Preparation of a toothpaste containing theobromine and fluoridated bioactive glass and its effect on surface micro-hardness and roughness of enamel

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The aim was to synthesize a toothpaste and analyze its effect on surface micro-hardness and roughness of enamel. Basic paste was prepared by using basic ingredients. Theobromine (0.2 wt%) and laboratory synthesized fluoridated-bioactive glass (F-BG, 4 wt%) were added to it. Post-demineralization, 36 enamel blocks were divided into six groups that were brushed with their respective toothpaste+artificial saliva (AS): group 1 (control): basic paste; group 2: basic paste+theobromine; group 3: commercial theobromine toothpaste; group 4: commercial BG toothpaste; group 5: basic paste+F-BG; and group 6: basic paste+theobromine+F-BG. On micro-hardness analysis, group 6 performed best, followed by group 4. Surface roughness results showed the maximum decrease in roughness values for group 6, followed by group 5. Treatment with toothpaste composition containing theobromine+F-BG resulted in the enamel's increased micro-hardness and decreased surface roughness.

Keywords: Biomaterial, Dental enamel, Fluoride, Theobromine, Toothpastes

INTRODUCTION

The oral cavity routinely goes through cycles of demineralization and remineralization¹. White spot lesions on the enamel surface caused by loss of minerals during the carious process are reversible². The recommended method to prevent and reverse initial lesions is to use toothpastes, mouthwashes, and gels with appropriate active ingredients³.

Toothpastes are everyday oral products that contain an active ingredient along with other basic components⁴. Various active ingredients that have a role in promoting enamel remineralization are added to toothpastes, the most common among these being fluoride and bioactive glasses (BG)⁴. The role of fluoride in reducing dental caries is well documented in the literature⁵. Fluoride ions help to reduce crystal volume by replacing hydroxyl in the apatite structure, resulting in overall stability and structural strength⁶. This replacement changes the hydroxyapatite (HA) into fluorapatite (FA), which is more resistant to dental caries and acid attacks⁷. BG was first introduced by Larry Hench in 1969 for bone osseointegration, and it contains calcium, sodium, and phosphosilicate⁸. The tremendous bone-bonding ability of BG could be attributed to HA layer formation on bone's surface when BG dissolves in an aqueous solution⁹.

The BG is now routinely used in oral products like toothpastes, as it has similar composition to dental enamel⁴. The traditional BG composition (called Bioglass® or 45S5) lacks fluoride⁹, so some researchers have added fluoride to it and demonstrated superior remineralization competence to other toothpastes¹⁰.

Theobromine, an alkaloid, is readily available in chocolate products and cocoa¹¹. It is reported that its presence in an apatite-forming medium could enhance the medium's potential to reverse demineralization¹². Theobromine-containing toothpastes like Theodent® (New Orleans, LA, USA) are available in the market; however, they do not contain any fluoride¹².

The authors could not find any study where theobromine has been incorporated with BG in a toothpaste. The combination of theobromine with a fluoridated BG (F-BG) to develop a toothpaste would be interesting, as all three of these ingredients have proven remineralizing capabilities. Therefore, the aim of this study was to develop a novel toothpaste containing theobromine with F-BG and to investigate its effect on physical and mechanical properties of human enamel. The null hypothesis (H_0) was set to be that all the toothpastes in this study (commercial and experimental) would result in similar increase in enamel's micro-hardness and decrease in its surface roughness post-treatment.

MATERIALS AND METHODS

Ethical approval (Ref: EA 2019041) was obtained from the scientific research unit of the college, and all ethical protocols were strictly followed. The study was conducted in two stages, where the first stage involved preparation of a basic paste and then addition of various active ingredients to it to attain a novel toothpaste. The second stage involved its testing against various commercial brands of toothpaste on extracted teeth to determine its effect on physical and mechanical properties of enamel. The group containing basic paste only was used

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as a positive control group, whereas Theodent® and Sensodyne® (GSK, UK) were used as negative control groups for theobromine and BG, respectively.

