Review Article

AN IN VITRO STUDY ON THE EFFICACY OF FOUR REMINERALIZING AGENTS

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ABSTRACT

To equate and assess the four commercially potential remineralization available products namely GC Tooth Mousse, SHY-NM, Biomed calcimax, and Bentodent on demineralized human teeth. 72 upper premolars bonded with 3M premolar bracket were included in this study. Teeth were randomly selected and divided into six groups of 12 teeth each. Group 1: Stored in de-ionized water alone (Positive control); Group 2: Stored in Tencates demineralizing solution alone (Negative control); Group 3: Stored in Tencates demineralizing solution & coated with GC Tooth Mousse every 4 hours; Group 4: coated with SHY-NM; Group 5: coated with Bio med calcimax; Group 6: coated with Bentodent. After 96 hours teeth were sectioned using a diamond disc and then thinned to 150-200 um thick with carborundum stone. Every segment acquired was envisaged underneath a polarized light microscope and analyzed using Image J software. Bonferroni Post-hoc test for multiple pairwise comparisons for minimum demineralization depth reveals a significant difference in the depth of demineralization except, for Group 3 and group 4; Group 5 and group 4. Post-hoc test for maximum demineralization depth reveals a significant difference between, Group 2 and group 6; Group 3 and group 4; Group 4 and group 5; Group 5 and group 6. GC Tooth Mousse & SHY-NM are effective in remineralizing artificially induced caries lesions compared to Biomed calcimax and Bentodent.

Key words: Enamel demineralization, White spot lesion, Medicaments, Polarized light microscop.

Introduction

Enamel demineralization is an inevitable side effect associated with fixed orthodontic care and associated with poor oral hygiene in the form of white spot lesions. Acidogenic bacteria such as streptococcus mutans and lactobacilli in plaque are the causative factors that contribute to WSL. Fejerskov and Kidd *et al*. as the "initial clinical sign of caries lesion which occurs in the enamel that can be visualized with the naked eye" identified the term white spot lesion [1]. Such caries lesion usually occurs around the bracket near the gingival edge, according to Gorelick *et al*. 1982. The prevalence of white spot lesions among orthodontic patients ranges from 2% to 96%. The labio-gingival area is considered the most common site for lateral incisors WSL and the maxillary posterior segments account for the least common site. Furthermore, males are affected considerably compared to females [2].

The first stage of enamel demineralization involves the softening of the surface, marked by the preferential loss of interprismatic substances that cause mineral loss on the surface of the enamel. The second stage (active lesion) includes dissolution with marked subsurface lesions confined only to deeper enamel areas, but porous mineral-rich layers cover the lesion body. The active lesion shows a stronger prognosis than arrested because it allows calcium and phosphate ions to quickly penetrate the enamel facilitating remineralization.

Remineralization is known as the natural non-cavitated lesion repair process and relies on the availability of calcium, and fluoride-supported phosphate ions to reconstruct a new surface on existing crystal residues in sub-surface lesions lasting after demineralization [3]. These crystals of remineralized hydroxy apatite (HAP) are less soluble in acid than the initial minerals. Glycoproteins adsorb onto the tooth structure in natural remineralization to form the protective pellicle layer & phosphoproteins control saliva calcium saturation. Glycoproteins, proline-rich protein, statherin, histatin, cystatin facilitate enamel remineralization by attracting calcium ions [4].

In 2011, Shah *et al*. stated that orthodontic brackets modified with photocatalytic titanium oxide (TiO2) coating of stainless steel decreased bacterial adherence and accumulation of biofilm [5]. In 2012, Hadler-Olsen *et al*. reported that 60 percent of orthodontic patients developed one or few WSLs at the end of orthodontic treatment, despite the use of prophylactic steps [6]. Hence the main objective of modern orthodontics is to manage white spot lesions non-invasively and skilfully restrict disease progression and enhance aesthetics, function, and strength.

