Comparative evaluation of the remineralization potential of Theobromine and Fluoride containing dentifrices using Scanning Electron Microscopy with Energy Dispersive X-Ray Analysis: An in-vitro Study

Nithya Annie Thomas[1], Priya Shetty[2], Charisma Thimmaiah[3]*, Sowmya B. Shetty[4], Nimmy Sabu[5], Kavita Bekal Kripalani[6]

1. Reader, Department of Pediatric and Preventive dentistry. Indira Gandhi institute of dental sciences, Nellikuzhi,Kothamanagalam, Kerala
2. Department of Pediatric and Preventive dentistry, A.J. Institute of dental sciences, Mangalore, India
3. Assistant Professor, Department of Pediatric and Preventive dentistry, Manipal College of Dental Sciences, Mangalore, Manipal Academy of Higher Education, Manipal, Karnataka, India.
4. Department of Pediatric and Preventive dentistry, A.J. Institute of dental sciences, Mangalore, India
5. Department of Pediatric and Preventive dentistry. Indira Gandhi institute of dental sciences, Nellikuzhi,Kothamanagalam, Kerala
6. Pediatric Dentist, Hiranandani hospital powai.

Abstract

The goal of modern dentistry is to prevent caries progression by non-invasive management of non-cavitated carious lesions through remineralization. Theobromine, an alkaloid extract from chocolate has shown a significant remineralizing potential in early carious lesions.

To comparatively evaluate the remineralizing efficacy of Theobromine containing dentifrice (Theodent™)Fluoride containing dentifrice (Clinpro™).

In an in-vitro randomized controlled study, 30 sound enamel specimens (4x4x1mm) with a 2x2mm window, prepared from freshly extracted premolars were demineralized using acetate buffer solution (pH 4.2). Specimens were then randomly divided into three groups: Group I- Artificial saliva (control), Group II- Theodent™, Group III- Clinpro™ having 10 specimens each and subjected to remineralisation for a period of 20 and 40 days. Structural and Elemental analysis (Ca/P) was done using Scanning electron microscopy-Energy-dispersive X-ray analysis (SEM-EDAX) at baseline, after demineralization and remineralization.

Compared to artificial saliva, Theodent™ and Clinpro™ showed significant remineralization as after 20 and 40 days. However, difference between the test groups was not significant. Remineralization in each group was dose-dependent, with more mineralization evident at 40 days. However, none of the groups achieved baseline Ca/P ratios. While, the SEM analysis at 40 days showed homogenous and distinct amorphous deposits on the enamel surface in the test groups, no deposits were seen on the enamel surfaces in the control group.

Remineralization potential of Theodent™ is similar to that of Clinpro™. Hence, Theobromine can be considered as a safer future alternative to Fluoride.

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Introduction

The current concept of the aetiology of dental caries states that, it is a dynamic and reversible process with alternating periods of demineralisation and remineralisation. Early stages of demineralization are widely known as “early carious lesion” or “white spot lesion”, is characterized by a whitish colour, rough and opaque appearance on enamel. These early carious lesions, up to the point of progression to cavitation, can be reversed and arrested by altering the oral environment[1-2].

While, decreased pH in the tooth biofilm results in efflux of calcium (Ca^{2+}), Phosphate (PO_4^{3-}) ions from tooth into saliva causing demineralization, increased pH causes influx of the salivary buffers supersaturating the plaque fluid with Ca^{2+}, PO_4^{3-} and Fluoride (F^-) ions, forming new hydroxyapatite crystals and

Corresponding author:
Assistant Professor Dr. Charisma Thimmaiah
Department of Pediatric and Preventive dentistry, Manipal College of Dental Sciences, Mangalore, Manipal Academy of Higher Education, Manipal, Karnataka, India.
E-mail: charisma.t@manipal.edu

remineralization[3]. With a shift in paradigm, modern dentistry now focuses on non-invasive management of non cavitated caries lesions using various remineralization systems to repair the enamel in an attempt to prevent caries progression and improve aesthetics, strength, and function[4].

