



Original Article

Comparison of Remineralization Potential of Theobromine and Nano-Hydroxyapatite to Sodium Monofluorophosphate: An *In Vitro* Study

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ABSTRACT

Objectives: The present *in vitro* study evaluated and compared the effect of theobromine gel and nano-hydroxyapatite (NHA) paste to conventional sodium monofluorophosphate-containing toothpaste on demineralized enamel. The study's objectives were to investigate the remineralizing potential and effect of the materials on the microhardness and surface roughness (SR) of demineralized enamel.

Material and Methods: The present *in vitro* study was conducted in Bharati Vidyapeeth (Deemed to be) Dental College and Hospital, Pune. The laboratory imaging and analysis were performed at Praj Metallurgical laboratory, Pune, over one month from December 2022 to January 2023. A total of 30 ($n = 30$) extracted human premolars were decoronated and embedded in customized acrylic resin molds. A window of 5×5 mm on the buccal surface was demineralized for three days and then subjected to a 21-day pH cycle, following which it was remineralized. Surface microhardness (SMH) and SR were recorded at baseline, after demineralization and remineralization by respective agents ($n = 10$ samples each). Scanning electron microscopy was also performed for the samples.

Results: A significant increase ($P < 0.05$) was observed in the SMH values in all three groups, although none of the groups reached the original microhardness values that were present at the baseline. The SMH values were highest in the sodium fluoride group after remineralization while the lowest increase was noted with theobromine. The specimens treated with NHA exhibited the highest sedimentation of the porosities and the least SR under scanning electron microscopy.

Conclusion: Sodium monofluorophosphate, theobromine, and NHA are effective remineralizing agents. While sodium monofluorophosphate achieved the highest microhardness increase, its improvement in SR was not at par with the other two materials. NHA excelled in improving the SMH and roughness.

Keywords: Dental caries, Electron, Microscopy, Scanning, Sodium fluoride, Theobromine, Toothpastes

INTRODUCTION

The classic definition of dental caries states that it is “an irreversible microbial disease of the calcified tissues of the teeth, characterized by demineralization of the inorganic portion and destruction of the organic substance of the tooth, which often leads to cavitation.”^[1] The inorganic portion of tooth structure in both enamel and dentin is made up of calcium hydroxyapatite

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(CHA) crystals. With the evolution of knowledge concerning the pathogenesis of caries, it was understood that the disease is not merely a unidirectional process of continuous demineralization but also involves intermittent periods of remineralization.^[2]

This remineralization can be attributed to the exposure of the tooth's outermost surface, whether decayed or intact, to the saliva and plaque fluids that are super-saturated with calcium and phosphates. Through these conditions encouraging remineralization, the caries progression can be retarded or even arrested. The addition of fluoride or water to the diet has been demonstrated to reduce the prevalence of dental caries due to the formation of calcium fluorapatite (CFA) crystals.^[3] These CFA crystals are even more resistant to acid disintegration than the naturally existing CHA.^[1] Therefore, dentifrices containing fluoride in the form of sodium fluoride or sodium monofluorophosphate are popularly used today.

However, fluoride is tolerable only to a certain dosage within the body, and the harmful effects of greater concentrations of fluoride cannot be overlooked. Concentrations of fluoride exceeding one ppm begin manifesting clinically as hypoplastic areas on teeth in the form of dark pits, stripes, or a corroded appearance of the crown. Higher dosages can also cause systemic effects such as early aging, decreased intelligence, and irritation of the gastrointestinal tract.^[4,5] Due to these adverse effects, modern-day manufacturers have attempted to formulate fluoride-free dental healthcare products that aim to remineralize teeth.

Theobromine, with the chemical formula 3,7-dimethylxanthine, is a white crystal powder that has been used to prevent enamel demineralization.^[6] It causes calcium and phosphate from the saliva to merge into a crystal unit that is four times bigger than hydroxyapatite, causing regrowth of enamel crystals. The combination of mineral placement as new enamel growth takes place increases the enamel hardness.^[7] Although gels and dentifrices containing theobromine have already been developed and marketed, they have yet to gain popularity.