The orthodontist must be conscious of the possible decalcification and take preventive steps to avoid or restrict the process of demineralization by creating an atmosphere conducive to remineralization by different remineralizing agents. The classical remineralizing agents provide the carious lesion with appropriate quantities of phosphate &

calcium ions and will not precipitate or increase calculus formation on the tooth surface. Hence, the current research is undertaken to equate and evaluate the efficacy of four remineralizing agents namely CPP-ACP, NovaMin, calcium hydroxyapatite, and calcium bentonite

Materials and Methods

The study was reviewed and approved by the Institutional Review Board (approval number 200/IHEC/1-19). The sample size was formulated from G*Power software (version 3.1.9.7). Effect size f = 0.45 with α err prob = 0.05 and Power (1-β err prob) = 0.8. The estimated sample size = 72(12 each group), actual power = 0.8202265. Hence, the study sample includes 72 extracted maxillary first premolars. These samples were garthered from the patients who have endured therapeutic extraction for orthodontic correction of teeth satiating the subsequent exclusion criteria: 1. Teeth with caries, restorations, stains, hypoplastic or white spot lesions 2. Crown with enamel fracture or structural defects. The Inclusion criteria are 1. Patient with age 13-16 years. 2. Teeth with intact buccal enamel & without decalcification 3. Teeth that were not previously bonded 4. Vital & Fully erupted teeth at the time of extraction 5. Anatomically and morphologically described maxillary premolar teeth 6, and extraction forceps caused no cracks.

The teeth were randomly assigned to six groups of 12 each. Group 1 (n=12): Stored in de-ionized water alone (Positive control) Group 2 (n=12): Stored in Tencates demineralizing solution alone (Negative control) Group 3 (n=12): Stored in Tencates demineralizing solution & coated with GC Tooth Mousse every 4 hours (CPP-ACP) Group 4 (n=12): Stored in Tencates demineralizing solution & coated with SHY-NM every 4 hours (calcium sodium phospho silicate or Novamin) Group 5 (n=12): Stored in Tencates demineralising solution & coated with Bio med calcimax every 4 hours (calcium hydroxy apatite and L Arginine) Group 6 (n=12): Stored in Tencates demineralising solution & coated with Bentodent every 4 hours (calcium bentonite).

The protocol for the Collection of extracted teeth, effective sterilization, perfect storage, and handling were followed according to the recommendations and guidelines given by OSHA and CDC. The stored teeth were thoroughly washed with de-ionized water and bonded with 3M Bracket using scotch bond etchant, Transbond XT adhesive, and primer (3M Unitek) according to the manufacturers' instructions. The apical third portion of the tooth was segmented off with a diamond disc. Then the teeth were attached to the inner surface of the plastic lid of a 20 ml polypropylene jar using yellow adhesive wax in such a way that the crowns could be immersed in the corresponding solution.

Teeth in groups 2, and 3,4,5,6 were subjected to an artificial caries challenge. Group 1 & 2 received no treatment and served as positive and negative Control; Group 3, Group 4, Group 5, and Group 6 received 0.5 ml applications of GC Tooth Mousse, SHY-NM, Biomed Calcimax, Bentodent respectively using paintbrushes. To ensure constant movement of the solution, all the groups were kept in a vibrator with a polypropylene jar for 30 secs for every four hours. After every 4 hours, teeth were taken out from the solution, cleaned with deionized water, and given brushing strokes using a toothbrush for 5 seconds to simulate normal tooth brushing. Then GC Tooth Mousse, SHY-NM, Biomed Calcimax, and Bentodent were applied over Group 3, Group 4, Group 5, and Group 6 for 40 seconds for every 4 hours for the total study period of 96 hours. After each application of remineralising agent, the teeth were washed with de-ionized water, dried and then placed in the demineralising solution.

