Original Research Paper

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Remineralization potential of GC Tooth Mousse and GC Tooth Mousse plus on initial caries like lesion of primary Teeth – An in-vitro comparative evaluation ”

ABSTRACT:

Aim: Teeth are constantly going through cycles of demineralization and remineralization. The ultimate goal of clinical intervention is the preservation of tooth structure and the prevention of lesion progression to the point where restoration is required. Thus promoting remineralization is the ultimate goal of clinical prevention of caries lesion. The present in vitro study aimed to investigate the efficacy of GC Tooth Mousse (CPP-ACP) and GC Tooth Mousse Plus (CPP-ACP)F on artificial enamel caries in primary human teeth.

Methods and Material: Sixty freshly extracted human primary anterior teeth were used in this study.

The root portion of 60 primary anterior teeth was separated from the crown portion at the cemento-enamel junction (CEJ)

Teeth samples were divided into 3 Groups (n=20 each). Group 1 as a control group, Group 2 GC Tooth Mousse,and Group 3 Tooth Mousse Plus containing dentifrices were used. Samples were subjected to 10 days of pH cycling protocol.

The changes were analyzed using Vickers Hardness Testing Machine and SEM.

Pre and post groups were compared by paired t-test. Independent groups were compared by one-way analysis of variance.

Result: Micro-morphological observations of the enamel surfaces with SEM : Group 1 the enamel scanning showed shallow depressions and fine porosities within these depressions, Group 2 showed numerous granular particles and amorphous crystals which were arranged on the enamel surface. Smooth, homogeneous surface, and no irregularities were seen in Group 3. Surface Microhardness Evaluation After treatment, the mean hardness Group III was the highest followed by Group II and Group I (i.e. Group I <Group II < Group III).

Key-word: Enamel, Remineralization SEM Tooth Mousse, Tooth Mousse Plus, VHN

Introduction:

Tooth structure in the oral environment is exposed to frequent demineralization and remineralization and if the balance is lost for any reason, it leads tothe destruction of dental structure.[1] Primary enamel lesions can remineralize, especially using boosting remineralization treatment.[2] White-spot lesions are the earliest macroscopic evidence of enamel caries.[3] Typically, the enamel surface layer stays intact during subsurface demineralization, but, without treatment, will eventually collapse into a full cavity.[4] Near the neutral pH of saliva, it is endowed with a natural buffering capacity. Natural demineralization of the tooth at an early stage is reversed by saliva, which contains calcium ions, phosphate ions, buffering agents, fluoride, and other substances.[5] Several studies have indicated that milk and its derivatives, such as cheese, has anti-caries properties in human beings and animal models, its functional mechanism is due to the chemical effects of phosphor protein casein and the calcium component of cheese.[6] Casein phosphopeptide amorphous calcium phosphate, briefly called CPP-ACP, has anti-caries protective effects through inhibition of demineralization and a combination of an increase in

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remineralization and a decrease in demineralization.[7,8] Every functional potential of CPP-ACP is similar to the effects of the most common anti-caries substance, i.e. Fluoride.

Different technologies are used for remineralization of early enamel caries lesions. Considering these factors, and in vitro, a clinical trial was planned to compare the remineralizing potential of GC Tooth Mousse (CPP-ACP) and GC Tooth Mousse Plus (CPP-ACP)F on artificial carious like lesion on primary teeth by using Vickers hardness testing Machine and scanning electron microscopy (SEM).

Subjects and Methods:

The sample size was scientifically obtained by the statistician. 60 freshly extracted sound human primary anterior teethdue to physiological mobility or retained in permanent dentition were used in this study after clinical and radiographic examinations. The study was carried out in the Department of Pediatric & Preventive Dentistry, IDS, Bareilly after getting ethical clearance. Teeth with any visible/ discoloration/ detectable caries / with hypoplastic /white spot lesion, enamel cracks/fracture, developmental defects and with any restoration were not used in the study

Sample preparation :

The root teeth were separated from the crown portion at the cemento-enamel junction (CEJ) using a diamond-coated disc. The labial surface of all samples was progressively ground flat and hand-polished with the aqueous slurry of progressively finer grades of silicon carbide, up to 4000 grit. Acid-resistant nail varnish was applied around the enamel surface, leaving a window (4 x 4 mm) at the center. Then, the baseline enamel SMH was measured.