Since the discovery of fluoride and its effect on teeth by Dr. Mckay, fluoride has become the mainstay facet of dentistry and remains so till date [3]. Commercially available fluoride toothpastes Clinpro™ 5000 toothpaste and Clinpro™ Tooth Crème contains 5000 parts per million of fluoride, and are safe for all teeth. Tricalcium phosphate (TCP) present in these fluoride dentifrices provide catalytic amounts of calcium and phosphate to boost fluoride efficacy when it comes in contact with the tooth[5].

Although, fluoridated dentifrices are believed to stimulate remineralization and protect against decay, however, due to its many adverse effects related to serendipitous ingestion and over-usage, is still under scrutiny[6]. As the use of fluoridated dentifrice on a daily basis is debatable, researchers still anticipate a real breakthrough in using alternate and more effective agents for caries prevention.

Theobromine, an alkaloid extract of cocoa beans has significant remineralizing potential. Studies have conferred that improved mineral gain and increased apatite crystal size with theobromine, strengthens the enamel against future acid erosion[7-8]. Theodent classic™, a commercially available non-fluoride prototype toothpaste is composed of theobromine, calcium, and phosphate in a proprietary formula designed to maximize rapid enamel remineralization. Although, remineralization efficacy of fluoride and theobromine against placebo is established, however, literature on comparison of Theobromine containing dentifrices (Theodent™) and fluoridated dentifrices (Clinpro™) is limited. Therefore, the current in vitro study was undertaken with an objective to comparatively evaluate the remineralizing efficacy of artificial saliva, Theodent™ and Clinpro Tooth Creme™ using Scanning electron microscopy-Energy-dispersive X-ray analysis (SEM-EDAX).

Materials and methods

A randomized controlled *in-vitro* trial was carried out in 30 sound enamel samples, which were subjected to demineralization and remineralization. Institutional Ethics Committee approval (Ref no. AJEC/Rev/88/2014-2015 dated 30.10.2014) was obtained prior to the study.

$$f=d \times \frac{1}{2} \frac{\sqrt{(k+1)}}{3(k-1)}$$

By taking Hegde et al. (2012) [9] as reference, and using the formula, , where k= number of groups, d =difference between the highest and the lowest means, 95% confidence interval, 80% power, 0.05 level of significance, a total of 30 enamel specimens with 10 specimens in each group were needed. Sound premolar teeth with all intact surfaces without any evidence of caries, extracted for orthodontic purpose were included in the study. Those with dental restoration or sealant, with enamel irregularities such as cracks, hypoplasia or any enamel malformations were excluded.

All the extracted teeth were cleaned by removal of soft tissue debris and stored in 0.1% Thymol (w/v) solution. The outline of the study design is depicted in figure 1. The enamel specimens, 4x4x1 mm in size with a 2x2mm window, were prepared from the buccal surfaces of extracted human premolar teeth. Following the baseline elemental (Ca/P) and structural analysis of the enamel specimens using SEM-EDAX, the specimens were immersed in the demineralizing solution (5 mL/specimen) for 5 days to produce artificial enamel carious lesions.

Qualitative and quantitative evaluations were performed using SEM-EDAX for each specimen, to estimation of loss of mineral content and surface morphology after demineralization (DML). Using a table of random numbers method, the specimens were then randomly assigned to three groups with 10 specimens each: Group 1- Control (artificial saliva); Group II- Theodent ™ Classic (Theodent, LLC, New Orleans, Lousiana); and Group III- Clinpro ™ Tooth Crème (0.21% w/w NaF paste with TCP). The respective remineralizing agent was applied twice daily with a cotton applicator tip for two minutes. The specimens were washed with de ionized water for removing the remnants of the remineralizing agents, followed by immersion in artificial saliva. The artificial saliva was changed every 24 hours and each specimen was kept in a separate container to avoid contamination and stored in an incubator at 37˚C, mimicking the oral

temperature. After completion of mineralization cycle, elemental (Ca/P) and structural analysis of remineralized enamel was done with SEM-EDAX at 20 days (RML1) and 40 days (RML2) respectively.