Preparation of basic paste

All the chemicals and precursors were of analytical grade and were commercially purchased from Sigma-Aldrich, USA. In this study, initially, a basic paste was prepared by using basic ingredients. In 100 mL of distilled water, 1.3 g of calcium carbonate was mixed inside a magnetic stirrer at room temperature. The mixture was left in stirring mode for 2 h. Glycerol (16 mL) was then added to the mixture, which was stirred for 1 h. After complete mixing, 0.5 g of sodium benzoate was added, and the mixture was stirred for 40 min. Then, 0.5 g of methylcellulose was added, and the mixture was again stirred for 40 min. After the addition of all basic ingredients, a flavouring agent (0.5 mL of peppermint oil) was added, and the components were mixed overnight for complete mixing of powders in the paste.

Preparation of active ingredients

Two active ingredients were added to the basic paste: theobromine and F-BG nanoparticles. Theobromine (AKSci®, CA, USA) was commercially acquired, whereas F-BG was synthesized by using calcium nitrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$) and diammonium hydrogen phosphate ($(\text{NH}_4)_2\text{HPO}_4$) as sources of calcium and phosphate precursors, respectively. Initially, 11.7 mmol of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and 23.1 mmol of triethyl orthosilicate (TEOS) were dispersed in 25 mL ethanol solution. During this reaction, the pH was maintained at 1–2 by adding nitric acid. Then, 2.47 mmol of sodium fluoride (NaF) was added to the reaction and allowed to mix. Later, this solution was added slowly to 2.86 mmol diammonium hydrogen phosphate ($(\text{NH}_4)_2\text{HPO}_4$) solution. The pH of solution was kept at 11 during the reaction using ammonia. After stirring for 48 h, the solution was filtered, washed to neutralize the pH, and dried at 70°C. The obtained sample was heat treated at 500°C for 6 h at the heating rate of 2°C.min⁻¹.

Addition of active ingredients to basic paste

Theobromine (0.2 wt%) and freshly synthesized F-BG (4 wt%) were added separately to the basic paste in increments, and the mixture was stirred overnight

in a magnetic stirrer at room temperature. For the experimental toothpaste, 0.2 wt% theobromine was added to the basic paste and allowed to stir for 24 h to ensure uniform mixing. Then, F-BG (4 wt%) was added in increments and stirred overnight for another 24 h.

Preparation of enamel blocks

Thirty-six freshly extracted teeth were collected from the Oral Surgery department of the institute. Teeth which were free from white spot lesions, caries, restorations, and other evident defects were selected for this study. The anatomical crowns of the teeth were obtained by cutting the teeth over the cemento-enamel junction utilizing a water-cooled diamond saw (Isomet® 5000 Linear Precision Saw, Buehler, IL, USA). After discarding the roots, the crowns were embedded in self-cure acrylic resin inside a pre-fabricated square mold to facilitate standardization. The crowns were placed in such a manner that their buccal/labial enamel surfaces were exposed, whereas the lingual surface was in contact with the self-cure resin. On the exposed enamel surface, a window of approximately 4×4 mm was created with a nail varnish to ensure that all brushing experiments and analysis were done only in that particular area. The exposed enamel surfaces were polished with 600-grit wet silicon-carbide paper for 1 min. Then, the enamel samples were subjected to citric acid challenge to induce demineralization. The enamel samples were immersed in 6 wt% citric acid (pH=2.0) for 5 min inside a glass beaker, which was placed on top of a rotating orbital shaker (CO-Z® Orbital Shaker, USA) to mimic *in-vivo* conditions. After the acidic challenge, the enamel samples were washed with distilled water for 1 min and then left to air-dry.

These enamel blocks were randomly divided into six groups, with each group receiving six specimens that received brushing with 1.0 g of their respective toothpaste mixed with AS (in a 1:2 ratio) as shown in Table 1.