Ten selected samples from each group (total 60) were also assessed by scanning electron microscope (SEM) (Zeiss, India).

Artificial carious lesions preparation[9]

A demineralizing solution and artificial saliva were then prepared in the Department of Biochemistry, Rohilkhand Medical College, Bareilly.

Demineralizing solution was prepared using 2.2mM CaCl2.2H2O (calcium chloride), 2.2mM NaH2PO4.7H2O (monosodium phosphate) and 0.05M Lactic Acid. Each ingredient was added separately to deionized water under continuous stirring and was allowed to dissolve completely before the next ingredient was added. The solution was maintained at 37°C and the pH was adjusted to 4.5 using 50% NaOH solution.

Artificial saliva was prepared by mixing 2.200g/L Gastric Mucin, 0.381g/L NaCl (sodium chloride), 0.213g/L CaCl2.2H2O (calcium chloride), 0.738g/L K2HPO4.3H2O (potassium hydrogen phosphate) and 1.114g/L KCl (potassium chloride). Each ingredient was added separately to Deionized water under continuous stirring and was allowed to dissolve completely before the next ingredient was added. The solution was maintained at 37°C and the pH was adjusted to 7.00 using 85% lactic acid.

The specimens of the enamel blocks were immersed in the 40 ml of demineralized solution. The solution was stirred and the demineralization was performed at 37 0 C for 48 hr, in an incubator to induce artificial caries formation, simulating an active area of demineralization. After demineralization, SMH and SEM were recorded.

The samples were divided into following groups (20 each): Group I -Control (brushed with DI water); Group II -GC Tooth Mousse (CPP-ACP) and Group III -GC Tooth Mousse Plus (CPP-ACP-F)

pH Cycling Protocol10:

The specimens underwent the remineralization process twice a day (09:00 am, 4:00 pm) for 10 days.

- 09:00 am: All the teeth were removed from artificial saliva, brushed using a soft-bristled powered toothbrush with respective remineralizing agents for 2-minutes, and gently rinsed with deionized (DI) water.
- 09:30am -4:00 pm: All teeth soaked in artificial saliva at 37°C.
- 4:00 pm: All teeth were removed from artificial saliva, brushed using a soft-bristled powered toothbrush with respective remineralizingagents 2-minutes, and gently rinsed with deionized (DI) water.
- 04:30pm -09:00 am: All teeth were again soaked in artificial saliva at 37°C.

The evaluation of remineralized samples was based on surface microhardness and SEM appearance of the enamel surface.

Hardness testing: Vicker's hardness test to check the microhardness of the enamel surface was done. The testing

was done with an FIE microhardness tester India. Thirty samples out of sixty(n=10) were placed on the tester after leveling the dental stone block so that a plane is achieved. The diamond tip that was used to create a nano indent. Under a 100x microscope, the sample positioning was done so that the indent falls on the enamel portion of the section. A load of 100 g for 15 sec was applied, and the rhomboid indent is measured for length and depth. Five indentations were placed on the surface and the average value was considered. Precision microscopes of magnification of ×400 were used to measure the indentations. The diagonal length of the indentation was measured by a built-in scaled microscope and Vickers values were converted to microhardness values. [11]

SEM Observation: At the end of the 10-day pH cycle, the remaining thirty teeth(n=10) were mounted for scanning electron microscopy (SEM) analysis (80000X magnification). All the teeth from each treatment group were mounted on carbon mounts and coated with a gold/palladium alloy coating by a process called sputtering. SEM images obtained at 80000X magnification. The sound enamel had an orderly rod appearance, and enamel crystals were homogeneously arranged with a clear outline.

The demineralized enamel had a smaller number of enamel rods with variable rod widths. The surface was however not flat. Some enamel crystals were irregularly arranged, some were even fused together and some rod-like crystals were disorderly distributed on the surface of the enamel.