Preparation of enamel specimen: By using a diamond disc (Axis dental, Texas) with a slow speed straight handpiece (NSK Japan) at 15,000 rpm, 30 enamel specimens, measuring 4x4x1 mm in size, were prepared from crowns of extracted human premolar teeth. The specimen was then coated with an acid resistant nail varnish (Revlon, India) leaving a narrow square window of approximately 2x 2mm size, on the intact buccal surface.

Preparation of demineralizing solution: The buffered demineralizing solutions were prepared according to Ten cate and Duijsters(1982) model using analytical grade chemicals and deionized water. The ingredients used for the preparation of demineralizing solution were: 2.2mM Calcium Chloride, 2.2mM Monopotassium phosphate and 50 mM acetic acid. The pH of the solution was measured with digital pH meter and adjusted to 4.2 using 1M Potassium hydroxide.

Composition of the artificial saliva solution: 3.90 mM Trisodium Phosphate, 4.29 mM Sodium Chloride, 17.98 mM Potassium Chloride, 1.10 mM Calcium Chloride - 0.08 mM Magnesium Chloride, 0.50 mM Sulfuric acid, 3.27 mM Sodium Bicarbonate and distilled water. The pH of the solution was measured with digital pH meter and adjusted to 7.2 using Potassium hydroxide.

Composition of Theodent™: Along with Rennou™ (naturally occurring chocolate extract blended with other minerals), the toothpaste contains the traditional hydrated silica, glycerin, sodium bicarbonate, Titanium dioxide and Xylitol.

Clinpro™ Tooth Crème: Each gram contains 5 mg of fluoride ion in a neutral pH base, consisting of water, sorbitol, hydrated silica, glycerin, polyethylene-polypropylene glycol, flavor, polyethylene glycol, sodium lauryl sulfate, titanium dioxide, carboxymethyl cellulose, sodium saccharin and tri-calcium phosphate.

SEM-EDAX examination: Prior to the SEM-EDAX evaluation, the enamel specimens were dried in the hot air oven for 15 minutes at 110℃ after which the samples were placed in a vacuum dessicator. The specimens were then sputter coated with 300A° layer of gold. The microstructural surface morphology was evaluated using a SEM (JEOL JSM 6380LA, Tokyo, Japan) and the surface composition determined using Energy Dispersive Spectroscopy (EDS). Images (2000x magnification) were captured electronically with the JEOL Analytical SEM software and SEM images of enamel were generated. Calcium and Phosphorus content was determined in percentage by weight in EDAX and the digital outputs were interpreted numerically as Ca/P ratios at all durations. The examiner was blinded for SEM-EDAX examination.

Statistical analysis: The identified data was entered and stored on excel spreadsheets and imported to Statistical Package for Social Sciences software (SPSS version 20, Chicago, Illinois, USA) for analysis. The normality of the data distribution was assessed graphically using Graphics test (Histogram). Paired T test, one way ANOVA and post hoc Bonferroni test were used wherever appropriate. The significance level was set at a level of $p < 0.05$.

Results

Elemental analysis of study groups and intergroup comparisons are summarized in table 1.

Time period	Artificial saliva (n=10)	Theodent™ (n=10)	Clinpro™ (n=10)	F value	P value
Baseline	2.06±0.05	2.08±0.07	2.20±0.11	16.994	0.001*
Demineralisation	1.92±0.01	1.83±0.06	1.84±0.06	13.416	0.002*
Remineralisation (After 20 days)	1.93±0.02	1.97±0.02	1.96±0.03	6.564	0.005*
Remineralisation (After 40 days)	1.96±0.02	2.02±0.05	2.02±0.03	9.987	0.001*

Table 1. Intergroup comparisons of Mean Ca/P values.
Test used: One-Way ANOVA. Values are expressed as mean± standard deviation.

