Original Article

Remineralization Potential of Theobromine on Artificial Carious Lesions

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Abstract

Background and Aims: This study aimed to investigate the remineralization potential of two concentrations of theobromine (100 mg/L and 200 mmg/L) with fluoridated dentifrice, NovaMin, and nanohydroxyapatite using DIAGNOdent, scanning electron microscopy (SEM), and energy dispersive X-ray (EDX) analysis. **Materials and Methods:** Two sections were taken from 50 teeth each. Artificial carious lesions were induced using demineralizing solution. Evaluation using DIAGNOdent, SEM, and EDX analysis for elemental evaluation of Ca/P ratio and fluoride ion was carried out. Teeth sections were then randomly assigned to five different groups: (1) fluoridated dentifrice (Colgate™, Colgate –Palmolive, India), Novamine- Shy NM™, Group pharamaceuticals, India), 3. Nano-hydroxyapatite- Remin Pro™, Voco, Germany) 4. 100mg and 5. 200mg of Theobromine toothpaste (Theodent classic™, Rennou, UK-853069003006). Remineralization was carried out for 14 days with two applications per day. Samples were reanalyzed using DIAGNOdent, SEM, and EDX. **Results:** A Tukey *post-hoc* test revealed statistically significant difference between NovaMin and all the other toothpastes ($P < 0.001$) for DIAGNOdent readings. On performing SEM-EDX analysis, it was seen that all agents had remineralization potential; however, no significant difference was found. **Conclusion:** Theobromine can be used as an effective novel remineralizing agent alternative to the already-available agents.

Keywords: *Nanohydroxyapatite, NovaMin, remineralization, theobromine*

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Introduction

Regardless of the age, gender, and ethnicity, dental caries is a complex disease affecting a majority population of the world. Dental caries is a continuous process, which starts from the first atomic level of demineralization, progresses to initial white spot, and often can cause dentinal involvement eventually leading to cavitation. The dynamic balance between demineralization and remineralization, as determined by pathological factors and protective factors, determines the end result. As the disease of dental caries is reversible so if diagnosed early enough, various antibacterial and chemical methods can be used to facilitate remineralization and reduce demineralization.[1]

Remineralization may be as simple as the immediate repair of recently acid damaged enamel and occur on need basis with no clinical evidence of a lesion. Conversely, the repair process may require prolonged mineral deposition in order to reverse a clinically detectable white-spot lesion (WSL). It is important to realize that remineralization is an ongoing process in the oral cavity. The fate of the lesion, whether it will progress to cavitate or remineralize, depends on biological factors in the plaque and saliva, composition of enamel, oral hygiene, dietary habits, and exposure to preventive agents.

Calcium, phosphate, and fluoride can act together and reverse demineralization, by depositing a new layer on

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the crystal remnants. The new mineral surface is more acid resistant as compared with the original carbonated hydroxyapatite mineral. The demineralization–remineralization cycle occurs numerous times daily, leading either to cavitation or to repair and reversal. The mainstay in caries prevention and remineralization is frequent exposure to low levels of fluoride. Thus, many novel remineralizing agents are being introduced for the well-being of the society.

Few novel remineralizing agents are nanohydroxyapatite, NovaMin, and theobromine, which help in remineralization by various mechanisms.

Nanohydroxyapatite (n-HAp) is considered one of the most biocompatible and bioactive materials, and has gained wide acceptance in dentistry in recent years. Hydroxyapatite acts as a calcium–phosphate reservoir, which helps to maintain a state of supersaturation with respect to enamel minerals and hence reduces enamel demineralization and enhances remineralization.[2]

NovaMin is a bioactive glass that acts as a biomimetic mineralizer matching the body's own mineralizing traits while also affecting cell signals in a way that benefits the restoration of tissue structure and function.[3]

According to Arman Sadeghpour from Tulane University, New Orleans, LA, one of the alkaloids found in chocolate (240 mg/cup) and cocoa (1.89%), theobromine, can be used to prevent demineralization of enamel. Theobromine (3,7 dimethylxanthine) is a chemical compound of the alkaloid group in the form of white crystalline powder and only differs from the caffeine molecule by one methyl group (1,3,7 dimethylxanthine). It decreases caries or promotes remineralization by enhancing crystallinity.[4] It is a nontoxic, natural, and an effective remineralizing agent. As theobromine causes calcium and phosphate from saliva to combine into a crystal unit four times larger than hydroxyapatite, growth of new enamel is stimulated.[5] Another theory states that adding theobromine to apatite material can increase the size of crystallite size.[6] Theobromine had shown increased hardness of enamel after its application followed by experimental demineralization of enamel.[7-9]