Nano-hydroxyapatite (NHA), a biomimetic material, can reconstruct hypomineralized enamel. Dentifrices consisting of NHA were first introduced in the 1980s and were approved by the Japanese government as anti-caries agents in 1993.^[8] It has been the subject of various studies; however, most of these studies were conducted at the manufacturer's request, and the outcomes were primarily disseminated in Japanese journals.^[9] Studies describing the remineralization benefits of NHA-containing toothpaste are scarce, particularly in the Indian context.

Despite the advancements in dental care products, there is a significant gap in the literature regarding the comparative effectiveness of emerging fluoride-free alternatives such

as theobromine gel and NHA paste against conventional fluoride-containing toothpaste, specifically in the context of the Indian population. Most studies on NHA have been limited in scope or sample size, making it difficult to generalize their findings globally. In addition, while theobromine-based products have shown promise in enhancing enamel hardness, their acceptance and widespread use remain limited due to a lack of comprehensive, comparative studies.

Hence, the present *in vitro* study evaluated and compared the effect of theobromine gel and NHA paste to that of conventional sodium monofluorophosphate-containing toothpaste on demineralized enamel. The study aims to investigate these materials' remineralizing potential and the effect on the microhardness and surface roughness (SR) of demineralized enamel. By addressing this research gap, the study seeks to provide more precise insights into the efficacy of these fluoride-free alternatives, potentially guiding better dental care practices and product development.

MATERIAL AND METHODS

The present *in vitro* study was conducted in Bharati Vidyapeeth (to be deemed) Dental College and Hospital, Pune, and its laboratory analysis was done at Praj Metallurgical Laboratory, Pune. The study commenced around December 2020 and continued for a month until January 2021. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Institutional Ethical Review Board (BVDU/DCH/620-1/2020-2021).

Sample size calculation

The sample size for this study was calculated using GPower software to ensure sufficient power to detect statistically significant differences between the groups. The effect size was estimated using data from a previous study conducted by Yuanita *et al.* (2020).^[10] For a two-tailed test comparing the means of two independent groups, with an effect size DDD of 1.9624797, an α error probability of 0.05, a power ($1-\beta$ error probability) of 0.95, and an allocation ratio of 1, the required sample size per group was determined to be 10. This calculation yielded a non-centrality parameter δ of 4.3882380, a critical t -value of 2.1009220, and 18 degrees of freedom, resulting in an actual power of 0.9555191. Since the study involves three groups (theobromine gel, NHA paste, and conventional sodium monofluorophosphate-containing toothpaste), the total sample size was set to 30.

Sample preparation

A total of 30 structurally sound human maxillary or mandibular premolars extracted for orthodontic purposes were utilized for the present study. Teeth with incipient white

spot lesions, visible caries, restorations, hypoplasia, or cracks were excluded from the study. Teeth with stains or calculus buildup were also excluded from the study.

The samples were disinfected and stored in fresh, renewed, deionized water till the beginning of the experimental procedures. The teeth were decoronated by moving a diamond disc through the cervical region in a buccolingual direction [Figure 1a]. Copious cooling by a continuous jet of cold water was performed to avoid excessive heat generation that would cause damage to the tooth structure.

The teeth were embedded in self-cured acrylic resin poured into customized plastic molds [Figure 1b]. It was ensured that the buccal surface of the tooth was facing upwards which was then subjected to sequential flattening and polishing by 400, 800, 1000, and 1200 grit abrasive papers. All the remaining surfaces of the acrylic blocks were painted with two coats of acid-resistant nail varnish.

On the buccal surface, an adhesive tape of 5 mm × 5 mm dimensions was applied to the central areas while a uniform coat of nail varnish was applied to the remainder of the buccal surface to render it resistant to acid demineralization. After thoroughly drying the samples, the adhesive tape was removed to expose a window of enamel surface susceptible to demineralization by acids.