At baseline, the mean Ca/P ratio of specimens in artificial saliva, Theodent™ and Clinpro™ was 2.06±0.05, 2.08±0.07 and 2.20±0.11, respectively. Significant difference the Ca/P values at baseline (p=0.001) and after demineralization (p=0.002) between different groups were noted. At the end of RML1 and RML2, higher mineral content was noted in specimens of Theodent™ group (1.97±0.02 and 2.02±0.05, respectively) followed by Clinpro™ (1.96±0.03 and 2.02±0.03, respectively) and

artificial saliva (1.93±0.02 and 1.96±0.02, respectively). The difference was statistically significant (p<0.05). Table 2 summarizes the changes in the mineral content within group at different time intervals. While, significant improvement in the Ca/P values from DML to RML1 was seen in Theodent™ (p<0.001) and Clinpro™ (p<0.001) groups, comparison of Ca/P values from DML to RML2 and from RML1 to RML2 was statistically significant in all groups (p<0.05).

Ca/P Mass %	Timeline	Paired Differences		T value	P value
		Mean Difference	Std. Deviation		
Artificial Saliva	BASELINE* DML	0.14	0.06	7.333	<0.001
	DML* RML1	-0.02	0.02	-2.62	0.028
	DML* RML2	-0.04	0.03	-4.957	0.001
	RML1* RML2	-0.03	0.03	-3.591	0.006
THEODENT™	BASELINE* DML	0.25	0.09	8.67	<0.001
	DML* RML1	-0.13	0.07	-6.355	<0.001
	DML* RML2	-0.19	0.08	-7.441	<0.001
	RML1* RML2	-0.05	0.04	-4.359	0.002
CLINPRO™	BASELINE* DML	0.36	0.15	7.707	<0.001
	DML* RML1	-0.12	0.06	-6.433	<0.001
	DML* RML2	-0.18	0.06	-9.801	<0.001
	RML1* RML2	-0.06	0.02	-11.583	<0.001

Table 2. Intra group comparison of mineral content at different timelines.
Test used: paired t test; DML= Demineralization, RML1=Remineralization after 20 days, RML2= remineralization after 40 days.

Ca/P Mass %	(I) group	(J) group	Mean Difference (I-J)	Std. Error	P VALUE
RML1	Artificial Saliva	Theodent™	-0.04*	0.01	0.007
		Clinpro™	-0.03*	0.01	0.018
	Theodent™	Clinpro™	0.004	0.01	0.928
RML2	Artificial Saliva	Theodent™	-0.06*	0.02	0.002
		Clinpro™	-0.06*	0.02	0.002
	Theodent™	Clinpro™	0.00	0.02	0.999
RML1*Baseline difference	Artificial Saliva	Theodent™	-0.01	0.03	0.927
		Clinpro™	0.11*	0.03	0.007
	Theodent™	Clinpro™	0.12*	0.03	0.003
RML2*Baseline difference	Artificial Saliva	Theodent™	-0.04	0.03	0.486
		Clinpro™	0.08	0.03	0.054
	Theodent™	Clinpro™	0.12*	0.03	0.003

TABLE 3. Remineralization efficacy of artificial saliva, Theodent™ and Clinpro™.
Test used: post hoc Bonferroni test; DML= Demineralization, RML1=Remineralization after 20 days, RML2= remineralization after 40 days.

Intergroup comparison with Post hoc analysis of RML 1 and RML 2 showed a significant difference in the Ca/P values between Artificial Saliva and Theodent™(p<0.05), and Artificial Saliva and Clinpro™ (p<0.05), the difference between Theodent™ and Clinpro™ was non-significant (p=0.9). On the other hand, comparison of mean difference between RML2 and baseline showed significant difference in Ca/P values between Theodent™ and Clinpro™ (p = 0.003), and while the difference between Artificial Saliva and Theodent™ (p=0.486), and Artificial Saliva and Clinpro™ (p=0.054) groups were statistically insignificant (Table3). However, neither Theodent™ nor Clinpro™ was able to achieve the Ca/P values (after remineralisation) equivalent to the baseline values.