As there is a limited literature available on remineralization potential of theobromine, this study was designed to determine its efficacy in remineralizing the artificially created incipient enamel lesions. The aim of this study was to investigate the remineralization potential of two concentrations of theobromine (100 mg/L and 200 mmg/L) with fluoridated dentifrice, NovaMin, and n-HAp using DIAGNOdent, scanning electron microscopy (SEM), and energy dispersive X-ray (EDX) analysis.

Hypothesis

Theobromine may have a similar remineralizing effect as that of fluoridated dentrifrice and other remineralizing agents used in this study.

Materials and Methods

Type Of Study

This is an *in vitro* experimental study. Sample size was estimated using the power calculation $\alpha = 0.05$ and $\beta = 0.20$ with 80% being the power of the study, based on previous findings reported by Hegde and Moany:[10]

$$n = (z_\alpha + z_\beta)^2 o'^2 / d^2$$

A sample size of 50 was deemed essential. A total of 200 primary molars indicated for extraction were obtained from patients aged 3–14 years who reported to the Department of Pedodontics and Preventive Dentistry, Manipal College of Dental Sciences (MCODS), Manipal, Karnataka, India. Owing to the difficulty in obtaining intact teeth, teeth with at least two surfaces intact were selected, 50 such teeth were selected, and these were then divided randomly into five groups: Group 1: flouride; Group 2: NovaMin; Group 3: n-HAp; Group 4: theobromine 100 mg/mL; and Group 5: theobromine 200 mg/mL. Samples were cleaned off any debris. With the help of micromotor hand piece and diamond disk, the samples were sectioned and roots were separated. Crowns were sectioned into two parts and were grouped as demineralized or remineralized sample. Two halves of same tooth were used for both deminearlization and remineraization. Total duration of the study was two months.

Induction Of Artificial Carious Lesions

All the samples were immersed in 12-mL demineralizing solution for 72 h. The composition of demineralizing solution used is as follows: 2.2-mM $CaCl_2$, 2.2-mM NaH_2PO_4, 0.05-M lactic acid, and 0.2-ppm fluoride. The pH was adjusted to 3 with 50% NaOH.[11] One sample was immersed in each bottle and then stored for 72 h at 37°C.[12]

Demineralization readings

DIAGNOdent recordings were taken at this stage as per the manufacturer's instructions [Table 1].[13]

Table 1: Standard readings of the DIAGNOdent

DIAGNOdent readings	Interpretation
0–12	Healthy tooth
12–20	Initial enamel lesion
>20	Dentinal lesion

Further for a better surface morphology analysis, all the samples were gold sputtered. For surface morphology evaluation, samples were subjected to SEM analysis. Following this, for elemental analysis for calcium, phosphate, and fluoride, EDX analysis was carried out (SEM-EDX, ZEISS EVO MA18, Oxford EDS, and X-act) at 15 kV and ×500, ×1000, ×1500, ×2000, and ×2500 magnification [Figure 1].

