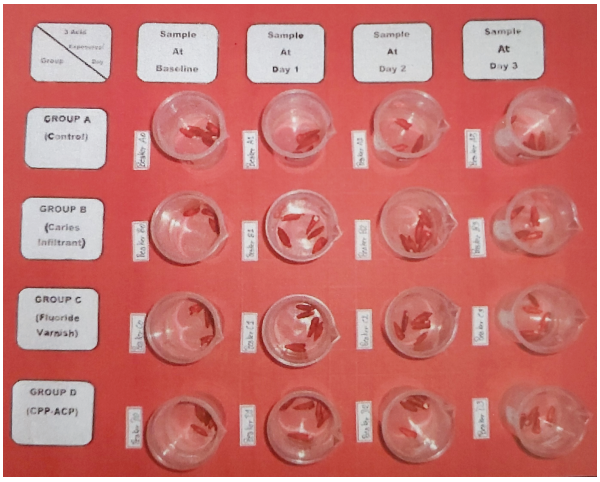


Fig. 3: Teeth from all groups placed in whole saliva solution at baseline, day 1, day 2, and day 3

Artificial Carious Lesion Preparation in Teeth

Out of the prepared 85 teeth specimens, 80 specimens were subsequently immersed for 96 hours in a demineralizing solution (100 mL) to artificially develop initial carious enamel lesions. The depth of the lesions produced by this method is 120–200 µm as established by Kumar et al. in their study.[20] Clinically chalky white areas similar to that produced in initial enamel carious lesions were visible on the 5 × 5 mm window created on the enamel surface of 80 specimens.

Test Groups

Eighty specimens of the total sample size (i.e., 85 specimens) had to be distributed to four groups, with 20 teeth in each group. Table 1 shows the details of the dental products used in various groups.

Randomization

In order to minimize bias, four envelopes for four groups were used to obtain a random distribution sequence before teeth were processed for the intervention, since each tooth has distinct anatomical features that can be a possible confounding factor in the estimation of effects. This procedure was done by a coinvestigator.

Blinding

This was a single-blinded study where allocation concealment of different groups was done by the coinvestigator, who coded all

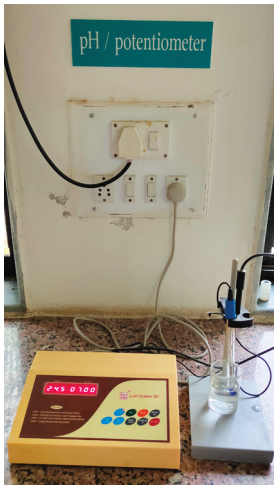


Fig. 2: Digital pH meter used for adjusting the PH of remineralizing solution at 7.0

Table 1: Distribution of 85 specimens with product details for four groups

Group	*Material*	*Trade name*	*Batch code*
I	Negative control	–	–
II	Caries infiltrant	Icon smooth surface, DMG, Hamburg, Germany	707406
III	Fluoride varnish	Bifluorid 12, Voco, Cuxhaven, Germany	1232043
IV	CPP-ACP	Tooth Mousse, GC Corp, Tokyo, Japan	120319S

the specimens using sealed envelopes after the remineralizing agents were applied by the principal investigator. The principal investigator was blinded to who recorded the readings and after collection of all the data, finally decoding of the samples was done by the coinvestigator.

Group I (20 Teeth)

Negative control; where no remineralizing agent was applied on the initial carious lesion.

Group II (20 Teeth)

Resin infiltrant (Icon smooth surface, DMG, Hamburg, Germany) was applied on the initial carious lesion.

Procedure: Icon-etch, which is 15% hydrochloric acid gel, was applied to the 5 × 5 mm window of the prepared specimen and placed for 2 minutes and rinsed. This erodes the lesion's surface region and opens the body's lesion pore system. Then pore system was dried with ethanol. The etchant was rinsed off with water for 30 seconds and dried with oil- and water-free air. With the application aid, the Icon-Infiltrant was applied to the lesion body for 3 minutes and the moist lesion surface was preserved by periodically twisting the syringe. The Icon resin's incredibly high penetration coefficient allows it to penetrate into the pores of the lesion. Excess material was discarded and light-curing of the material lasted 40 seconds. The applicator tip was replaced and Icon-Infiltrant was applied for 1 minute and light-cured for 40 seconds.

Group III (20 Teeth)

Fluoride varnish (Bifluorid 12, Voco, Cuxhaven, Germany) was applied on the initial carious lesion.

Procedure: On the 5 × 5 mm window of the prepared specimen, a thin layer of fluoride varnish (Bifluorid 12) containing 6% sodium fluoride and 6% calcium fluoride was applied with Pele Tim that is foam pellets for 10–20 seconds after properly shaking the bottle till the balls become separated from the sediment and were dried with air.

Group IV (20 Teeth)

CPP-ACP as a topical coating (Tooth Mousse, GC Corp, Tokyo, Japan) was applied on the initial carious lesion.

Procedure: On the 5 × 5 mm window of the prepared specimen, CPP-ACP (Tooth Mousse) was taken in sufficient quantity on the gloved finger and applied twice for 3 minutes.