Results:

Under the limitations of the study, the following observations were made on evaluating scanning electron microscope:

In the sound enamel the crystals were homogeneously arranged with a clear outline and rods were orderly placed. (Fig 1)

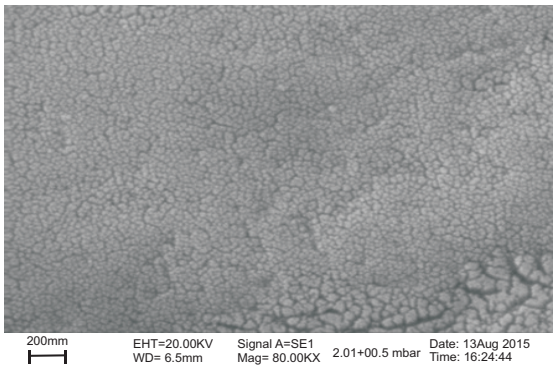


Fig. 1 SEM Image Of Baseline Enamel

- The demineralized enamel showed a rough surface with a honeycomb appearance, which is a peculiar characteristic of carious enamel. (Fig 2)

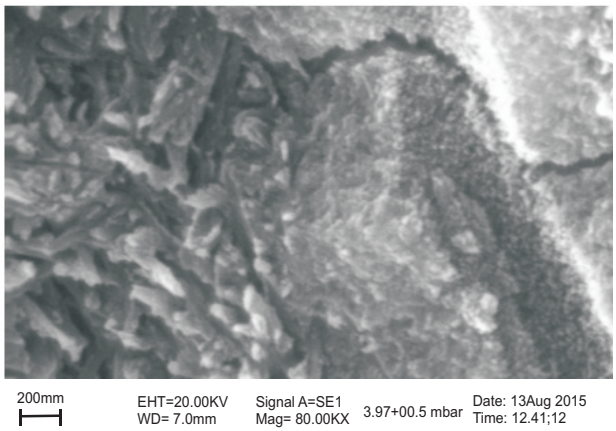


Fig 2 SEM Image Of Demineralized Enamel

- Shallow depressions and fine porosities within these depressions were observed in group I.(Fig 3)

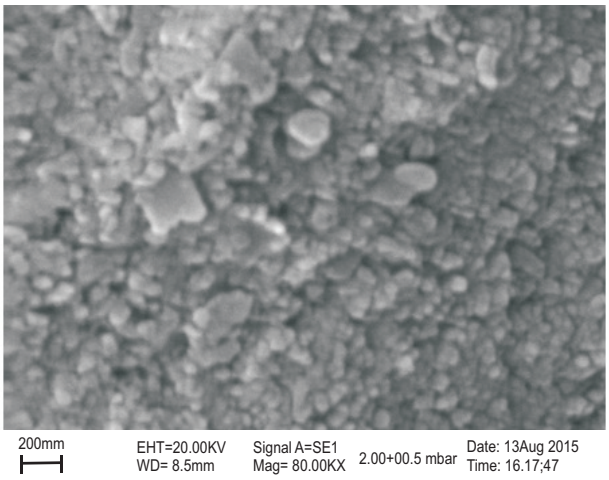


Fig 3 SEM Image Of Enamel Group 1(Control)

o In group II, the SEM image of the enamel surface treated with GC Tooth Mousse numerous granular particles and amorphous crystals were arranged on the enamel surface, those crystals seemed to be homogeneous, and there was no obvious intercrystalline space. (Fig 4)

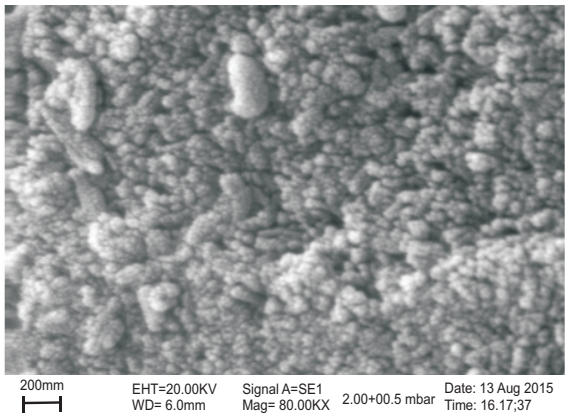


Fig 4 SEM Image Of Enamel Group 2(CPP-ACP)

o In group III, GC Tooth Mousse Plus samples a relatively smooth, more homogeneous surface was observed.(Fig 5)