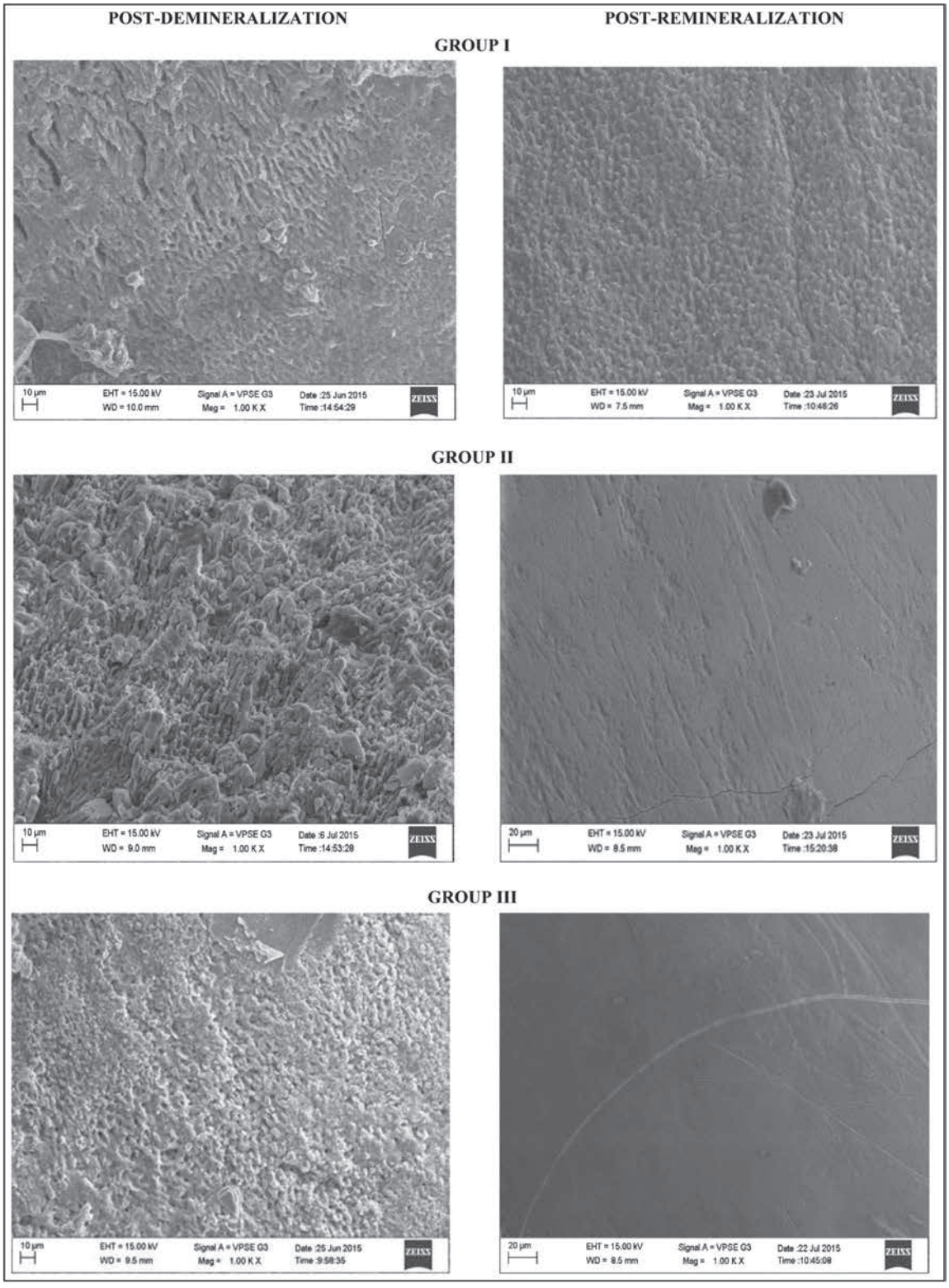

Figure 1: SEM images after demineralization and remineralization in all the groups

Application Of Test Agents

Test agents, Group I (fluoride dentrifrice: Colgate™, Colgate-Palmolive, Mumbai, India), Group II (Novamine: SHY-NM™, Group Pharmaceuticals, Bangalore, India), and Group III (n-HAp: Remin Pro™, Voco, Cuxhaven, Germany), were used in paste form. Groups IV and V were prepared by dissolving 100 and 200 mg of theobromine toothpaste (Theodent Classic™, Rennou, London, UK) measured in 1 L of artificial saliva. Respective test agents were applied on the samples at 24-h interval for 14 days. A cotton tip applicator was used to apply the respective agents on the samples according to manufacturer's instructions. Samples were washed and stored in deionized water itself. Deionized water was changed every 24 h.

After 14 days of remineralization regime, the surface was assessed using DIAGNOdent (KaVo DIAGNOdent 2095, Kaltenbach & Voigt GmbH & Co. KG, Germany) to record the values. The samples were also assessed using SEM-EDX (ZEISS EVO MA18, Oxford EDS, X-act) to study the change in surface characteristics and estimate the mineral content of calcium, phosphate, and fluoride [Figures 1 and 2].