Exposure of the Specimens to Acid Attack

The test groups were subjected to three acid exposures each day for 3 days accounting for a total of nine acid exposures. Total 15 teeth from each group were divided into three batches, with five teeth in each batch for placing in artificial demineralizing solution for 10 minutes at the interval of 2 hours three times/day, simulating artificial carious attack.

On day 1, five teeth from each group were exposed to the three acid attacks by placing them in beakers AA1 (group I), AB I (group II), AC1 (group III), and AD1 (group IV) three times for 10 minutes at the interval of 2 hours. These teeth were then subjected to physical and chemical evaluation after three acid attacks for microhardness and enamel solubility (ES) by means of digital microhardness testing and atomic absorption spectrophotometry (AAS), respectively.

On day 2, five teeth from each group were exposed to a total of the six acid attacks (three attacks/day for 10 minutes each at the interval of 2 hours) by placing them in beakers AA2 (group I), AB2 (group II), AC2 (group III), and AD2 (group IV). These teeth were also subjected to physical and chemical evaluation after six acid attacks for microhardness and ES as mentioned above. On day 3, remaining five teeth from each group were exposed to a total of the nine acid attacks (three attacks/day for 10 minutes each at the interval of 2 hours) by placing them in beakers AA3 (group I), AB3 (group II), AC3 (group III), and AD3 (group IV). These teeth were also subjected to physical and chemical evaluation after nine acid attacks for microhardness and ES. Each day after the acid attacks, all groups were stored separately in a remineralizing solution (artificial whole saliva solution) (Fig. 3). ES from the remineralizing solution was also checked for 3 days.

Evaluation Techniques

Physical Evaluation by Digital Microhardness Testing

Preparation of standard resin blocks: The specimens were embedded and blocks from epoxy resins (DPI-RR Cold Cure, DPI, Mumbai, India) were prepared of size 34 mm × 15 mm × 6 mm so as to subject them to microhardness testing. Resin blocks were prepared in such a way that the window of 5 × 5 mm prepared on labial or lingual surfaces is, seen which will be subjected to digital microhardness testing (Banbros, Ghaziabad, India).

Surface microhardness (SMH) testing of prepared specimens in resin acrylic blocks: The SMH of each sound sample was calculated using digital monitor of the Vickers hardness tester (Banbros, Ghaziabad, India) at TCR Advanced Engineering Pvt. Ltd., Vadodara. The pyramid diamond instrument applied a load of 50 g to the enamel surface at three points. The average was taken as the standard reading of the three points. The intensity of SMH was measured using the Vickers index unit and the Vickers hardness number (VHN) readings were obtained.

The microhardness of five sound teeth was checked that were not subjected to demineralization so as to know the SMH of intact sound teeth.

After application of the above remineralizing agents, the microhardness of five teeth from each group (20 teeth) was checked, providing the information of the microhardness at the baseline. At day 1 after the exposure of the specimens to acid attack, the preparation of specimens was done by embedding them in resin blocks and microhardness of 5 teeth from each group (20 teeth) was checked, providing the information of the microhardness after three acid attacks. At day 2 the microhardness of five teeth from each group (20 teeth) was checked after the exposure of the specimens to acid attack, providing the information of the microhardness after six acid attacks. At day 3, the microhardness of five teeth from each group (20 teeth) was checked after the exposure of the specimens to acid attack, providing the information of the microhardness after nine acid attacks.

Chemical Evaluation by Atomic Absorption Spectrophotometer

Equipment Used

AAS (NovAA 350 Analytikjena, Jena, Germany), at Choksi Laboratories Limited, Vadodara, India, was used to check the ES of the treated surface enamel with the remineralizing agents by evaluating the amount of calcium ion loss (ACa), calculated by inspecting the difference in the amount of calcium ions in the demineralizing solution and remineralizing solution, pre- and post-acid attack.

AAS Principle

novAA 350 is a fully automated double-beam mode flame system and an automatic eight-lamp turret. A vapor of atoms of metal is formed when a solution of metallic salt (some other metallic compound) is aspirated through a flame (e.g., acetylene burning in the air). A much greater percentage of gaseous metal atoms, though, would usually remain at the ground level. This ground state atoms are capable of receiving radiant energy from their own individual resonance wavelength, which, in general, is the radiation wavelength released by the atoms if excited from the ground state. Therefore, if light is passed into the flame containing the atoms in question through the resonance wavelength, part of the light will be absorbed, with the degree of absorption being equal to the amount of atoms of the ground state present in the flame. The quantity of light energy absorption by the specific element is measured by AAS.

The ES of treated surface enamel of four groups for all three days was evaluated. The amount of calcium present in the demineralizing solution (D) and remineralizing solution (R) is calculated before starting the experiment. By subtracting the calcium ions present in the post-attack solution at each day (1, 2, 3) from D, calcium loss (ACa) during acidogenic exposure can be calculated. The range of the instrument is 185–900 nm wavelength and the values of calcium ions were obtained in parts per million (ppm).