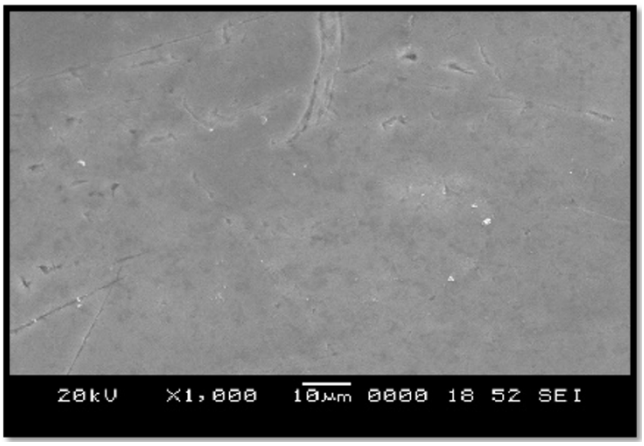

Figure 1. SEM image of the enamel specimen at baseline.

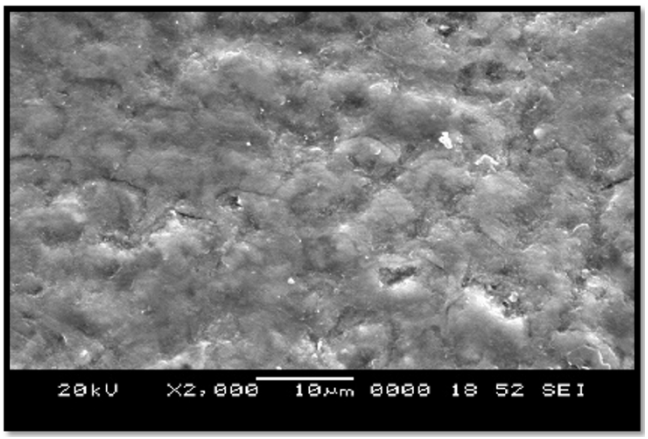

Figure 2. SEM image of the demineralized enamel surface showing evidence of micro porosities.

The SEM images of the enamel surfaces at baseline were generally smooth as shown in figure 01. However, after demineralisation, there was evidence of micro porosities on the surface

(figure 02), which could be due to the acidic challenge. After remineralisation, the SEM images revealed homogenous and distinct amorphous deposits on the enamel surface remineralisation of Clinpro™ and the Theodent™, while no deposits were seen on the enamel surface of the control group (Artificial saliva) as shown in the figure 03 and 04.

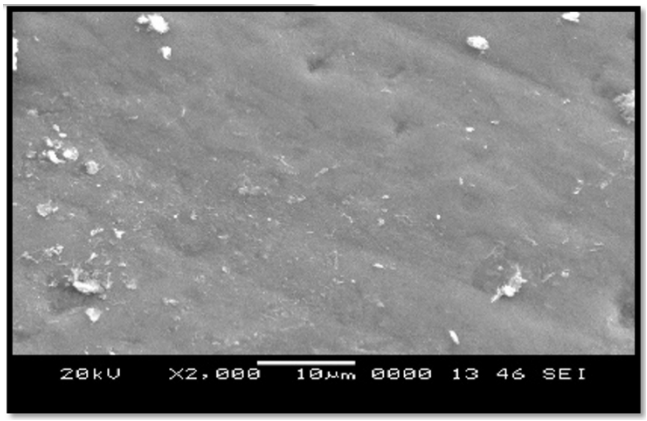

Figure 3. SEM image of the enamel subsurface lesion area treated with Theodent™ for 20 days.