Statistical Analysis

Data were entered in a dataspread sheet using the Statistical Package for the Social Sciences software version 18.0 (SPSS, Chicago, IL). Statistical analysis for DIAGNOdent readings was performed using *post-hoc* Tukey's honestly significant difference and one-way analysis of variance (ANOVA). A value of $P < 0.001$ was considered statistically significant. Elemental analysis was carried out using Kruskal–Wallis ANOVA and Wilcoxon signed rank test. A value of $P < 0.05$ was considered statistically significant.

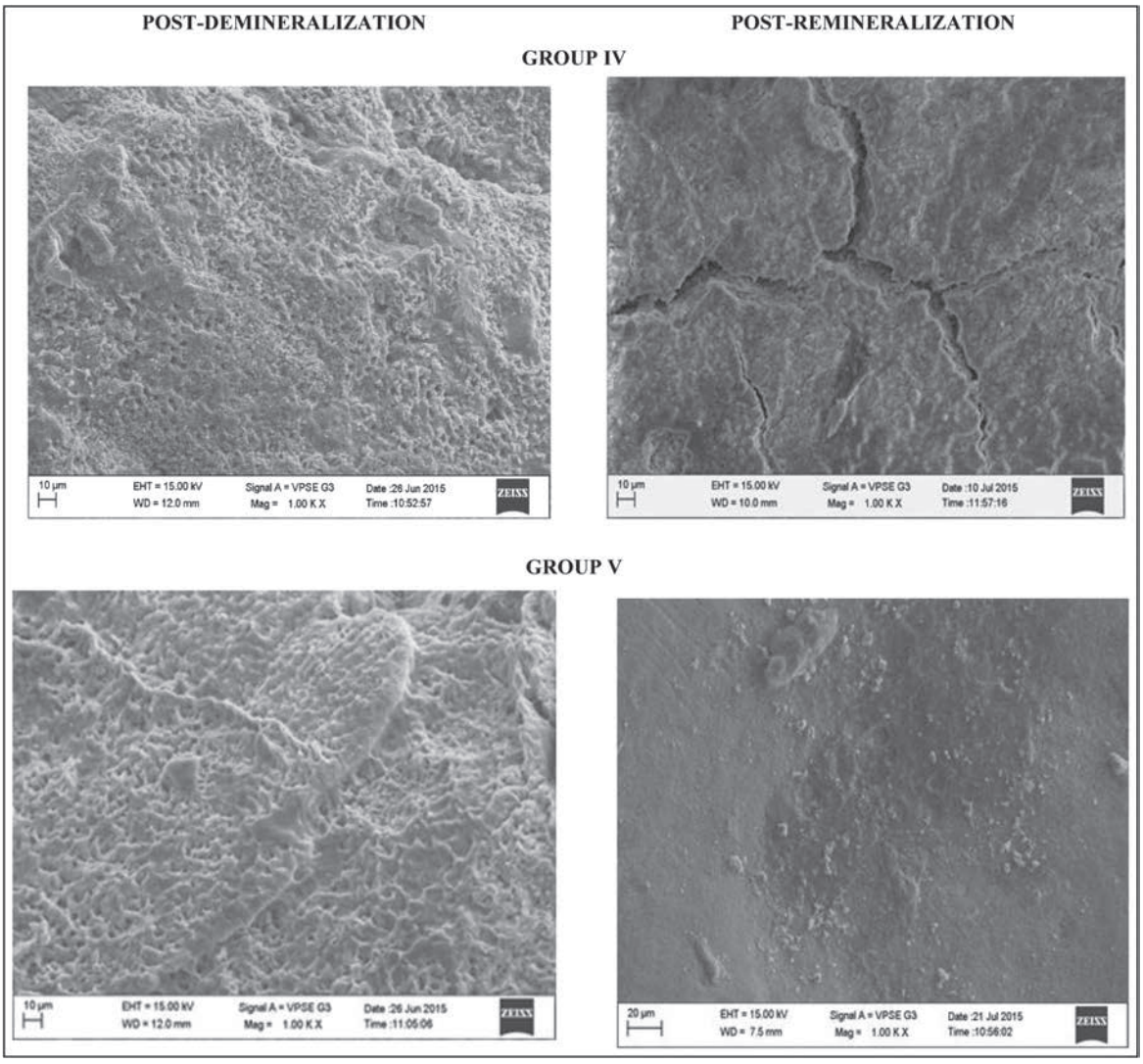

Figure 2: SEM images after demineralization and remineralization in all the groups