Statistical Analysis

For testing the hypothesis, the collected data were entered in Microsoft Excel (2007) spreadsheet. Descriptive statistical tests were computed using Excel statistical operations. Inferential statistics was done by using the SPSS 17.5 version for Microsoft Windows. The statistical tests that were applied for analyzing the data between the study groups and controls were ANOVA, Tuckey's post-hoc test,

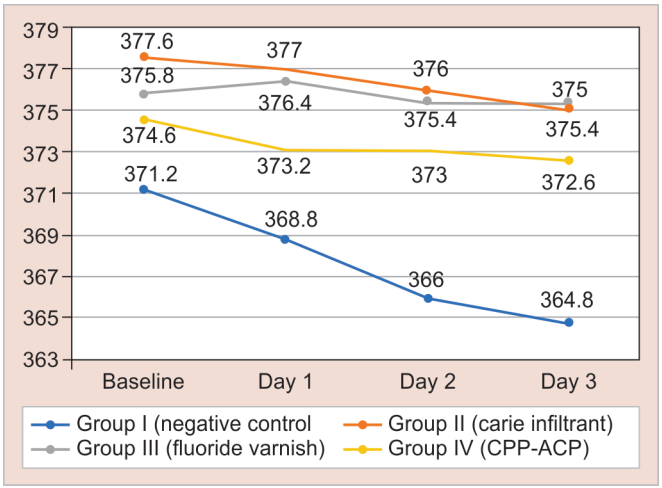
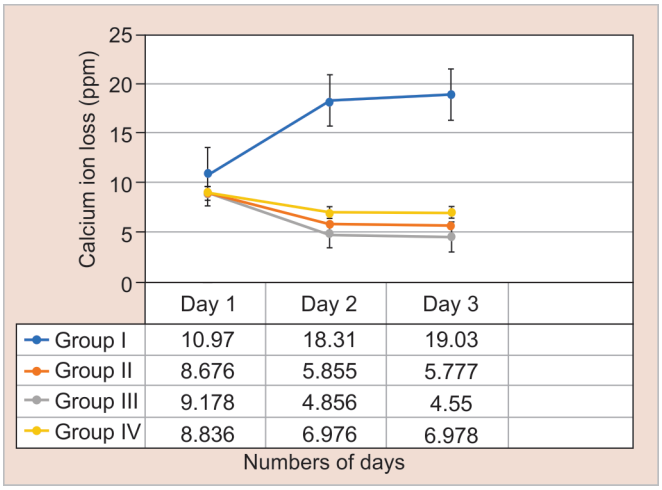


Fig. 4: Line graph showing mean microhardness (VHN) values of four groups at baseline, day 1, day 2, and day 3



	Day 1	Day 2	Day 3
Group I	10.97	18.31	19.03
Group II	8.676	5.855	5.777
Group III	9.178	4.856	4.55
Group IV	8.836	6.976	6.978

Fig. 5: Line graph showing amount of calcium loss (ppm) of all groups from the artificial cariogenic solution baseline, day 1, day 2, and day 3

and for the interpretation of the data within the group, the paired *t*-test was applied. The *p* value of <0.005 was considered statistically relevant for all the experiments performed. The level of confidence interval was kept as 95%.

Results

Figure 4 shows line graph indicating mean microhardness value of group I (negative control), group II (resin infiltrant), group III (fluoride varnish), and group IV (CPP-ACP) (resin infiltrant) at baseline, day 1, day 2, and day 3. Microhardness of group I keeps on decreasing as the number of days (acid exposure) advances. For group II and group IV, the mean values decrease with the increase in number of acid exposures, whereas for group III an increase in mean microhardness was seen from baseline to day 1 (375.80–376.40), further decrease at day 2, and gradually stabilize by day 3.

Figure 5 shows the line graph denoting the amount of calcium loss (ppm) of all four groups from the artificial cariogenic solution at day 1, day 2, and day 3. The amount of calcium ion (ppm) leach out for group I increases from day 1 to day 3 (10.97–19.03 ppm), whereas for group II, group III, and group IV the calcium (ppm) leach out decreases from day 1 to day 3 in the artificial cariogenic solution.

Figure 6 depicts the amount of calcium loss (ppm) from the whole saliva solution at day 1, day 2, and day 3 for four groups. Each value denotes the total amount of calcium ion loss for five teeth. The amount of calcium ion (ppm) leach out for group I increases from day 1 to day 3 (1.166–3.341 ppm), whereas for group II, group III, and group IV, the calcium (ppm) leach out decreases from day 1 to day 3 in the whole saliva solution.

Discussion

Very few studies have been done to evaluate and compare microhardness as well as ES of surface enamel caries with the various remineralizing agents. The present research is one of a kind in which the evaluation and comparison of microhardness and ES of treated surface enamel with resin infiltrant, fluoride varnish, and CPP-ACP is done. For checking the remineralizing ability, it is necessary to know how much mineral has been lost or acquired to check the remineralizing potential, and direct or indirect mineral quantification methods are used to evaluate how much the loss or gain has been resulted. In the present study, two methods used to evaluate and compare remineralizing ability of the above-mentioned remineralizing agents are Vickers microhardness testing and atomic absorption spectrophotometry.