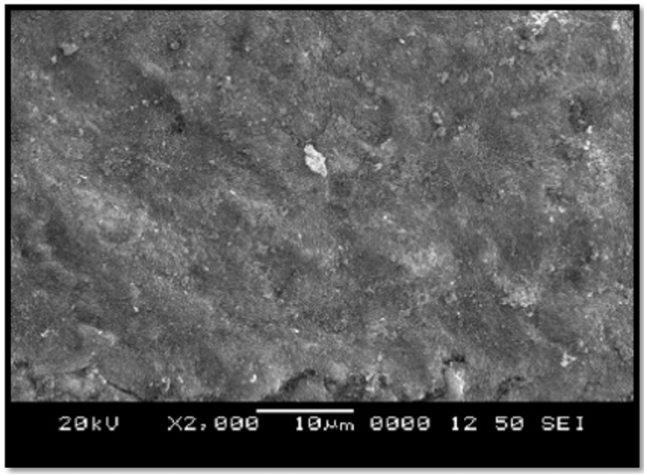

Figure 4. SEM image of the enamel subsurface lesion area treated with Theodent™ for 40 days.

Discussion

Early carious lesion with its potential for reversal is an interesting area of research since ages. Several studies have been carried out to assess the effect of various preparations of calcium, phosphate, and fluoride on enamel remineralization [3, 10]. Although, fluoride containing dentifrices are widely used, however due to reported local and systemic adverse effects, its use as a daily usage dentifrice is controversial. In the present study, remineralizing efficacy of Theobromine containing dentifrice (Theodent™) and fluoridated dentifrices (Clinpro™) was compared with artificial saliva (control) in an *in vitro* model. The *in vitro* model key advantage of providing the ability to carry out single variable experiments under highly controlled conditions [11].

As remineralizing property of saliva is well known in the field of caries research, and considering the *in vitro* nature of study, artificial saliva was used as a control and as a storage medium for the samples in our study. In addition to replicating the composition of natural saliva, artificial saliva also creates an artificial oral environment and prevents bacterial contamination of enamel samples.The pH-cycling regime by Ten Cate and Duijsters (1982) was used in the current study in order to replicate the continuous process of demineralisation and remineralisation in the oral environment [12]. All specimens were demineralized using buffered acidic solution at a pH 4.8 to create subsurface lesions with mineral profiles similar to the typical natural initial caries lesions and subjected to remineralisation for 20 and 40 days using the experimental materials.

Demineralization and remineralization efficacy of the experimental materials was scrutinized using SEM- EDAX, which reliably detects ion levels at very low concentrations within a highly mineralized tissue [13]. In our study, EDAX examination showed significant improvements in the Ca/P values at RML1 and RML2 in all three groups as compared to DML values, however, none of the groups attained the baseline mineral densities. The remineralization achieved was maximal after 40 days of remineralization suggesting that the effect is dose-dependent [7]. It is evident that lesions with increased Ca/P at baseline have a marked decrease in further mineral loss and a concomitant increase in further mineral gain. In this study, the Ca/P at baseline was maximum for Clinpro™ and least for artificial saliva, after demineralization, the maximum mineral loss found in artificial saliva and Theodent™ had the least mineral loss. With 20 and 40 days of exposing the specimen to the remineralising agents, Theodent™ achieved the maximum mineral gain and artificial saliva demonstrated the least remineralisation.

Interestingly, significant improvement in Ca/P values in the control group was also

observed. We believe that, the presence of an organic buffer and supersaturated concentrations of calcium and phosphate in the artificial saliva aided in remineralization over time conferring the remineralizing effect of saliva in early carious lesion. However, as compared to the test groups, Ca/P values were least in the control group. This could be related to the theory, which suggests that the presence of organic substances in the saliva blocks the underlying pores in carious lesion or cause precipitation of calcium causing surface remineralization and hinders remineralization at the body of the lesion[14]. In our study, presence of mucine in the artificial saliva would have had the same effects over enamel; however, it is a distant possibility. Moreover, mineral gain is a slow process and is dependent on the ability of saliva to supply bioavailable calcium and phosphate ions to the tooth[15].