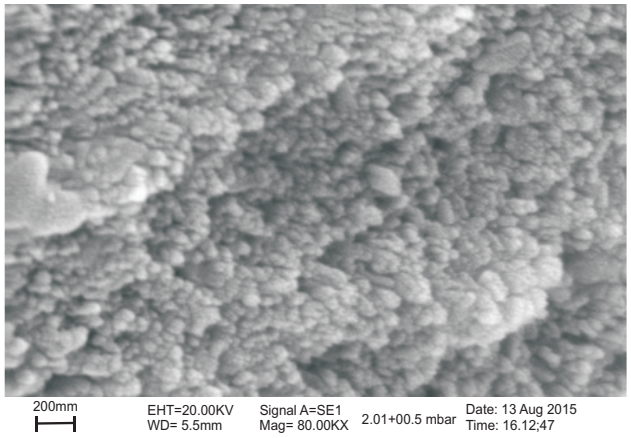


fig 5 SEM Image Of Enamel Group 3 (CPP-ACP)F
Surface microhardness evaluation

The surface hardness of baseline enamel (i.e. before demineralization) and after demineralization is summarized in Table 1 and also shown graphically in Graph1, the hardness of all teeth samples before demineralization ranged from 315-349 kg/mm2 with a mean (± SD) 331.43 ± 9.09 kg/mm2 while after demineralization it ranged from 234-261 kg/mm2 with a mean (± SD) 244.65 ± 6.93 kg/mm2.

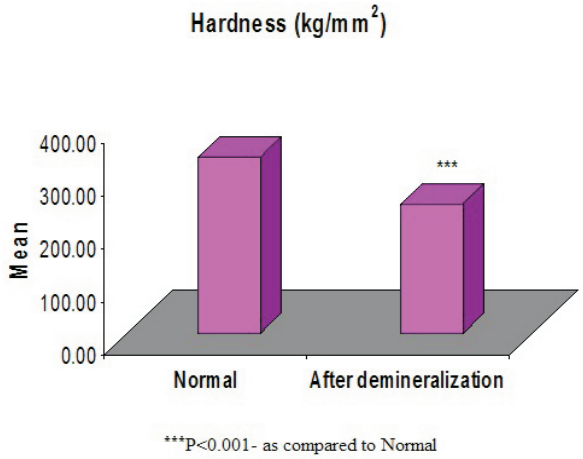
The initial hardness decreased comparatively after demineralization

Comparing the mean hardness before and after demineralization, the paired t-test showed a significant decrease (26.2%) in hardness after demineralization (331.43 ± 9.09 vs 244.65 ± 6.93, t=52.35, p<0.001).

Table 1: Vicker's Hardness (Mean ± SD) of extracted normal teeth before and after Demineralization

Normal (before demineralization) Initial	After demineralization	T value	P value
331.43 ± 9.09 (315-349)	244.65 ± 6.93 (234-261)	52.35	<0.001

Numbers in parenthesis indicate the range (min -max)



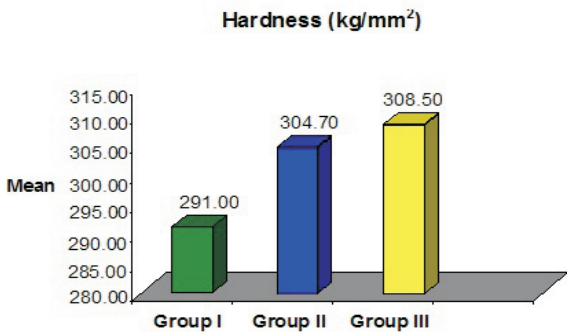
Graph 1 Mean hardness of normal teeth before and after Demineralization.