After 96 hours the teeth were removed from the solution and thoroughly washed with de-ionized water. Longitudinal slicing of the tooth surface was done in a linguobuccal direction much closer to the brackets, with a diamond disc under de-ionized water cooling. Then the sections were thinned to 150–200 µm thick using carborundum stone. The enamel sections of the samples were viewed under OLYMPUS CX41 microscope with crossed polarizer and analyzer plates to measure the demineralization depth. Photography was done using a Canon 200 D digital camera at 2x zoom under a 5×10 x magnification. The demineralization depth was calculated using the software image J (Java based image processing program). For each sample, the linear distance from the tooth surface adjacent to the bracket to its maximum and minimal demineralization depth was reported in micrometers **(Figure 1)**.

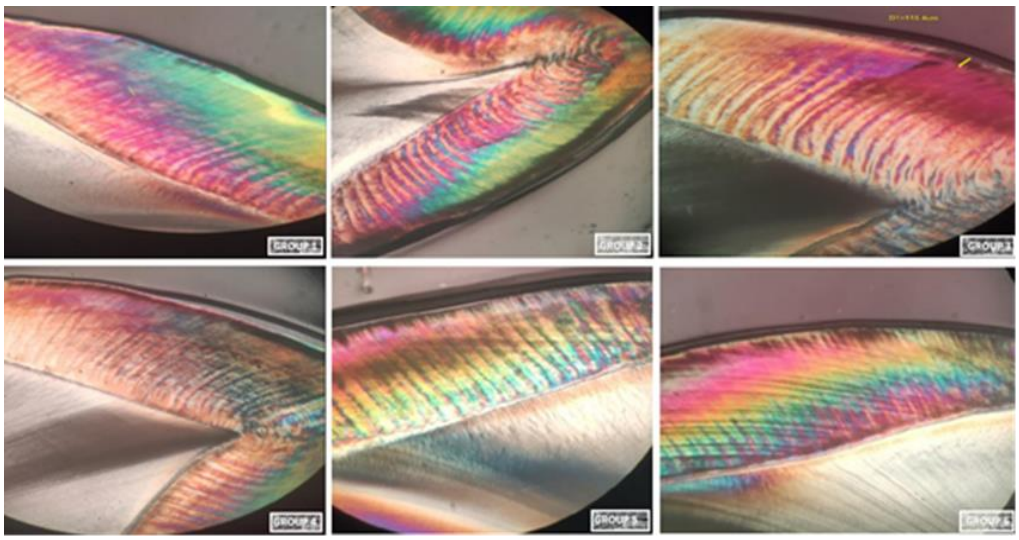

Figure 1. Demineralization Depth Measurement Using Image J Software

Table 1. Comparison of demineralization (minimum) between the groups

Variable	Group	Mean	Std. Deviation	95 % Confidence Interval		P value
Max demineralization	1	32.73	8.12	27.56	37.89	0.041
	2	256.91	17.76	245.62	268.19	
	3	213.46	9.25	207.58	219.33	
	4	223.24	10.10	216.83	229.66	
	5	233.29	10.61	226.55	240.03	
	6	244.51	10.16	238.06	250.96	

Statistical Analysis

Descriptive and Inferential statistics were analyzed by IBM SPSS version 20.0 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp). Quantitative data were represented as Mean and SD. Parametric methods (One-way ANOVA with Bonferroni post-hoc test - for comparison of parameters between 6 groups were used for intergroup comparison. Throughout the study, a P-value of <0.05 was considered a statistically significant difference.

Results and Discussion

A comparison of demineralization (minimum and maximum) between the groups was done using one-way ANOVA. It was found that there was a substantial dissimilarity in depth of demineralization (minimum) between the groups **(Table 1)**. The post-hoc test is done after a significant P-value is obtained in the corresponding ANOVA test. The test shows that there was a substantial dissimilarity in the depth of demineralization (minimum) in every group when compared with each other groups, except, Group 3 and group 4, Group 5, and Group 4 **(Table 2)**. There was a substantial change in depth of demineralization (maximum) between the groups **(Table 3)** Post-hoc test shows that there was a significant difference in the depth of demineralization (maximum) in every group when compared with each other groups, except for Group 2 and group 6, Group 3 and group 4, Group 4 and group 5, Group 5 and group 6.