Microhardness Testing

Enamel demineralization due to pH drop below the critical point leads to the breakdown of hydroxyapatite and the diffusion to the enamel surface of calcium and phosphate ions.[21,22] Microhardness determinations may provide indirect evidence of mineral loss or gain by evaluating enamel and dentin hardness as far as de- and remineralization are concerned.[23] During caries development process, there is loss of minerals from the tooth structure and as microhardness indentation measurements have been used since Koulourides' first *in situ* experiments (1966)[24] to assess demineralization and remineralization effects, by providing an evaluation of mineral loss; this procedure was used to evaluate the mechanical properties of the surface enamel, its caries-related and remineralizing process-related alterations. Various other authors have utilized the same method for estimation of carious process-related alterations in their studies.[25–27]

Microhardness testing assessment can be conducted by SMH where the indenter load is perpendicular to the surface of the polished tissue or cross-section microhardness (CSMH) assessment where the indenter load is parallel to the anatomical surface of the tissue. In the first case, as opposed to a homogeneous one in the second case, the indenter experiences an inhomogeneous tissue. In the current research, SMH testing was performed to determine the quantity of remineralization on artificially developed surface enamel caries as topical application of the three remineralizing agents was performed on the enamel surface, and since the findings about the difference in hardness from one tooth segment to another were often not used for each other, so CSMH was not used.[28]

Knoop hardness and Vickers hardness are the two most commonly used methods to evaluate microhardness of tooth. The difference between measures of Knoop and Vickers hardness is primarily the penetration depth of the indenter. The Knoop indenter penetrates about 3.5 μm for an indentation length of 100 pm, while the Vickers diamond reaches a depth of around 14 μm.[21] In this study, enamel demineralization was assessed using the digital display Vickers hardness tester. The tissue has lost mineral if the indentation length values increase; if the indentation length values

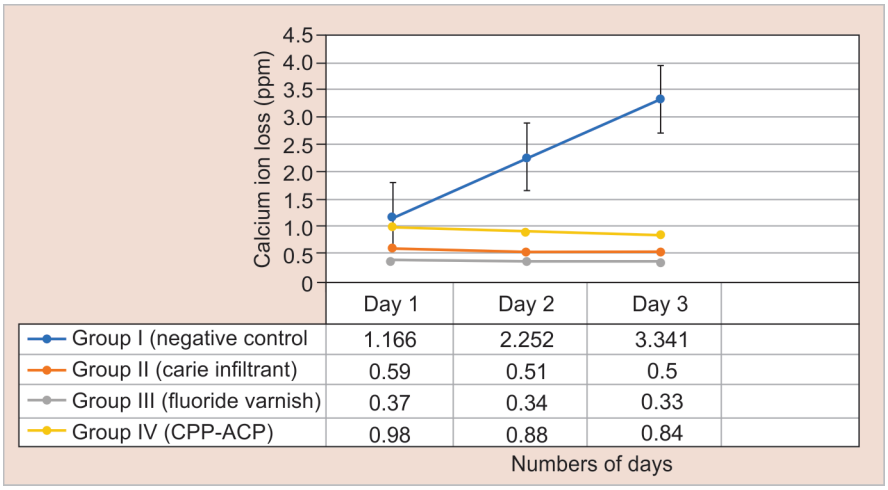


Fig. 6: Line graph showing amount of calcium loss (ppm) of all groups from the whole saliva solution at day 1, day 2, and day 3

decrease in magnitude, the mineral is more likely acquired by the tissue. Therefore, higher Vickers hardness number (VI-IN) shows slight mineral loss; lower microhardness values show mineral loss. A linear relationship with a correlation coefficient of 0.95 by White[29] between the Vickers hardness indentation length and lesion depth has been found.

Microhardness at Baseline

The mean microhardness values of four experimental groups, group I (negative control), group II (resin infiltrant), group III (fluoride varnish), and group IV (CPP-ACP) at baseline that is after application of remineralizing agents, have the following order: group II > group III > group IV > group I (Fig. 4). For experimental groups (group II, group III, and Group IV), the values of mean microhardness are higher compared to negative control (group I) owing to the topical application of remineralizing agents above the surface enamel lesions in them.

The mean microhardness value of intact sound teeth is 381.6 VHN, but mean microhardness values of none of the experimental groups attained the values of sound intact teeth. Group II that is with application of resin infiltrant was the closest to achieving the mean microhardness value of intact sound teeth with 377.60 VHN followed by group III (fluoride varnish) with 375.80 VHN and group IV (CPP-ACP) with 374.60 VHN.