Artificial saliva (AS) preparation

The AS was prepared by mixing 0.4 g NaCl, 0.4 g KCl, 0.6 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.79 g $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, and 0.005 g $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ in 1,000 mL of deionized water, as suggested earlier by Fusayama *et al.*¹³. The pH of AS was 5.5, which was adjusted to a neutral pH of 7 by utilizing NaOH.

Table1 Showing brushing treatment of enamel blocks by their respective toothpastes for various groups in this study

Groups	Brushing treatment of enamel blocks by their respective toothpastes
Group 1	Enamel blocks brushed with basic paste
Group 2	Enamel blocks brushed with basic paste+0.2 wt% theobromine toothpaste
Group 3	Enamel blocks brushed with commercial theobromine toothpaste
Group 4	Enamel blocks brushed with commercial BG toothpaste
Group 5	Enamel blocks brushed with basic paste+4 wt% F-BG toothpaste
Group 6	Enamel blocks brushed with basic paste+0.2 wt% theobromine+4 wt% F-BG toothpaste

Tooth brushing protocol

Manual toothbrushes belonging to one commercial brand (Trisa®, Switzerland) were used. A toothbrushing simulator (Toothbrush Simulator; model ZM-3.8, Germany) was used to replicate toothbrushing, giving each sample 5,000 linear strokes, which is equivalent to 6 months of *in vivo* brushing¹⁾. The applied load was 250 g, and the linear distance covered by toothbrushes was set to 50 mm. After every 500 strokes, 1.0 g of respective toothpaste mixed with AS in a 1:2 ratio was added to the sample. After the brushing strokes, the samples were washed with distilled water for 1 min and were left to air-dry before further analysis.

Surface micro-hardness analysis

The Vickers hardness data of the enamel surface were collected at three time intervals: pre-treatment, post-demineralization, and post-treatment. The specimens from each group were evaluated for changes in surface micro-hardness values. The Vickers hardness was measured using a digital micro-hardness tester (FM-ARS 9000; Future-Tech, Kawasaki, Japan). Three indents were made on the treated surface of each specimen using a Vickers diamond indenter under a 100 g load applied for 10 s.

Surface roughness measurement

Surface roughness was measured three times with a Contour GT Optical Microscopes profilometer (Bruker, Tucson, AZ, USA) at three intervals (pre-treatment, post-demineralization, and post-treatment), just like in the surface micro-hardness analysis. Each specimen was placed in a marked fixed position so that the scan was performed in the exposed enamel window area only. The mean surface roughness values (Ra) (which are the mean of the height of peaks and the depth of valleys from the mean line along the measuring length) were then calculated for all the samples.

Statistical analysis

The results were analyzed using Statistical Package for the Social Sciences (SPSS) software (version 19.0; SPSS, Chicago, IL, USA). The Wilcoxon sign-rank test was applied to evaluate significant differences within each group comparing pre-treatment *vs* post-demineralization values and pre-treatment *vs* post-treatment values. A p -value ≤ 0.05 was considered statistically significant.

RESULTS

Surface micro-hardness analysis

The Vickers hardness number (VHN) was obtained by subtracting the post-treatment values from post-demineralization values. The results indicate that group 6 performed best, followed by group 4, group 2, and group 5 (Table 2). Groups 1 and 3 showed a further decrease in micro-hardness of enamel samples (Table 2). All intra-group comparisons were statistically significant when pre-treatment *vs* post-demineralization values were paralleled, whereas post-demineralization *vs* post-treatment values were significant for groups 1–3 and 6.

Surface roughness analysis

The enamel surface profile was revealed by profilometry for groups 1–3, as shown in Fig. 1, whereas for groups 4–6, it is shown in Fig. 2. These images demonstrate enamel surface profile post-polishing (a), post-demineralization (b), and post-treatment (c). The surface roughness results (values in μm) show the maximum decrease in roughness values for group 6, followed by group 5 and group 1 (Table 3). For all the other groups, roughness values were increased post-brushing, as tabulated in Table 3. Intra-group comparisons were significant for groups 2–6 when pre-treatment *vs* post-demineralization values were compared, whereas they were significant for groups 2–5 when post-demineralization values were matched against post-treatment values.