The remaining crown enamel samples were further randomized equally to treat with one of four treatment groups [Group I: Control (brushed with DI water), Group II: GC Tooth Mousse (CPP-ACP) and Group III: GC Tooth Mousse Plus (CPP-ACP- F) (fluoridated)]. The hardness of four groups after treatment is summarized in Table 2 and also depicted in Graph 2. After treatment, the hardness of Group I, Group II and Group III ranged from 283-299 kg/mm2, 300-310 kg/mm2 and 302-314 kg/mm2, respectively with mean (± SD) 291.00 ± 5.50 kg/mm2, 304.70 ± 3.59 kg/mm2 and 308.50 ± 3.84 kg/mm2, respectively. After treatment, the mean hardness Group III was the highest followed by, Group II and Group I (i.e. Group I < Group II < Group III).

Table 2: Hardness (Mean ± SD) of teeth of three groups after treatments

Group I	Group II	Group III
291.00 ± 5.50 (283-299)	304.70 ± 3.59 (300-310)	308.50 ± 3.84 (302-314)

Numbers in parenthesis indicate the range (min-max)



Graph 2 - Mean hardness of different groups after remineralization

Evaluating the effect of treatments (groups) on the hardness of teeth, ANOVA revealed a significant effect of treatments on the hardness of teeth (F=49.08, P<0.001) (Table 3).

Table 3: Evaluation of the effect of treatments (groups) on the hardness of teeth using ANOVA

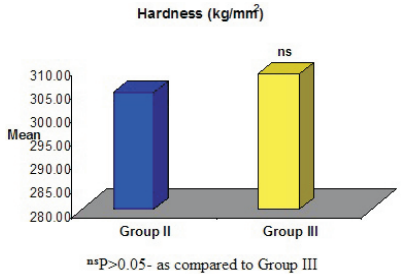
Source of variation (SV)	Sum of square (SS)	Degree of freedom (DF)	Mean square (MS)	F Value	P value
Groups	2569.00	3	856.50	49.08	<0.001
Residual	628.20	36	17.45		
Total	3198.00	39	873.95		

Intergroup comparison (Table 4 and Graph 3) also indicated that CPP-ACP-F exhibited better hardness hence superior remineralization.

Table 4: Comparison (p-value) of mean difference in hardness of teeth between the groups by Tukey post hoc test

Comparisons	Mean difference	Q Value	P Value	95% CI of difference
Group I vs. Group II	13.70	10.37	P <0.001	8.66 to 18.74
Group I vs. Group III	17.50	13.	P <0.001	12.46 to 22.54
Group II vs. Group III	3.80	2.88	P >0.05	1.24 to 8.84

CI=confidence interval***P<0.001- as compared to Group-I



nsP>0.05- as compared to Group III

Graph 3- Meanhardness of teeth of Group III as compared to Group II.

Discussion:

Tooth caries is known as the most prevalent chronic disease with its etiology being quite complex involving interaction between the agent, host, time, and environmental factors. Prevention of dental caries is very essential as it affects a person's self-esteem, quality of life, and also indirectly contributes to the decrease in the nation's productivity.

The term white spot lesion(WSL) was defined by Fejerskov et al as "the first sign of a carious lesion on enamel that can be detected with the naked eye".[12]

However, WSLs can persist, resulting in an esthetically and structurally unacceptable condition.

The focus in caries has recently shifted to the development of methodologies for the detection of the early stages of caries lesions and the non- invasive treatment of these lesions. The non- invasive treatment of early lesions by remineralization has the potential to be a major advance in the clinical management of the disease. Remineralization of white-spot lesions may be possible with a variety of currently available agents.

Fluoride is known to promote remineralization but is dependent on calcium and phosphate ions from saliva to accomplish this.

Recent investigations have primarily focused on various calcium phosphate-based technologies that are designed to supplement and enhance fluoride's ability to restore tooth mineral. This new calcium phosphate-based technology is the alternative to fluorides and introduced as a "Non fluoridated remineralizing agent." These are Complexes of casein phosphopeptides-amorphous calcium phosphate, Amorphous calcium phosphate, Sodium calciumphosphosilicate (bioactive glass), Nanohydroxyapatite,Calcium carbonate carrier-Sensistat, trimetaphosphate ion Alpha-tricalcium phosphate, Dicalcium phosphate dihydrate, Xylitol carrier.