Results

DIAGNOdent readings were seen to increase following induction of WSLs and reduced following remineralization. The mean percentage difference between DIAGNOdent readings for Groups I, II, III, IV, and V increased to 60.74 ±10.94, 31.99 ± 10.05, 65.48 ± 11.66, 66.27 ± 10.00, and 62.97 ± 9.41 [Table 2].

Table 2: Mean percentage difference in DIAGNOdent readings post-demineralization and post-remineralization in various groups

Toothpaste	*N*	Mean percentage change	Std. deviation
Fluoride	10	60.74	10.94
Novamine	10	31.99	10.05
Reminpro	10	65.48	11.66
Theobromine 100 mg/mL	10	66.27	10.00
Theobromine 200 mg/mL	10	62.97	9.41

A Tukey *post-hoc* test revealed that the mean percentage change was statistically significant between Novamine and all the other toothpastes ($P < 0.001$) with 95% confidence interval, but there was no statistically significant difference in mean percentage change between n-HAp, theobromine (100 mg/mL), fluoride, and theobromine (200 mg/mL), indicating that n-HAp, theobromine (100 mg/mL), fluoride, and theobromine (200 mg/mL) were broadly similar in terms of effectiveness [Table 3]. Figure 1 shows the SEM analysis.

Post-demineralization

After 72 h of demineralization, SEM images were taken. There was a loss of surface integrity in all the study groups. Enamel showed irregular surface like a honeycomb pattern. Porous defects could be seen, thus proving loss of aprismatic enamel and presence of destructed enamel rods.

Post-Remineralization

After a 14-day remineralization regimen, samples were again subjected to SEM examination. The porous

Table 3: Intergroup comparison of DIAGNOdent readings post-demineralization and post-remineralization in various groups

		Mean difference	*P* value
Fluoride	Novamine	28.752	$P < 0.001$
	Reminpro	–4.731	0.848
	Theobromine 100 mg/mL	–5.522	0.761
	Theobromine 200 mg/mL	–2.220	0.989
Novamine	Fluoride	–28.752	$P < 0.001$
	Reminpro	–33.483	$P < 0.001$
	Theobromine 100 mg/mL	–34.274	$P < 0.001$
	Theobromine 200 mg/mL	–30.972	$P < 0.001$
Reminpro	Fluoride	4.731	0.848
	Novamine	33.483	$P < 0.001$
	Theobromine 100 mg/mL	–0.791	0.999
	Theobromine 200 mg/mL	2.511	0.983
Theobromine 100 mg/mL	Fluoride	5.522	0.761
	Novamine	34.274	$P < 0.001$
	Reminpro	0.791	0.999
	Theobromine 200 mg/mL	3.302	0.954
Theobromine 200 mg/mL	Fluoride	2.220	0.989
	Novamine	30.972	$P < 0.001$
	Reminpro	–2.511	0.983
	Theobromine 100 mg/mL	–3.302	0.954

Table 4: Intergroup comparison of mean percentage difference of Ca/P ratio post-demineralization and post-remineralization

	1 Mean ± SD	2 Mean ± SD	3 Mean ± SD	4 Mean ± SD	5 Mean ± SD	*P* value
Ca/P[a] difference	1.06 ± 0.30	0.85 ± 0.44	1.01 ± 0.45	0.68 ± 0.37	0.84 ± 0.42	0.21

SD = standard deviation

[a]The mean calcium and phosphorus ratio difference after demineralization and remineralization between five groups

defects were all filled; thus, the surface integrity was reestablished. However, no distinct difference could be seen among the various groups, with regard to the surface coatings that filled the porosities.

Energy Dispersive X-Ray Analysis

EDX analysis was used to determine calcium, phosphorous, and fluoride contents in weight percent (wt%) of demineralized and remineralized enamel in all samples of each experimental group. The calcium and phosphorus content was then converted into Ca/P ratios for each group from the obtained data [Tables 4 and 5].