This might be because group I: resin infiltrant (Icon smooth surface, DMG, Hamburg, Germany) stabilizes the porous lesion body by achieving an impervious barrier, consisting of a preferably broad, hemogeneous resin layer within the lesion body. Caries infiltration utilizes capillary forces to transport resins into enamel porosities with high penetration coefficients (so-called infiltrants). The infiltrant occludes diffusion pathways for cariogenic acids and dissolved minerals after polymerization.[6] In comparison to caries sealing, the diffusion barrier is mainly created inside the lesion body and not at the lesion surface in caries infiltration. Clinically, such microinvasive treatment of enamel caries has been shown to be successful in stopping and stabilizing lesions,[7–9] while increasing microhardness compared to untreated or remineralized carious lesions.[30,31]

In case of group II: fluoride varnish (Bifluorid 12, Voco, Cuxhaven, Germany), the greater microhardness mean values may be because it aids in the formation of long-lasting intraoral fluoride reservoirs[32] and because of its ability of forming fluorhydroxyapatite to enhance the remineralization of incipient carious lesions.[24] Second, the varnish delivery system allows ion prolonged contact between fluoride and dental hard tissues, therefore providing ideal conditions for their interaction and at the same time preventing the immediate loss or fluoride alter application. Various fluoride varnishes differ greatly in their ability, to reharden, delivery of fluoride to carious lesions and in their fluoride release characteristics into artificial saliva, which depends on the contact time with the lesions according to Lippert.[33] Bifluorid 12 (6% NaF and 6% CaF_2) showed better remineralizing ability among other fluoride varnishes in studies done by Stamm[34] and Biswas.[35]

Sustained release mechanisms, such as varnishes, typically display an initial burst, with rapid release of the active agent, followed by a slower release period.[36] The presence of low-level fluoride affects the conversion of less-stable, more-soluble mineral stages such as dicalcium phosphate dehydrate [DCPD], tricalcium phosphate [TCP], octacalcium phosphate [OCP], to more-stable, less-soluble mineral phases, such as hydroxyapatite [HAP], fluorhydroxyapatite [FHAP], and fluorapatite [FAP].[37]

In case of group IV, the higher mean microhardness values may be due to CCP's ability to locate ACP at the tooth surface when CPP-ACP is used as a topical coating (Tooth Mousse, GC Corp, Tokyo, Japan). In this way, the CCP-ACP can serve as a reservoir of calcium phosphate, buffering the free activities of calcium and phosphate ions, thereby helping to sustain a state of supersaturation with regard to the demineralization and enhancement of remineralization of tooth mineral depressing enamel (Reynolds; Huq et al.).[14,38]

It has been revealed by Reynolds[39] and Cross et al.[40] that casein phosphopeptide (CPP) has the potential to stabilize calcium phosphate in solution by forming colloidal casein and phosphopeptide amorphous calcium phosphate complexes. The CPP molecules are a cluster of residues—Ser(P)–Ser(P)-Ser(P)-Glu-Glu—which, by stabilizing amorphous calcium phosphate under neutral and alkaline conditions, greatly improve the apparent solubility of calcium phosphate. The CPP's multiple phosphoseryl residues bind to ACP nanodusters in supersaturated solutions, thus preventing growth to the critical size needed for phase transformations (Reynolds).[39]

Microhardness at Day 1

The mean microhardness values of four experimental groups at day 1 that is with three acid attacks for the time period of 10 minutes/

attack, after application of remineralizing agents, have the following order: group II > group III > group IV > group I (Fig. 4). Three acid attacks/day were given since a person has minimum of three meals/day simulating oral conditions in the present *in vitro* study. Time period of 10 minutes/attack was kept as the fall in pH below the critical pH (5.5) occurs for 10 minutes during eating.[41] It is mostly due to the formation of lactic acid in plaque intraorally, with acetic and propionic acids being lost from the plaque at the same time.[42,43] If more acidogenic, aciduric bacteria are present in plaque, the pH will decrease more rapidly. The rate of pH decrease also depends on the rate at which plaque bacteria will metabolize the dietary carbohydrate. As sucrose is rapidly metabolized, creating a quicker decrease, bigger molecules, such as starch, can move more slowly through plaque, and before plaque bacteria will be assimilated, it will have to be broken down.

For the groups, group I, group II, and group IV, the values of mean microhardness at day 1 fell as compared to the values at baseline with difference being of 2.4 VHN, 0.6 VHN, and 1.4 VI-IN, respectively, owing to the three acid exposures at day 1. For group II, the fall in microhardness values after acidogenic challenge is similar to that reported by Torres et al.[30] stating surface hardness reduction when infiltrated lesions were submitted to a new cariogenic solution.

For group III, there was an increase in mean microhardness value by 0.6 VHN from baseline to day 1. Partially bound fluoride is also known as soluble or alkali-soluble fluoride potassium hydroxide (KOH), which prevents enamel crystal demineralization. The intraorally accessible calcium fluoride-like globules and ionic fluoride are loosely attached fluorides. The other group is strongly bound fluoride integrated into the apatite crystals, which is also known as alkali-insoluble fluoride, KOH-insoluble fluoride, or apatitically bound fluoride. During *in vitro* testing following short exposures to topical fluorides, Cruz et al.[41,44] found this to be minimal in sound enamel. The acquisition of securely bound fluoride often involves time, which first includes the diffusion of available fluoride into the enamel, explaining the increase in microhardness values from baseline to day 1.