Outcome recording

The surface microhardness (SMH) of the samples was tested by a Microhardness Tester (Reichert Austria Make, Sr.No.363798, Load: 50 g, Reference Standard: ISO 6507); [Figure 1c]. The SR measurement was carried out by Stylus profilometer [Figure 1d], Mitutoyo, Japan. Model: SJ 210. Scanning electron microscopic (SEM) images were also obtained for the specimens.

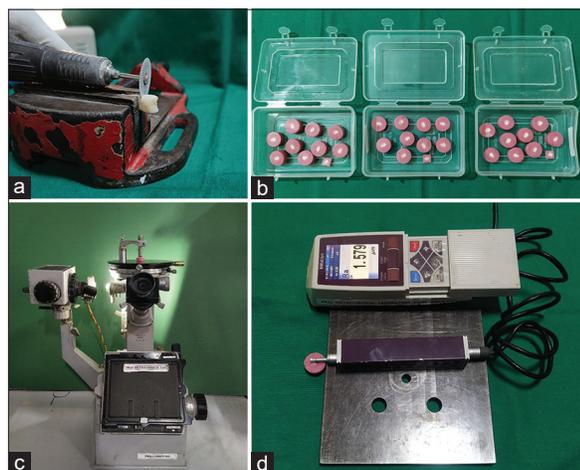


Figure 1: (a) De-coronation of teeth; (b) prepared samples embedded in resin; (c) microhardness tester; and (d) stylus profilometer.

Preparation of solutions

The solutions were prepared as described in an earlier study.^[11] The demineralizing solution was prepared with 50 mM acetic acid (pH 4.5), 2.2 mM potassium dihydrogen phosphate (KH_2PO_4), 2.2 mM calcium nitrate, and 0.1 ppm sodium fluoride. The remineralization solution used in pH-cycling contained 20 mMol l-1 HEPES(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 130 mM potassium chloride, 1.5 mM calcium chloride, 0.9 mM KH_2PO_4 , and 1 mM sodium azide. The pH was adjusted to 7.0 with potassium hydroxide.

Demineralization procedure

The samples were then immersed individually in 20 mL demineralizing solution (pH 4.4) for 96 h to develop incipient carious lesions in the enamel characterized by demineralization and SR. The SMH and SR values were recorded again along with SEM imaging, following which a 21-day pH cycling model was commenced to simulate environmental conditions of the oral cavity.

Remineralization-demineralization pH cycling model

The samples were equally divided into three groups comprising $n = 10$ samples each, which included Group A (Sodium fluoride paste), Group B (Theobromine gel), and Group C (NHA paste). The respective remineralizing agents were then applied to the samples with applicator tips. After two minutes, the samples were cleaned with deionized water and then immersed in 20 mL of demineralizing solution for three hours. After thorough washing with deionized water, the remineralizing agents were again applied for two minutes. The samples were then submerged in 20 mL of the remineralizing solutions (pH 7) for 17 h. The solutions were replaced every 48 hours, and these procedures were continued cyclically for 21 days. After remineralization, the final SMH and SR recording was done after the completion of the 21-day pH cycling model, along with SEM imaging of the samples.

Statistical analysis

The data were recorded in a Microsoft Excel Sheet and subjected to statistical analysis using IBM Statistical Package for the Social Sciences v23. An intra-group comparison of means of microhardness and SH was performed using a paired *t*-test. Inter-group comparison of the parameters was done by one-way Analysis of Variance (ANOVA) and *post hoc* Tukey test.

RESULTS

The microhardness [Table 1] and SR [Table 2] values of the samples at baseline, demineralization, and post-remineralization

Table 1: Intra-group change in microhardness.

Group	Interval	Mean	SD	Difference	P-value
Group A (Fluoride dentifrice)	Baseline	326.80	5.05	165.10	<0.001*
	Demineralization	161.70	7.92		
	Demineralization	161.70	7.92	-82.70	<0.001*
	Remineralization	244.40	6.80		
Group B (Theobromine gel)	Baseline	330.80	7.66	198.70	<0.001*
	Demineralization	132.10	5.38		
	Demineralization	132.10	5.38	-49.10	<0.001*
	Remineralization	181.20	6.49		
Group C (Nano-hydroxyapatite paste)	Baseline	332.00	4.97	183.10	<0.001*
	Demineralization	148.90	5.26		
	Demineralization	148.90	5.26	-70.00	<0.001*
	Remineralization	218.90	5.53		

Paired *t*-test; * indicates significant difference at $P \leq 0.05$. SD: Standard deviation

Table 2: Intra-group change in surface roughness.