At a concentration of 1.1 mmol/l, Theobromine reduces the calcium, phosphorus and magnesium dissolution from apatite of the enamel, enhances the crystallinity of the apatite and further improves resistance against the acid dissolution [12]. Remineralized teeth with theobromine have better resistance to demineralization as compared to fluoride-treated teeth suggesting better enamel hardness in those surfaces [16, 17]. While, Nasution et al [18] reported increased enamel hardness with fluoride as compared to the theobromine, in our study, Theodent™ showed a higher healing effect on remineralizing artificial carious lesions when compared to Clinpro™, similar to a study done by Amaechi et al [16]. However, the comparison of the remineralisation at the end of the study period of the two study groups was not statistically significant. This could be due to the variable mineral concentrations in of the study groups.

Karlinsey et al.[19] reported that the combination of sodium fluoride with fTCP in an aqueous solution produces significantly greater surface and subsurface rehardening of white spot lesions than with the use of fluoride alone. The high magnitude of remineralization seen with the Clinpro™ was similar to the studies by Pulido et al [20] and AL-Mulla et al [21]. This observed response can be explained by assuming that the deposition of fluoride during the treatment depends on lesion depth, improved surface microhardness with Clinpro™ is related to the presence of fluoride compatible TCP which imparts superior remineralisation at both the enamel surface and within the subsurface lesions, thereby boosting the enamel surface strength [22].

In the SEM images, the enamel surfaces at baseline were generally smooth. However, after demineralization, there was evidence of microporosities on the surface which could be due to the acidic challenge. After remineralization, the enamel surfaces of Theodent™ group were slightly less smooth, showing shallow lines or pits and had globules/ amorphous deposits on the enamel surface similar to those found in the study by Kargul et al [7] and Nakamoto et al [12] who reported formation of larger HAP crystals (acid resistant) and a smooth enamel surface with application of theobromine. In the Clinpro™ group, reduction in the surface alteration with the presence of a diffuse mineral coating was found. This remineralization could be related to blocking of underlying pores in the carious lesion, caused by the presence of organic substances that attach to the enamel surface, or by the precipitation of calcium mineral phases within the superficial aspect of the lesion [18,19].

Although, fluoride is an excellent remineralizing agent, however is associated with potential toxic side-effects [23]. On the other hand, the natural compound, Theobromine is safe and does not produce chemical dependency[24]. Moreover, despite the difference in the basic formulations, we along with previous results [25-27] have observed comparable remineralization efficacy in both Theodent™ and Clinpro™. Thus, with a better understanding of the health benefits of cacao consumption in man, especially the potential of theobromine in the inhibition of dental caries, this FDA approved non-fluoride prototype (Theodent classic™) can be used has a major step forward in oral health maintenance and prevention of dental caries.

Despite the clinical significance, the study has its set of limitations. Although we tried to replicate demineralized surfaces *in vitro,* it may not be true representation of early enamel caries due to the inability to replicate the complex biological processes involved in caries. Moreover, the artificial saliva used in the study lacks important protective biochemical processes available in the oral environment including the pellicle formation on the enamel. Upon demineralization, the specimens were not subjected to multiple daily acidic challenges that

would normally occur in the oral environment. Additionally, as SEM-EDAX is heavily dependent upon sample preparation, variation in polishing or sample tilt could overshadow any differences in the mineral content. Further studies with use of microCT can be used to validate results of caries related research. This experiment was also limited in time, which added to setbacks with the mineral content estimation. Moreover, it is evident that remineralization not only re-establishes the hardness of the sound enamel, but also produces greater resistance to further acid dissolution. However, the same was not assessed in our study. Further research on remineralized surfaces to various acid insults can be interesting.

Conclusions

Although, tooth brushing with fluoridated toothpaste can be considered as one of the most common recommendations from dental professionals to patients with white spot lesions, in light of the findings presented in this study, it is possible to conclude that the topical application of theobromine containing dentifrices are effective in the prevention and management of early carious lesions. Hence, Theodent™ can be used as an alternative to the conventional fluoride containing agents in remineralizing white spot lesions. However, further in-vivo clinical trials comparing Theobromine containing dentifrices is warranted to justify its clinical effectiveness in managing early carious lesions.

Declaration of Interest

The authors report no conflict of interest.

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