Microhardness at Day 2

The mean microhardness values of four experimental groups at day 2 that is with six acid attacks for the time period of 10 minutes/attack, after application of remineralizing agents, have the following order: group II > group III > group IV > group I (Fig. 4). For all the groups, group I, group II, group III, and group IV, the values of mean microhardness at day 2 fell as compared to the values at day 1 with difference being of 2.8, 1, 1, and 0.2 VHN, respectively, owing to the total of six acid exposures at day 2 suggesting avoidance of acid exposure for 24 hours after topical application of remineralizing agents.

Microhardness at Day 3

The mean microhardness values of four experimental groups at day 3 that is with nine acid attacks for the time period of 10 minutes/attack, after application of remineralizing agents, have the following order: group II = group III > group IV > group I (Fig. 4). For the groups, group I, group II, and group IV, the values of mean microhardness at day 3 fell as compared to the values at day 2 with difference being of 2.2, 0.6, and 0.4 VHN, respectively, owing to the total of nine acid exposures at day 3. For group III, the mean microhardness value remained same as day 2 at day 3 indicative of greater resistance to acid attack. Moreover, the values for group III coincided with group II (Fig. 4) suggesting that eventually as the days pass the resistance to acid attack increases on account of the sustained release of fluoride from the fluoride varnish, which leads to formation of fluorapatite crystals.

Intergroup Comparison of Microhardness at Baseline

The difference in microhardness between group I and all other groups was extremely significant (Table 2) demonstrating porous enamel prism structure after production of enamel caries requiring immediate attention and remineralization. The difference between group I and group IV was very significant (*p* value 0.003) because resin infiltrant fills the porous prismatic enamel structure whereas CPP-ACP has topical effect and it does not fill the porous carious enamel.

Intergroup Comparison of Microhardness at Day 1

The difference in microhardness between group I and all other groups was extremely significant signifying increase of the porous enamel prism structure of untreated surface enamel caries by three acid attacks (Table 2 and Fig. 4). As resin infiltrant is a low-viscosity resin that fills the spaces between the remaining porous lesion crystals and the demineralized tissue, increasing the mechanical strength of the infiltrated enamel and reducing the progression of the lesion[7–9,45–47] due to which the difference between group II–group IV and group III–group IV was also extremely significant. Rehardening of surface enamel caries by fluoride varnish depends on the various factors such as incubation time, fluoride uptake, and fluoride release. In the present study fluoride release was visible at day 1, whereas the rate of remineralization for CPP-ACP was 1.5 to 3.9×10^{-8} mol hydroxyapatite m^{-2} s^{-1} as stated by Reynolds.[39]

Manufacturers typically recommend children to eat only soft food during the first 2 hours after FV application, refrain from eating hard and abrasive foods, and avoid oral hygiene measures such as toothbrushing or flossing for at least 6 hours after application. No prompt dietary restrictions guidelines exist for the tested fluoride varnish and thus from the finding of the present study it can be stated that dietary restrictions for the food substances that fall the critical PH for a longer periods of time should be minimized for first 24 hours such as sucrose-containing food; it is the most cariogenic sugar since it can form glucan that allows for firm bacterial adhesion to teeth and restricts acid and buffer diffusion in the plaque. It can be interpreted that avoidance of regular intake of juice or other sugar-containing beverages in the bottle or sippy cup and reduction of steadily consumed sugar-containing snacks (e.g., sweets, cough drops, lollipops, suckers) should be accomplished for an initial 24 hours.

Intergroup Comparison of Microhardness at Day 2

The difference in microhardness between group I and all other groups was extremely significant, signifying increase of the porous enamel prism structure of untreated surface enamel caries by six acid attacks. The difference between group II and group IV was extremely significant (*p* value < 0.0001), whereas between group III and group IV was very significant (*p* value 0.002) (Table 2 and Fig. 4) because of the same reasons mentioned above.

Intergroup Comparison of Microhardness at Day 3

The difference in microhardness between group I and all other groups was extremely significant signifying increase of the porous enamel prism structure of untreated surface enamel caries by nine

acid attacks. The disparity between group II and group IV and group III and group IV (*p* value 0.004) reduces on day 3 relative to day 2 (Table 2 and Fig. 4) because the CPP stabilizes more than 100 times more calcium phosphate than is usually available in aqueous solution before spontaneous precipitation at neutral and alkaline P1-1 (Holt and van Kemenade)[48] and precipitation occurs on the 3rd day symbolizing remineralization.

The ACP and the crystalline phases, dicalcium phosphate dihydrate (DCPD) and octacalcium phosphate (OCP), were involved as intermediates in the formation of hydroxyapatite (HA) depending on pH and saturation. Assuming that the deposited mineral in remineralized lesions was mainly HA, the maximum average remineralization rate for the 10-day duration was $3.9 \pm 0.8 \times 10^{-8}$ moles HA/m^2.[49] This value is equal to the maximum rate of remineralization of enamel subsurface lesions obtained using a constant-composition method by de Rooij and Nancollas.[50]

Intragroup Comparison of Microhardness

Group I

The difference in microhardness between baseline and day 1 and day 1 and day 2 was very significant, whereas between baseline and day 2, baseline and day 3, and day 1 and day 3 is extremely significant (*p* value < 0.0001) (Table 3 and Fig. 4) symbolizing increased porosity of the surface enamel with the increase in number of acid exposures.