The present study utilized an in vitro model to compare the remineralizing potential of two sugar-free, cream-based RML agents i.e. tooth mousse (CPP-ACP) and Tooth Mousse Plus (CPP-ACPF) on artificial enamel carious lesion by using Vickers hardness testing Machine and scanning electron microscopy (SEM).

Under the limitations of the present study, it was observed that significant remineralization was elicited amongst all groups.

Assessment of in vitro demineralization and remineralization can be done using different methods. Many studies have been conducted using one or a combination of different methods like the SEM/ESEM,[7,13,14] Diagnodent,14surface microhardness, [15,16] etc. The present study utilized both SEM and surface microhardness to assess remineralization.

Teeth for the study were obtained after proper consent. Subjects were selected after a thorough clinical and radiographical examination.

Teeth were noncarious and the subjects were not suffering from chronic and or systemic diseases. The primary anterior teeth were chosen in this study for the induction of artificial caries like lesion and surface hardness because anterior teeth are flat on the surface and straight in profiles that are mandatory for proper contact of the indenter tip.[17] The added advantage of using nanoindentation is that it checks the breakage of the enamel rods without causing any further damage to the enamel surface.

The organic content of the primary tooth enamel is higher than that of the permanent tooth making the primary tooth enamel softer and more porous and consequently more susceptible to caries compared to permanent enamel.Zhang Q etal (2011)[15],VeerittaYimcharoen (2011) [18], Mirkarimi M et al (2013)[19] N Agrawal et al (2014)[20] Aminabadi NA et al (2015) [21] performed similar study on primary teeth.

The mid coronal site was chosen for the windows preparation to avoid both the enhanced fluoride surfaces of cervical enamel and the reduced fluoride surfaces coronally. A similar methodology was employed by Shetty S et al (2014).[11]

In the present study, the samples of all the groups were exposed to pH Cycling for 10 days to measure the resistance of samples to demineralization, and then, the hardness measurement test was performed. After 10 days of pH cycling protocol, samples were subjected for enamel topography and hardness. Results from several studies have shown that artificial saliva can reharden demineralized enamel,[17] so each sample in the present study was immersed in artificial saliva for 6 hours as recommended by Muratha and colleagues to simulate oral environment.[22]

Vicker's hardness method was used to check microhardness because it was non-destructive, very reliable, rapid, and economical as compared to otherhardness tests. The square-shaped indent obtained was more easy and accurate to measure and detect visually and digitally.

In our study, the mean values for enamel microhardness at baseline were in the range from 315 VHN to 349 VHN which is within the standard range of 250 VHN to 360 VHN and there was a decrease in hardness after demineralization ($p<0.001$) and after remineralization by Group I (control), Group II (Tooth Mousse) and Group III (Tooth MoussePlus), the microhardness of teeth increased significantly.

The surface topographic changes,analyzed by SEM, showed that the enamel surface treated by Tooth Mousse Plus has a much smoother and uniform surface compared to that of Tooth mousse.

The values of surface microhardness indicate that the remineralization of enamel is more in samples of group III. This may be because of the presence of Fluoride in CPP-ACP makes it more capable to remineralize the enamel.
The active ingredient of tooth mousse and tooth mousse plus is casein phosphopeptide – amorphous calcium phosphate.

CPP are peptides that are derived from the milk protein casein that is complexed with calcium and phosphate.Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP) was introduced as a remineralizing agent in the year 1998. Caseins are a heterogeneous family of proteins predominated by alpha 1 and 2 and beta- caseins. CPPs are phosphorylated casein-derived peptides produced by the tryptic digestion of casein. CPP contains the –Ser (P)–Ser (P)–Ser (P) -Glu-Glu active cluster sequence which has a remarkable ability to stabilize calcium and phosphate in a metastable solution. In neutral and alkaline supersaturated calcium phosphate solutions, amorphous calcium phosphate (ACP) nuclei form spontaneously. The above phosphopeptide through the -Ser- sequence is able to bind to the forming ACP nanoclusters in metastable solutions.