Table 2 Showing mean VHN values (SD) for all groups pre-treatment, post-demineralization, post-treatment

	Group statistics mean VHN (SD)					
	Group-1	Group-2	Group-3	Group-4	Group-5	Group-6
Pre-treatment	520 (83)	558 (52)	546 (49)	577 (123)	540 (75)	554 (29)
Post-demineralization	406 (73)	330 (38)	398 (75)	480 (131)	473 (35)	399 (34)
Post-treatment	404 (65)	357 (53)	359 (62)	522 (90)	492 (42)	482 (52)
$\wedge p$ -value	0.028	0.028	0.028	0.028	0.028	0.028
$\wedge\wedge p$ -value	0.046	0.028	0.028	0.345	0.600	0.028
Difference (post-treatment–post demineralization)	–2	27	–39	42	19	83

$\wedge p$ -value: Within group significance pre-treatment *versus* post-demineralization

$\wedge\wedge p$ -value: Within group significance pre-treatment *versus* post-treatment

A “–ve” value demonstrates reduction in micro-hardness

Fig. 1 Three dimensional (3D) profilometric images belonging to groups 1–3.
a) Pre-treatment, b) Post-demineralization, and c) Post-treatment and showing Ra values

Fig. 2 3D profilometric images belonging to groups 4–6.
a) Pre-treatment, b) Post-demineralization, and c) Post-treatment and showing Ra values

Table 3 Showing mean Ra values for all groups pre-treatment, post-demineralization, post-treatment

	Group statistics mean Ra (μm) (SD)					
	Group-1	Group-2	Group-3	Group-4	Group-5	Group-6
Pre-treatment	0.86 (0.86)	0.44 (0.20)	0.52 (0.25)	0.39 (0.08)	0.59 (0.18)	0.62 (0.17)
Post-demineralization	0.93 (0.41)	1.09 (0.33)	1.51(0.92)	1.54 (0.91)	1.65 (1)	1.96 (1.66)
Post-treatment	0.74 (0.29)	1.15 (0.63)	1.63 (0.78)	2.11 (2.65)	1.12 (0.42)	0.55 (0.08)
$^{\wedge}p$ -value	0.345	0.028	0.028	0.028	0.028	0.028
$^{\wedge\wedge}p$ -value	0.345	0.028	0.028	0.028	0.046	0.463
Difference (post treatment–post demineralization)	–0.19	0.06	0.12	0.53	–0.53	–1.41

$^{\wedge}p$ -value: Within group significance pre-treatment versus post-demineralization.

$^{\wedge\wedge}p$ -value: Within group significance pre-treatment versus post-treatment.

A “-ve” value demonstrates reduction in surface roughness.

DISCUSSION

Based on the results of this study, H_0 was rejected, as the results indicate superior performance of the experimental toothpaste with theobromine and F-BG as compared with others in terms of increasing micro-hardness and decreasing surface roughness of demineralized human enamel.

The purpose of this study was to prepare a toothpaste containing theobromine with F-BG and then evaluate its competence to increase microhardness and decrease surface roughness following demineralization. In this study, 0.2 wt% and 4 wt% of theobromine and F-BG were used in the experimental toothpaste respectively. These percentages were obtained from previously conducted studies that showed positive outcome of these materials on enamel and dentin tissues, without any toxic effect^{1,14}. Clinically, the appearance of early enamel carious lesion is white, which occurs as a result of loss of normal translucency of the enamel¹⁵. As a consequence of caries initiation, the enamel surface has lower mineral content in comparison to a sound enamel surface; therefore, the affected layer possesses a lower hardness value when compared with a sound enamel surface¹⁶. Hence, clinically, the surface of the enamel becomes fragile as a result of carious lesion and is quite vulnerable to breakage on probing.