Group II

The difference in microhardness between day 1 and day 3 was significant (*p* value 0.016) (Table 3 and Fig. 4) representing decreased microhardness with the increase in number of exposures to acidic challenge, which may be due to enamel dissolution that is not entirely impregnated by the adhesive or, thus, to crack dissolution that is on the surface during the photo-curing procedure as stated by Torres et al.[30]

Group III

No statistically significant difference was seen in mean microhardness values between baseline, day 1, day 2, and day 3 (Table 3 and Fig. 4), representing continuous and sustained release of fluoride from the fluoride varnish to the enamel surface lesions leading to rehardening of the enamel surface as considerably larger portion of fluoride will be incorporated in the form of structurally bound fluoride (e.g., fluoridated hydroxyapatite) in demineralized enamel than in sound enamel over time, where fluoride is primarily present as loosely bound fluoride (e.g., as calcium fluoride) and, therefore, is subject to removal.[32]

Group IV

The difference in microhardness between baseline and day 1, baseline and day 2, and baseline and day 3 is significant (*p* value 0.01, 0.05) (Table 3 and Fig. 4), indicating lower potential of remineralization with the increasing number of exposures to acidic challenge.

Enamel Solubility Testing by Atomic Absorption Spectrophotometer

However, the relationship between microhardness indentation and mineral content is empirical; quantitative mineral content can only be obtained when measured against quantitative technique.

Table 2: Intergroup comparison of all groups at baseline, day 1, day 2, and day 3 of all four groups

Comparison between groups		*Baseline*		*Day 1*		*Day 2*		*Day 3*	
		Mean difference	*p value*	*Mean difference*	*p value*	*Mean difference*	*p value*	*Mean difference*	*p value*
Group I	Group II	−6.400 (−8.42, −4.38)	<0.0001****	−8.200 (−9.69, −6.71)	<0.0001***	−10.000 (−11.51, −8.49)	<0.0001****	−10.600 (−12.54, −8.66)	<0.0001****
Group I	Group III	−4.600 (−6.62, −2.58)	<0.0001****	−7.600 (−9.09, −6.11)	<0.0001***	−9.400 (−10.91, −7.89)	<0.0001****	−10.600(−12.54, −8.66)	<0.0001****
Group I	Group IV	−3.400 (−5.42, −1.38)	0.001***	−4.400 (−5.89, −2.91)	<0.0001***	−7.000 (−8.51, −5.49)	<0.0001***	−7.800 (−9.74, −5.86)	<0.0001***
Group II	Group III	1.800 (−0.223.82)	0.090	0.600 (−089,2.09)	0.662	0.600 (−0.91, 2.11)	0.675	0.000 (−1.94, −1.94)	1.000
Group II	Group IV	3.000 (0.98, 5.02)	0.003**	3.800 (2.31,5.29)	<0.0001****	3.000 (1.49, 4.51)	<0.0001****	2.800 (0.86, 4.74)	0.004**
Group III	Group IV	1.200 (−0.82, 3.22)	0.357	3.200 (1.71. 4.69)	<0.0001****	2.400 (0.89, 3.91)	<0.002**	2.800 (0.86, 4.74)	0.004**

The grading of *p* value was done as follows: extremely significant when *p* value was <0.0001 (symbolized by ****); extremely significant when *p* value was between 0.0001–0.001 (***); very significant when *p* value was between 0.001-0.01 (**); significant when *p* value was between 0.01–0.05 (*) and when *p* value was ≥0.05 it was not considered to be statistically significant

Table 3: Intragroup comparison of microhardness foe all the groups at baseline, day 1, day 2, and day 3

	Group I		*Group II*		*Group III*		*Group IV*	
Comparison among days	*Mean difference*	*p value*	*Mean difference*	*p value*	*Mean difference*	*p value*	*Mean difference*	*p value*
Baseline Day 1	−2.40	0.004**	−0.60	0.305	0.60	0.305	−1.40	0.025*
Baseline Day 2	−5.20	0.001***	−1.60	0.035	−0.40	0.178	−1.60	0.035*
Baseline Day 3	−6.40	0.001***	−2.20	0.063	−0.40	0.374	−2.00	0.022*
Day 1 Day 2	−2.80	0.002**	−1.00	0.142	−0.40	0.230	−0.20	0.621
Day 1 Day 3	−4.00	0.001***	−1.60	0.016*	−1.00	0.189	−0.60	0.070
Day 2 Day 3	−1.20	0.109	−0.60	0.501	0.00	1.000	−0.40	0.477

The grading of *p* value was done as follows: extremely significant when *p* value was <0.0001 (symbolized by ****); extremely significant when *p* value was between 0.0001–0.001 (***); very significant when *p* value was between 0.001–0.01 (**); significant when *p* value was between 0.01–0.05 (*) and when *p* value was ≥0.05 it was not considered to be statistically significant