Fluoride Ion

Using the same EDX analysis increase in fluoride ion concentration following remineralization was calculated [Table 6]. Comparing the mean concentration difference of fluoride ion between various groups was carried out using Kruskal–Wallis ANOVA test. No statistically significant difference was seen for mean concentration difference of fluoride ion for all groups with *P*-value 0.75 with 95% confidence interval. Mean percentage difference of Ca/P ratio between demineralization and remineralization of each group using Wilcoxon signed rank test. Statistically significant increase was seen for Group 1 between demineralization and remineralization.

Discussion

Enamel remineralization has been studied for about 100 years, and it has been suggested that "the noninvasive" treatment of early caries lesions by remineralization has the potential to be the major advance in the clinical management of the disease. WSLs represent the first clinical observation of demineralization in enamel and their early diagnosis has been reported in the literature.[14] DIAGNOdent is one of the recent noninvasive methods to detect early WSLs. However, *in vitro* and *in vivo* studies conducted to evaluate the efficacy of this laser device have given substantially varying results.[15-17] For this study, probe B of DIAGNOdent was used. Prior to every measurement session, the instrument was calibrated against its own ceramic standards as recommended by manufacturer. The tip was moved over the entire tooth surface to collect the fluorescence from all directions and the maximum value was taken.

DIAGNOdent examination was carried out at room temperature 22°C which was according to a study by Shi *et al.*[16,19] In accordance with a study by Pinelli *et al.*,[17] samples were dried for 10 s before the examination. Following remineralization values decreased which was similar to results by Leila[18] where values decreased for both n-HAp and fluoride but no significant difference was seen between two. Shi *et al.*[16] have also showed similar results. No studies till date have evaluated the remineralization potential of NovaMin and theobromine with DIAGNOdent. According to this study, the values decreased for both the groups following remineralization. However, a significant difference was seen between NovaMin and other groups viz. fluoride, n-HAp, and both the concentrations of theobromine. No significant difference was found while comparing the other groups.

Various demineralizing agents have been used till date, in this study, demineralization was carried out as described by Lata *et al.*[8] The specimens were kept in the demineralization solution ($CaCl_2$, NaH_2PO_4, lactic acid, and fluoride) for 72 h at 37°C creating a subsurface demineralization of approximately 150 µm width with an intact surface simulating an early enamel lesion. In this study, fluoride, NovaMin, and n-HAp were used in commercially available paste form. This was carried out in view of replication of patient convenience in using the tooth creams like a toothpaste with toothbrushes. Theobromine was used in two concentrations, 100 mg/mL and 200 mg/mL, which were prepared by mixing requisite amount of Theodent toothpaste (Theodent Classic™) in artificial saliva.[4] The agents were applied with cotton tip applicator for 2 min twice daily at 12-h interval. Samples were immersed in deionized water after each treatment till the next cycle of agent

Table 5: Intragroup comparison of mean percentage difference of Ca/P ratio post-demineralization and post-remineralization

Group	Demineralization Mean ± SD	Remineralization Mean ± SD	*P* value
1	1 ± 0.15	2.06 ± 0.29	0.005
2	1.09 ± 0.33	1.95 ± 0.22	0.007
3	1.31 ± 0.29	2.15 ± 0.28	0.005
4	1.54 ± 0.29	2.23 ± 0.30	0.005
5	1.14 ± 0.35	1.98 ± 0.23	0.005

SD = standard deviation

Table 6: Intragroup comparison of mean percentage difference of fluoride ion concentration post-demineralization and post-remineralization

Group	Demineralization Mean ± SD	Remineralization Mean ± SD	*P* value
1	3.77 ± 0.65	4.87 ± 1.24	0.01
2	4.17 ± 3.40	4.58 ± 2.41	0.20
3	3.38 ± 0.68	4.69 ± 3.31	0.28
4	4.24 ± 2.48	5.02 ± 2.88	0.33
5	4.91 ± 4.21	5.73 ± 4.1	0.28