Group 3(CPP-ACP) showed significant remineralisation (p value =0.010) when associated with Group 5(Biomedcalcimax) and Group 6 (Bentodent) and insignificant remineralisation (p value = 0.561) when compared to Group 4(SHY NM). Group 4(SHY NM) showed significant remineralisation (p value = 0.010) when compared to Group 6 (Bentodent) and insignificant remineralisation when correlated with Group 3(CPP-ACP) (p value = 0.561) and Group 5(Biomedcalcimax) (p value = 1.000). Group 5(Biomedcalcimax) showed significant remineralisation (p value = 0.025) when compared to Group 6 (Bentodent) **(Table 4)**.

Table 2. Bonferroni Post-hoc test for multiple pairwise comparison (minimum)

(I) Group	(J) Group	Mean Difference(I-J)	Std. Error	P value.
1.00	2	-141.56667*	4.77399	.010

	3	-88.47500*	4.77399	.010
	4	-98.61667*	4.77399	.010
	5	-105.80000*	4.77399	.010
	6	-121.45000*	4.77399	.010
2.00	1	141.56667*	4.77399	.010
	3	53.09167*	4.77399	.010
	4	42.95000*	4.77399	.010
	5	35.76667*	4.77399	.010
	6	20.11667*	4.77399	.010
3.00	1	88.47500*	4.77399	.010
	2	-53.09167*	4.77399	.010
	4	-10.14167	4.77399	.561
	5	-17.32500*	4.77399	.008
	6	-32.97500*	4.77399	.010
4.00	1	98.61667*	4.77399	.010
	2	-42.95000*	4.77399	.010
	3	10.14167	4.77399	.561
	5	-7.18333	4.77399	1.000
	6	-22.83333*	4.77399	.010
5.00	1	105.80000*	4.77399	.010
	2	-35.76667*	4.77399	.010
	3	17.32500*	4.77399	.008
	4	7.18333	4.77399	1.000
	6	-15.65000*	4.77399	.025
6.00	1	121.45000*	4.77399	.010
	2	-20.11667*	4.77399	.010
	3	32.97500*	4.77399	.010
	4	22.83333*	4.77399	.010
	5	15.65000*	4.77399	.025

Table 3. Comparison of demineralization (maximum) between the groups

Variable	Group	Mean	Std. Deviation	95 % Confidence Interval		P value
Max demineralization	1	32.73	8.12	27.56	37.89	0.041
	2	256.91	17.76	245.62	268.19	
	3	213.46	9.25	207.58	219.33	
	4	223.24	10.10	216.83	229.66	
	5	233.29	10.61	226.55	240.03	
	6	244.51	10.16	238.06	250.96	

Table 4. Bonferroni Post-hoc test for multiple pairwise comparison (maximum)

(I) Group	(J) Group	Mean Difference(I-J)	Std. Error	Sig.
1.00	2.00	-224.18333*	4.66868	.010
	3.00	-180.73333*	4.66868	.010
	4.00	-190.51667*	4.66868	.010

	5.00	-200.56667*	4.66868	.010
	6.00	-211.78333*	4.66868	.010
2.00	1.00	224.18333*	4.66868	.010
	3.00	43.45000*	4.66868	.010
	4.00	33.66667*	4.66868	.010
	5.00	23.61667*	4.66868	.010
	6.00	12.40000	4.66868	.149
3.00	1.00	180.73333*	4.66868	.010
	2.00	-43.45000*	4.66868	.010
	4.00	-9.78333	4.66868	.599
	5.00	-19.83333*	4.66868	.001
	6.00	-31.05000*	4.66868	.010
4.00	1.00	190.51667*	4.66868	.010
	2.00	-33.66667*	4.66868	.010
	3.00	9.78333	4.66868	.599
	5.00	-10.05000	4.66868	.525
	6.00	-21.26667*	4.66868	.010
5.00	1.00	200.56667*	4.66868	.010
	2.00	-23.61667*	4.66868	.010
	3.00	19.83333*	4.66868	.001
	4.00	10.05000	4.66868	.525
	6.00	-11.21667	4.66868	.287
6.00	1.00	211.78333*	4.66868	.000
	2.00	-12.40000	4.66868	.149
	3.00	31.05000*	4.66868	.010
	4.00	21.26667*	4.66868	.010
	5.00	11.21667	4.66868	.287