Group	Interval	Mean	SD	Difference	P-value
Group A	Baseline	0.61	0.05	-0.81	<0.001*
	Demineralization	1.42	0.09		
	Demineralization	1.42	0.09	0.32	<0.001*
	Remineralization	1.10	0.04		
Group B	Baseline	0.65	0.04	-0.61	<0.001*
	Demineralization	1.26	0.09		
	Demineralization	1.26	0.09	0.36	<0.001*
	Remineralization	0.90	0.04		
Group C	Baseline	0.60	0.05	-0.56	<0.001*
	Demineralization	1.16	0.06		
	Demineralization	1.16	0.06	0.37	<0.001*
	Remineralization	0.79	0.06		

Paired *t*-test; * indicates significant difference at $P \leq 0.05$. SD: Standard deviation

time points are tabularized below. At baseline and after demineralization, there was no statistically significant difference ($P > 0.05$) in the microhardness and SR values of the three groups.

Change in microhardness

In all three groups, the microhardness value decreased significantly ($P < 0.001$) after demineralization and again increased significantly ($P < 0.001$) after remineralization [Figure 2]. However, after remineralization, the mean microhardness values among the three groups differed significantly from each other, being most significant for fluoride dentifrice ($244.4 + 6.8$) and lowest for the theobromine group ($181.2 + 6.49$).

The highest increase in microhardness percentage was seen in Group A, followed by Group C, and was lowest in Group B. Group B showed significantly less ($P < 0.05$) increase in

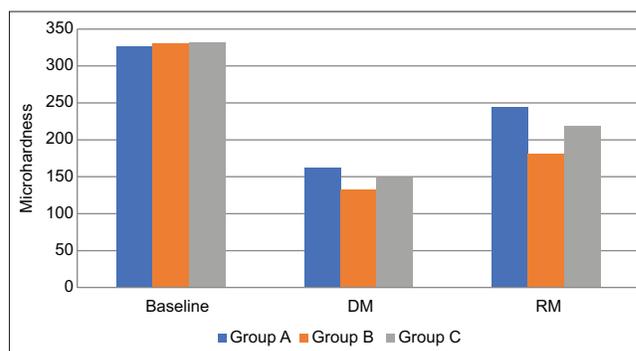


Figure 2: Intergroup comparison of microhardness of three groups (DM: Demineralization, RM: Remineralization).

microhardness as compared to Groups A and C. Pair-wise comparison by one-way ANOVA test and *post hoc* Tukey test [Tables 3 and 4] revealed that after remineralization, the microhardness value of Group A was significantly higher ($P < 0.05$) than other two groups, and the microhardness value of Group C was significantly higher ($P < 0.05$) than Group B.

Change in SR

In all three groups, the SR value increased significantly ($P < 0.001$) after demineralization and again decreased significantly ($P < 0.001$) after remineralization. The highest change was seen in Group C, followed by Group B, and the lowest in Group A. Group A showed significantly less change in SR score than Group B and Group C [Table 2]. After remineralization, the SR score of Group A was significantly higher than the other two groups, and the SR score of Group B was significantly higher than Group C.

The images obtained after SEM imaging at various time points are depicted in Figure 3.

Table 3: Intergroup comparison of surface roughness of three groups.