SD = standard deviation

application.[4] Samples were analyzed using SEM as it is one of the most sensitive, time-tested techniques to assess the demineralization and remineralization of the carious lesions *in vitro* as reported in earlier studies.[20,21] Samples were dried and gold sputtered. To avoid the loss of samples, samples were sectioned into two halves and were examined at different magnifications. At ×1000 magnification, numerous depressions in a honeycomb pattern were revealed, which corresponded to the observations made by previous study.[22] With the observation of SEM images, it could be said that DIAGNOdent also has similar credibility. Post-remineralization, all the samples showed reestablishment of surface integrity with increase in crystal size and occlusion of porous defects. However, there were minor differences among all the groups which could be because of their different mechanisms of remineralization. The SEM findings were in accordance with previous studies.[4,23,24]

Similar to a study by Huang *et al.*,[22] the porous defects formed on demineralization were filled on remineralization using NaF and n-HAp. Although Swaroop *et al.*[24] reported that n-HAP gave significantly better results, this can be attributed to preparation of fresh slurry in their study as compared with use of commercial toothpastes in this study. Similarly, Amechi [4] on evaluating SEM images post-remineralization claimed that crystal size increased post-remineralization on using theobromine as a remineralizing agent, thus giving the enamel surface a more uniform appearance. Although in a study by Kargul *et al.*[22] on comparing the surface topography of enamel following application of 200 mg/L of theobromine and 100 mg/L of theobromine, 200 mg/L was found to have better results; in our study, no such difference could be seen. This could be attributed to 5-min application in that study as compared with 2-min application in our study. Mony *et al.*[23] on interpreting SEM images of samples remineralized with NovaMin and fluoride concluded that following remineralization, NovaMin gave smoother and uniform enamel, whereas formation of Fluoroapatite following fluoride application resulted in irregular enamel where defects were not uniformly covered. There is a lack of remineralization studies assessing the fluoride gain by EDX analysis. In this study, increase in the fluoride content post-remineralization was seen in all the groups; however, there was a statistically significant increase in fluoride content post-remineralization for Group I and statistically nonsignificant increase was seen for Groups II, III, IV, and V with *P*-values 0.20, 0.28, 0.33, and 0.28, respectively. A significant difference was seen in the fluoride content between Group I and other groups. In this study, all the groups showed a significant increase in the Ca/P ratio following remineralization; however, no statistically significant difference was seen between the groups. This is in agreement with previous studies that showed the capability of the chosen test agents to induce remineralization of early enamel lesions. The results were in accordance with Mohanty *et al.*[25] where remineralization potential of NovaMin to a control was analyzed. The samples were analyzed at 0 days, 48 h, and 10 days. A significant increase for Ca/P ratio was seen on EDX evaluation for NovaMin. Contrary to the results of this study, Swaroop[24] in their study concluded that on performing the elemental analysis a significant increase was seen for n-HAp as compared with sodium fluoride. This could be attributed to the use of freshly prepared slurry of the agents as compared with the commercial toothpaste used in this study. A elemental analysis comparison carried out for theobromine and fluoride showed an increase in the Ca/P ratio without any significant difference, which was similar to result shown by Amechi[4] in *an in vitro* experimental study conducted by Irawan *et al.*[7] Suryana *et al.*[9] and Sulistianingsih *et al.*[26] revealed that there was an increase in enamel microhardness of enamel after application of theobromine gel. These findings are in consistent with our results where we found a significant increase in Ca/P ratio post application. Nakamoto *et al.*[27] stated that theobromine-containing toothpastes can be used as an effective alternative to fluoride-containing dentifrices.

A limitation of this study is the lack of SEM-EDX analysis for the baseline samples and small sample size owing to the cost constraint. SEM-EDX at baseline could have facilitated a better comparison of the ability of the test agents to induce remineralization and their potential to bring the mineral content closer to baseline levels. In this study, superiority of one agent could not be established. Further *in vivo* studies with large sample size are recommended for evaluation of remineralization potential of Theobromine.

Conclusion

The following conclusions can be drawn based on this study:

1. All test agents showed remineralization potential.
2. No significant difference was seen between the remineralization potential of the test agents.
3. Theobromine can be used as an effective alternative to the already-available remineralizing agents.

Data Availability Statement

Data will be available on request from corresponding author (Nekkanti Sridhar, drsri.pedo@gmail.com).

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Conflicts of interest

There are no conflicts of interest.

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