Fixed orthodontic appliances increase the prevalence of white spot lesions (WSLs) regardless of prophylactic measures & have a negative impact on the aesthetic appearance of the tooth [7]. The novel approach in caries management is the non-invasive method (i.e.) enamel remineralization which is one of the most exciting topics for researchers for the past 100 years. This method can change the lesion from an active to an inactive state. Toothpaste, varnishes, gels, and fluoride-releasing materials are commonly used professional delivery methods, to remineralize high-risk areas. The primary mechanism of remineralization occurs by diffusion of ions especially calcium & phosphate from saliva and topical agents to reconstruct an acid-resistant, hypermineralised layer on the existing crystal remnants which then act as remineralization nuclei [8]. There are many techniques to measure mineral changes in human enamel. These techniques include microhardness measurement of enamel cross-sections, different microradiography techniques, polarized light microscopy, iodine absorptiometry, and light scattering. In this study, Polarized light microscopy (PLM) analysis was selected as it is extremely sensitive to changes in hard tissues [9]. Various remineralizing agent has been presented in current years as additives for traditional fluoride-based systems [10]. Hence, this in vitro survey was assumed to assess and equate the remineralizing potential of four commercially available products namely GC Tooth Mousse, SHY-NM, Biomed calcimax, and Bentodent on demineralized human teeth. GC Tooth Mousse contains CPP-ACP, SHYNM contains bioactive glass, Biomed calcimax contains calcium hydroxyapatite and L-Arginine, and Bentodent toothpaste contains calcium bentonite. In this study, 72 Upper human premolar teeth indicated for therapeutic extraction were collected and stored in 10% formalin because it acts as the best disinfecting agent & also it resists demineralization by fixing proteins in the organic

pellicle attached to the surface of teeth during storage [11]. In experimental caries studies , Spontaneously formed WSLs to be used. Nevertheless, it is tough to produce a carious lesion in vivo in standard sizes. Consequently, a demineralization solution was selected in this study to form incipient caries lesions of standard size. The teeth were kept in this solution for 96 hours as described by Reynolds and Black [12].
To simulate the dynamic process of demineralization & remineralisation, Ph cycling model was adopted in many studies. However, Ph Cycling models have limitations like the inability to simulate the intraoral conditions progressing to caries development; to mimic plaque fluid & salivary composition experienced in vivo and to simulate topical use & clearance of product from the oral cavity[13].

Saumya Kakkar *et al*. used Tencates demineralization solution alone in their study [14]. Although the same limitations apply to the usage of chemical demineralization alone, it was the preferred method of choice due to its simplicity and practicality. Group 1 was immersed in de-ionized water due to previous studies published that de-ionized water does not alter the microhardness of the teeth significantly till 2 months [15], whereas the remaining five groups were immersed in Tencates demineralizing solution for 96 hrs at room temperature [16]. The lesion depth was evaluated using a Polarized light microscope due to the histological characteristics of enamel and dentin can be visualized better than transmitted light microscope due to its birefringence property,

In the present study, group 3(10% CPP-ACP -Recaldent- a water-based, lactose-free cream) showed increased remineralization when compared to group 4, group 5 & group 6. Recaldent technology was developed by Eric Reynolds and co-workers at the University of Melbourne, Australia. Previous studies have shown a short term remineralising effect and long-term caries-preventing effect in the in vivo clinical and randomized control trial [17].