Interval	Group A versus Group B	Group A versus Group C	Group B versus Group C
Baseline	0.314	0.149	0.897
DM	<0.001*	<0.001*	<0.001*
RM	<0.001*	<0.001*	<0.001*
Group	Mean	SD	P-value
Group A	51.40	7.03	<0.001*
Group B	37.31	6.02	
Group C	47.23	7.48	
Pairwise comparison			
Gr A versus Gr B: <0.001*			
Group A versus Gr C: 0.376			
Gr B versus Gr C: 0.009*			

One-way ANOVA test; *post hoc* Tukey test; * indicates significant difference at $P \leq 0.05$. DM: Demineralization, RM: Remineralization, SD: Standard deviation

Table 4: Intergroup and pairwise comparison of surface roughness of three groups.

Interval	Group A		Group B		Group C		P-value
	Mean	SD	Mean	SD	Mean	SD	
Baseline	0.61	0.05	0.65	0.04	0.60	0.05	0.130
DM	1.42	0.09	1.26	0.09	1.16	0.06	<0.001*
RM	1.10	0.04	0.90	0.04	0.79	0.06	<0.001*
Group	Mean	SD	P-value		Pairwise comparison		
Group A	22.27	4.60	0.001*		Gr A vs. Gr B: 0.023*		
Group B	28.63	4.88					
Group C	31.44	5.54					
					Group A vs. Gr C: 0.001*		
					Gr B vs. Gr C: 0.423		

One-way ANOVA test; *post hoc* Tukey test; * indicates significant difference at $P \leq 0.05$. DM: Demineralization, RM: Remineralization, SD: Standard deviation

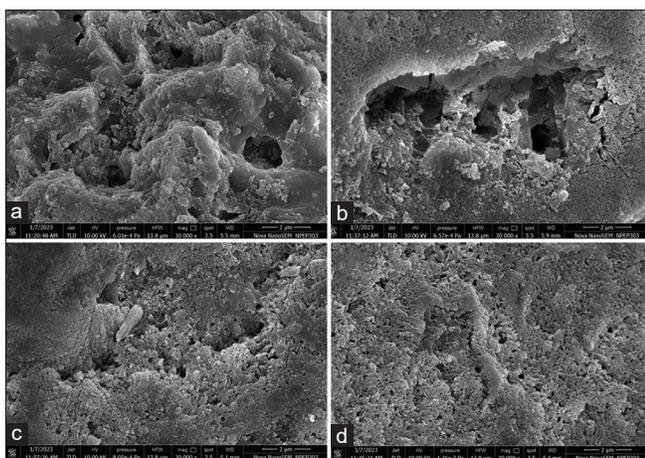


Figure 3: FE-SEM analysis at $\times 30,000$ – (a) demineralized surface, (b) remineralization with sodium fluoride, (c) theobromine gel, and (d) nano-hydroxyapatite paste. FE-SEM: Field emission scanning electron microscopy.

DISCUSSION

The present study investigated the effect of theobromine and NHA on the microhardness and SR of demineralized enamel as compared to sodium monofluorophosphate.

The choice of materials precluded the potential of theobromine for remineralization despite its unpopularity and NHA represents advancements in the technology of remineralization research. It was, therefore, necessary to simulate a clinical condition of demineralized enamel in the oral environment for which the precise above-mentioned methodology was employed. The pH cycling model used in the present study was adopted from the regimen described by Gocmen *et al.* based on the model introduced by Dunipace *et al.* for the evaluation of remineralization properties of enamel.^[12,13]

The microhardness was measured using Vicker’s microhardness test. The selection of this particular test was due to its versatility, accuracy, and reliability while requiring some effort for surface preparation of the sample. A similar selection was done in earlier studies that evaluated enamel remineralization of tooth samples.^[13,14]

The SR was measured using a stylus profilometer. It is a device used for analyzing topographical characteristics of the surface of materials but requires thorough contact and causes surface destruction.^[15] Since the present *in vitro* study was conducted on extracted teeth, this particular technique was suitable for measuring the SR. Likewise, earlier researchers

who used extracted teeth to study the enamel SR have successfully employed this technique.^[16,17]