In the present study, ES was also checked along with microhardness so as to provide direct quantitative assessment of remineralization as well. Atomic absorption spectrophotometer provides quantitative and precise measurement of the mineral lost or gained in parts per million. Enamel solubility of all groups was calculated by the difference in the amount of calcium loss (ppm) in the artificial cariogenic as well as whole saliva solution at day 1, day 2, and day 3 from the solution at baseline (D & R). Similar procedure was done by various author to check the remineralizing ability of the remineralizing agents.[51,52]

By evaluation of the amount of calcium loss (ppm) from the artificial cariogenic solution at day 1, day 2, and day 3, it was found that the amount of calcium ion (ppm) leach out for group I increases from day 1 to day 3, accounting for the total loss of 48.31 ppm calcium. For group II, group III, and group IV, the total calcium leach out is 20.308, 18.584, and 22.79 ppm, respectively, that decreases from day 1 to day 3 (Fig. 5). After group I (negative control), the maximum calcium leach out is seen in group IV (CPP-ACP) and the minimum is seen in relation to group III (fluoride varnish). The reason being that, since CPP-ACP was applied topically it may lead to the presence of increased calcium ion in the solution.

By evaluation of the amount of calcium loss (ppm) from the whole saliva solution at day 1, day 2, and day 3, it was found that the amount of calcium ion (ppm) leach out for group I increases from day 1 to day 3, accounting for the total loss of 6.759 ppm calcium. For group II, group III, and group IV the total calcium leach out is 1.6, 1.04, and 2.7 ppm, respectively, that decreases from day 1 to day 3 (Fig. 6). Even in this solution after group I (negative control), the maximum calcium leach out is seen in group IV (CPP-ACP) and the minimum is seen in relation to group III (fluoride varnish) suggesting fluoride varnish can provide better remineralization as compared to other agents.

The present study had few limitations as below:

First, it is an *in vitro* study, hence exact replication/simulation of oral conditions cannot be achieved. Second, the employed artificial saliva cannot completely mimic human saliva as saliva turnover and salivary flow is much greater *in vivo*. Third, CPP-ACP is applied as a topical coating whereas its mechanism of action as the remineralizing agent is by localizing ACP by CCP leading to increase in the level of calcium phosphate in plaque, and fourth, large-scale clinical trial needs to be done before applying the result in clinical practice.

Recommendations proposed for conduction of such a study in future are as follows:

Further model development is necessary to improvise the investigation that precisely simulates the oral conditions. Moreover, other remineralizing agents can also be evaluated and compared so as to identify the best remineralizing agent available.

Conclusion

The microhardness and ES of the treated surface enamel with resin infiltrant, fluoride varnish, and CPP-ACP were evaluated and compared in the following *in vitro* analysis.

Out of all the remineralizing agents used, microhardness of resin Infiltrant is higher as compared to other remineralizing agents but as the acid attacks increases microhardness of fluoride varnish approximates that of resin infiltrant with CPP-ACP having the least microhardness. Fluoride varnish has the least ES compared to the other remineralizing agents after acidogenic challenge, followed by resin infiltrant and CPP-ACP.

Following conclusive points can be drawn from the present study:

- The remineralizing agents such as resin infiltrant, fluoride varnish, and CPP-ACP all can remineralize initial carious lesion. This can be stated as after application of all above remineralizing agents on the initial enamel lesions, the microhardness values increased though the mean microhardness values of all the four groups did not match with the mean value of the sound intact teeth.
- With increase in number of acid exposures from one to nine, the microhardness for group I (negative control) decreased considerably, which was statistically extremely significant and the calcium leach out in the artificial acidogenic as well as the whole saliva solution was the maximum (55.069 ppm) among other groups.
- In group II (resin infiltrant) as the number of acid exposures increases from one to nine, microhardness decreased. The maximum calcium leach out occurred after three acid exposures (9.28 ppm), which stabilized after six acid exposures.

- The microhardness of group III (fluoride varnish) was least at the baseline but increased after three acid exposures, finally matching with microhardness value for group II (375.40 VHN) at the end of nine acid exposures. The maximum calcium leach out was seen after three acid exposures.
- For group IV (CPP-ACP), the initial fall after three acid exposures in mean microhardness value and calcium ion loss was high, which was later stabilized from four to nine acid exposures.
- Microhardness after nine acid exposures for the four groups has the following order: group II, group III (375.40 VHN) > group IV (372.60 VHN) > group I (364.80 VHN).
- Enamel solubility (calcium leach out) for the four groups after nine acid exposures has the following order: group I (55.069 ppm) > group IV (25.49 ppm) > group II (21.908 ppm) > group III (19.624 ppm).

Clinical Significance

Fluoride varnish can be regarded as the choice of material to be used for the treatment of incipient carious lesions because of the low application frequency (once every 3–6 months), requires minimal patient compliance as is a noninvasive procedure, and less time-consuming but avoidance of acidogenic challenge for 24 hours should be done after application to achieve best results.

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