Two studies concluded that CPP-ACP showed better remineralising potential than calcium sodium phosphosilicate using scanning electron microscope (SEM) and energy dispersive x-ray analysis (EDAX) [18, 19]. Four weeks of application of CPP-ACP, after debonding resulted in a significant reduction of white spot lesions. Hence CPP-ACP is effective before, during or after acid intervention [20, 21]. Moreover casein phospho peptide-amorphous calcium phosphate decreased mutans streptococci and showed a reduction in the bacterial count [22].

Following CPP-ACP, the effective material in the study was SHY-NM which contains bioactive glass (Novamin). A study using the Vickers micro hardness test concluded that initially Novamin showed superior remineralizing properties than CPP-ACP but eventually both have similar remineralizing potential [23]. On bleached enamel BAG act as a reservoir of ions in demineralized areas. A similar study concluded that novamin resulted in the formation of a protective layer on the enamel surface after 10 days of the remineralization phase [24]. A study using a scanning electron microscope revealed that BAG plugs are larger, angular, and intimately attached to the enamel surface compared to the plug formed by CPP-ACP [25].

The next remineralizing agent following CPP-ACP, SHY-NM was biomed calcimax toothpaste. Without additional additives, synthetic HAP particles are attached to pellicle-covered enamel surfaces and adhesion is directly proportional to the organic enamel content and inversely proportional to its size (1.3um). By filling small pores in demineralized tooth surfaces, the HAP (Hydroxy apatite) crystal stimulates remineralization [26].

The dipole properties of HAP and the resulting electrostatic forces are responsible for the cohesion of tooth surface crystallite clusters that provide the effect of antibiofilm and minimize solubility. HAP replaces fluoride and antimicrobials in a dentifrice & it is more effective for xerostomic patients [27]. HAP crystals improve enamel remineralization and resistance against acids even after three days and three months. A study using Vickers microhardness tester & SEM concluded that remineralizing efficacy of n-HAP toothpaste was higher than that of BAGs toothpaste due to the smaller particle size of nanohydroxyapatite (50-1000 nm) compared to the hydroxyapatite (1.3 um) [28].

Calcium bentonite is considered to have least remineralizing potential. A phyllosilicate group of minerals named after Montmorillon in France is bentonite or montmorillonite. Montmorillonite (MMT) is layered clay formed by silicon oxide (tetrahedral structure) and aluminum hydroxide (2:1) (octahedral structure).

These particles are plate-shaped with an average diameter of around 1 um. The replacement of lower valence cations, which leaves the adjacent oxygen atoms with a net negative charge that can attract cations, is known as cation exchange capacity (CEC). There is continuous attraction of calcium & phosphate ions via Cation Exchange Capacity (CEC), resulting in supersaturation & remineralization [29].

Recently Calcium montmorillonite is used as fillers in dental composites to improve remineralizing potential [30]. There are no studies to provide data about the remineralizing potential of calcium bentonite as toothpaste.

Limitations of this study includes inability to simulate the oral environment and the Protective potential .Only quantitative changes are observed and the duration of application of remineralizing agents is short than the recommended period.

Well designed & high-quality clinical studies in this area are still required before definitive recommendations. Hence

many in vitro and invivo studies should be done with huge sample size to justify the results.

Conclusion

GC Tooth Mousse & SHY-NM are effective in remineralizing artificially induced caries lesions compared to Biomed calcimax and Bentodent .

The use of CPP–ACP caused in a substantial decrease in lesion depth associated with the positive and negative control, Biomed calcimax & Bentodent; conversely, it was statistically insignificant when compared to SHY NM. No significant difference was found between Group 3 and group 4; Group 4 and group 5; Group 5 and group 6. Henceforth CPP-ACP in GC Tooth Mousse is superior to SHY-NM, Biomed calcimax & Bentodent tooth paste.

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Ethics statement: The study was reviewed and approved by the Institutional Review Board (approval number 200/IHEC/1-19).

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