At baseline and after demineralization, there was no difference in microhardness between the three groups, indicating that the groups were identical at the beginning of the study and after demineralization. In all three groups, the microhardness value decreased significantly, implying that the demineralization process was successfully performed. A significant increase in the microhardness after remineralization in all three groups indicated that all three agents are effectively achieved remineralization of the lost enamel structure. The highest increase in microhardness percentage was seen in the teeth treated with sodium monofluorophosphate followed by NHA and theobromine. In fact, theobromine achieved significantly less percentage increase in microhardness than the other two agents. Our findings corroborate those reported by Najibfard *et al.*, who stated that NHA and fluoride dentifrice achieved significant remineralization.^[18] A similar recent study reported that brushing with sodium monofluorophosphate provided the highest increase in microhardness among other dentifrices containing theobromine or caffeine.^[19] Premnath *et al.* found the remineralization potential of fluoride dentifrice to be higher than theobromine, similar to the the present research findings.^[20] The significant decrease in roughness achieved by the NHA group observed in Mielczarek *et al.*'s study is similar to our findings.^[21]

On the contrary, earlier studies compared theobromine to standard sodium fluoride dentifrices. The results showed that there was a greater increase in SMH value of enamel achieved by theobromine compared to the latter. In 2018 Suryana *et al.* also found that the enamel hardness resulting from the use of theobromine was significantly higher than that resulting from the use of NHA.^[22] Taneja *et al.*, on the other hand, did not find any significant difference in the remineralizing capacity of the three agents.^[7]

At baseline under SEM, the surface appeared to be almost smooth with very sparsely located pores, and after the demineralization, it appeared as a homogenous wide-scale pattern of honeycomb/micropores. Following remineralization in the case of the sodium monofluorophosphate, not all the pores were sedimented, especially when compared to theobromine and NHA, wherein the surface appeared smooth with filled pores. The better filling of the demineralized voids by NHA can be attributed to the nano-sized particles that clinically manifest as improved microhardness and a smoother surface.^[23] A similar correlation was found by Amaechi *et al.* wherein the authors reported that more of the tubules were occluded with the theobromine when compared to the sodium monofluorophosphate when observed under SEM.^[24]

While the present study offers plausible results, certain limitations of its design cannot be overlooked. The oral cavity harbors numerous biological and chemical entities

that cannot be entirely replicated *in vitro*.^[25,26] Since the teeth are bathed in varying quantities and combinations of salivary components, the pH cycling model used in the present study cannot accurately simulate the saliva and plaque fluids existing in the natural state.^[26,27] The demineralization process induced in the *in vitro* design is much faster than that which occurs in the oral cavity. Long-term *in vivo* studies with adequate follow-up can help overcome these limitations.

CONCLUSION

The present study demonstrates that sodium monofluorophosphate, theobromine, and NHA are all effective remineralizing agents for demineralized enamel. The results indicated that while all three agents significantly increased the microhardness of demineralized enamel, sodium monofluorophosphate achieved the greatest increase in microhardness, followed by NHA, with theobromine showing the slightest increase. Specifically, the mean microhardness values post-remineralization were highest for sodium monofluorophosphate, intermediate for NHA, and lowest for theobromine.

All three agents significantly decreased the SR after remineralization. NHA showed the most significant improvement in SR, followed by theobromine, with sodium monofluorophosphate showing the least improvement. The SR scores post-remineralization were highest for sodium monofluorophosphate, intermediate for theobromine, and lowest for NHA. The SEM images further supported these findings, showing that NHA and theobromine more effectively filled the demineralized pores compared to sodium monofluorophosphate, resulting in smoother surfaces.

These results suggest that while sodium monofluorophosphate is highly effective in enhancing microhardness, NHA excels in improving microhardness and reducing SR. Therefore, NHA may offer a superior balance of benefits in remineralizing demineralized enamel. Future *in vivo* studies with long-term follow-up are recommended to confirm these findings and better simulate the natural oral environment.

Ethical approval: The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Institutional Ethical Review Board (BVDU/DCH/620- 1/2020-2021).

Declaration of patient consent: Patient's consent was not required as there are no patients in this study.

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Conflicts of interest: There are no conflicts of interest.

Use of artificial intelligence (AI)-assisted technology for manuscript preparation: The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

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