TheseCPP-ACP nanocomplexes which are of around 1.5nm radius prevent the growth of the nanoclusters to the critical size required for nucleation and phase transformation. Hence, the calcium and phosphate are maintained in high concentration forms that are readily available at the tooth surface without allowing their precipitation into calculus.[23,24]

CPP will bind to surfaces such as plaque, bacteria, soft tissue, and dentin (owing to its sticky nature), providing a reservoir of bioavailable calcium and phosphate in the saliva and on the surface of the tooth. It has been proposed that the mechanism of anticariogenicity for CPP-ACP is that it substantially increases the level of calcium phosphate in plaque, which decreases enamel demineralization and enhances remineralization. Rose25 investigated the proposed mechanism of action of CPP-ACP and demonstrated that CPP-ACP and calcium competed for the same binding sites on Streptococcus mutans.

Many in vitro studies have proved that CPP-ACP has a remarkable ability to remineralize caries. While in one study authors have concluded that although CPP-ACP can remineralize surface lesion, it is not effective in remineralizing the early enamel caries at the subsurface level.[2]

Fluoride present in the oral fluids alters the continuously occurring dissolution and reprecipitation processes at the tooth–oral fluid interface. Remineralization of incipient caries lesions is accelerated by trace amounts of fluoride. High concentration fluoride therapies lead to the deposition of aggregates of calcium fluoride on the surface, which then acts as a reservoir of fluoride. The rate of fluoride release is enhanced at lower pH levels. A pH of less than 5 causes loss of adsorbed phosphate and triggers a slow dissolution of the calcium fluoride.[26-27]

Fluoride, when added to CPP-ACP, gives a synergistic effect on the remineralization of early carious lesions. Elsayadet al28 reported that the addition of fluoride to CPP-ACP could give a synergistic effect on enamel remineralization. Karlinseyet al29 found CPP-ACP +fluoride to be effective in remineralizing bovine enamel specimens. In the present research also CPP-ACP+ fluoride is found to be more effective than CPP-ACP alone with no significant difference.

CPP-ACPF is a supersaturated solution of amorphous and crystalline calcium phosphate phases. It has added fluoride content. It is a stabilized composition so that spontaneous precipitation of calcium phosphate is stopped. The remineralizing capacity is directly proportional to the levels of free calcium and phosphate ions that are stabilized by CPP. When CPP-ACPF is applied on the tooth surface, its sticky CPP part readily mixes with enamel and biofilm releasing the calcium and phosphate ions. The free calcium and phosphate ions enter the enamel rods and form the apatite crystals again. [30]

The Result of our in vitro study were in accordance to the ones conducted by Srinivasan N (2010)[31], Jayarajan et al(2011)[14], Patil N et al (2013)[9], Shetty S et al (2014)[11], Mettu S et al (2015)[17].

In contrast to these studies, Mehta et al 32 concluded that there was no significant difference when the remineralizing effect of CPP-ACP was compared with the remineralizing effect of CPP-ACFP.

The use of fluoride and the CPP-ACP in recent years have been the best possible method in the prevention of enamel caries and also in halting the progress of the existent enamel lesions.

However, It's important to note that compliance of patients in oral hygiene maintenance and in-home fluoride use is of utmost importance in the prevention of enamel caries.

Limitation of study:

The period of remineralization used in the study was 10 days, which could not remineralize artificial caries completely.Although surface remineralization was confirmed, enamel subsurface remineralization was not evaluated in the study. Thus, direct extrapolations to clinical conditions must be exercised with caution because of the obvious limitations of in vitro studies.

Conclusion:

It could be concluded that remineralizing agents Tooth Mousse and Tooth Mousse Plus are excellent delivery vehicles available in a slow-release amorphous form to localize calcium, phosphate, and fluoride at the tooth surface. As this study was conducted under in-vitro conditions, furthermore elaborated studies regarding different aspects of tested materials need to be undertaken before recommending the materials for clinical use under specified conditions.

Future scope : Further studies on enamel crystal formation and chemical structure using advanced quantification techniques and the resistance of acid solubility of these remineralized crystallites have to be investigated to achieve more conclusive results.

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