Considering the significance of the surface enamel layer in relation to caries development, the assessment of this layer is of utmost importance. Micro-hardness measurement is a tool that provides a simple, fast, and non-invasive way to evaluate the hardness of a surface that is prone to cracks and fractures, such as enamel¹⁷. The micro-hardness values analyzed by calculating VHN recorded that pre-treatment values in this study were in the range of 520–577, which was slightly higher than the microhardness value reported by other studies^{18,19}. This increased pre-treatment VHN value could be a result of teeth being used which might have been exposed to fluoridated toothpastes prior to extraction, as opposed to freshly erupted teeth. It should also be noted that high standard deviation values were observed on micro-hardness analysis. The probable reason for this could be non-homogeneity of material surface (enamel), as the teeth were collected from different patients in oral surgery department. Following the acidic challenge, the micro-hardness values of the enamel specimens from each group decreased, and this is in agreement with previously reported studies^{20,21}. The present study showed that the highest micro-hardness value was achieved by the experimental toothpaste containing theobromine with F-BG, although samples went through a demineralization process first, which is a fairly vigorous challenge. This could be the result of the presence of three remineralizing agents together in this experimental toothpaste, namely theobromine, fluoride, and BG. Theobromine can be inserted in the microtunnel that is found within hydroxyapatite crystals of enamel, thus causing an internal stress that requires a greater external stress to cause deformation²². This in

turn increases the strength and hardness of the enamel surface. Fluoride and BG have proven their ability to remineralize the enamel structure in various previously carried out studies^{16,20}. Although the presence of these agents alone in other groups caused an increase in micro-hardness, it was still less than in the experimental toothpaste group, accentuating that the presence of these agents together could lead to an improved and harder enamel surface that is resistant to acidic challenges.

Apart from surface micro-hardness, non-contact profilometry or surface roughness was also used in this study, as researchers believe that it is a good tool to assess dental hard tissue's surface loss or gain. In the present study, the maximum decrease in roughness was observed with the experimental toothpaste containing theobromine and F-BG. There is a scarcity of studies in the literature that have evaluated the effect of theobromine in reducing enamel's surface roughness, and to the best of the authors' knowledge, this is the first study to evaluate this. In the current study, neither a commercially available toothpaste containing theobromine nor its combination with basic paste reduced the roughness of the demineralized enamel surface. However, its combination with F-BG decreased surface roughness significantly, even more than the combination of BG with basic paste. The mechanism by which BG reduces surface roughness is well understood and exceedingly reported in the scientific literature^{23,24}, however, theobromine's exact mechanism of reducing enamel's roughness has not been reported. The only information available regarding it is in a previous study that stated that addition of theobromine could increase the enamel crystal size, subsequently inhibiting surface dissolution of ions¹⁴, thus making the surface less rough, as was observed in the present study.

This study had a few limitations, the first of which was its *in-vitro* nature. Although every attempt was made to ensure standardization of experiments, the results of this study should still be interpreted cautiously, as *in-vivo* conditions are dynamic and could be different from an *in-vitro* setting. Another limitation was that the hardness level and roughness values could be diverse in different enamel surfaces; still, an attempt was made to perform pre-treatment, post-demineralization, and post-treatment scans in the designated marked area on the enamel's surface. Future quantitative and clinical studies should be performed to test the efficacy of our experimental toothpaste under more vibrant *in-vivo* conditions.

CONCLUSION

Within the limitations of this study, it is concluded that toothpaste containing 0.2 wt% theobromine+4 wt% F-BG exhibited better results than the other groups in terms of increasing micro-hardness of enamel and decreasing its roughness. Therefore, toothpaste containing 0.2 wt% theobromine+4 wt% F-BG can be suggested as regular use toothpaste, especially for patients at high risk of caries and erosion.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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