EGYPTIAN DENTAL JOURNAL

VOL. 67, 119:126, JANUARY, 2021

PRINT ISSN 0070-9484 • ONLINE ISSN 2090-2360

ORTHODONTICS, PEDIATRIC AND PREVENTIVE DENTISTRY

www.eda-egypt.org • Codex : 129/21.01 • DOI : 10.21608/edj.2020.52781.1402

EVALUATION OF THE REMINERALIZING EFFECT OF THEOBROMINE AND FLUORIDE USING SCANNING ELECTRON MICROSCOPE

Rania Mostafa Shawky*, Nagwa Mohammed Khattab** *and* Mina Kamal Yassa***

ABSTRACT

Background: Theobromine is a natural alkaloid of plant origin; theobroma cacao. Attempts have been made to use it as a remineralizing agent.

Aim of the study: Assessment of the remineralizing potential of theobromine containing toothpastes compared to those with fluoride using scanning electron microscope.

Methods: 15 maxillary first bicuspids were selected. Crowns were sectioned and immersed in a demineralizing solution for 4 days. 30 specimens were divided into 3 groups (10 specimens each); group 1, specimens brushed with toothpastes containing theobromine, group 2, specimens brushed with toothpastes containing fluoride and group 3 for specimens brushed with toothpaste free of fluoride or theobromine. The brushing time was 9 minutes and 20 seconds each. Remineralization was carried out using a pH cycling model for 3 days using artificial saliva. The enamel was scanned before and after demineralization and after remineralization.

Results: A statistically significant difference was found in calcium and phosphate levels between group 1and 3 and also between group 2 and 3, however no statistical significant difference between group 1and 2.

Conclusion: Theobromine could be suitable alternative to fluoride for caries prevention in terms of safety and efficiency.

KEY WORDS: Theobromine, Fluoride, Remineralization, SEM

INTRODUCTION

Dental caries continues to be one of the most prevalent oral diseases in both children and adults. Only early demineralized lesions can be arrested and repaired. However, it often progresses until the tooth is cavitated and damaged [1,2].

In demineralization, minerals are removed from tooth enamel. Formation of bacterial acids lowers

* Dentist at Ministry of Health, B.D.S, Faculty of Dentistry, Minia University
** Professor in Pediatric Dentistry and Dental Public Health Department, Faculty of Dentistry, Ain Shams University.
*** Lecturer in Pediatric and Community Dentistry Department, Faculty of Dentistry, Minia University.

the pH to a level that dissolves hydroxyapatite resulting in future cavitation. Critical pH at which demineralization begins is an average of 5.5. On the other hand, in remineralization, mineral salts recrystallize early demineralized tooth enamel repairing early carious lesions [3].

Research on fluoride started in 1940s and since then the interaction between fluoride and dental hard tissue has fascinated the dentists around the globe. However, a rising concern is the fluoride toxicity in children due to ingestion of fluoride toothpaste or mouth rinse [4]. Moreover, recent studies associated decreased IQ scores with increased fluoride exposure in infancy [5, 6].

Theobromine is a natural alkaloid of plant origin; theobroma cacao. It differs from caffeine by only one methyl group. Recently, attempts have been performed to use theobromine as a remineralizing agent and as a potential substitute to fluoride.

Theobromine, a safer alternative to fluoride, forms enlarged hydroxyapatite crystallites in the calcium and phosphate rich medium. This reinforces enamel and makes it less vulnerable to acid attacks. Further, theobromine negatively interacts with streptococci mutans [7].

Hence, this study was conducted to assess the remineralizing potential of theobromine and fluoride containing toothpastes using scanning electron microscope (SEM) & Energy Dispersive X-Ray Analyzer (EDX).

MATERIALS AND METHODS

In the current in vitro study, 30 specimens were allocated in 3 parallel groups (10 specimens each). Calculation of the sample size was performed by G*Power software version 3.1.9.4. Where, the effect size was 0.40, and power (1-β) of 80% at a 5% significance level.

Teeth collection

Fifteen freshly extracted, for orthodontic purpose, human premolars were collected from orthodontic clinic of Minia University Dental Hospital. Selected teeth were free of caries, enamel malformations, cracks erosions or abrasions on buccal or lingual/palatal surfaces.

Teeth preparation

Teeth were cleaned and stored in thymol for maximum 2 months before use. A carborundum disk under coolant was used to section the crowns 2mm apical to the cemento-enamel junction. Then teeth crowns were mounted in metal rings using polymethylmethacrylate and each crown was sectioned mesiodistally into buccal and palatal sections.

Surface of each specimen was polished and covered with adhesive tape measuring 4x4 mm, the rest of the surface was covered with nail polish to standardize the area of treatment and evaluation.

Artificial caries was induced by dipping each specimen solely in a demineralizing solution for 4 days at 37°C. Demineralizing solution has the following composition 2.2mM Calcium chloride, 2.2mM Sodium dihydrogen phosphate, and 0.05M acetic acid; the pH was adjusted with 1M potassium hydroxide to a pH= 4.5 [8].

According to the toothpaste used for brushing, specimens were divided into 3 groups (10 specimens each): G1 (study group); theobromine containing tooth paste (Theodent-classic® toothpaste). G2 (positive control); fluoride containing tooth paste (Sensodyne f®) and G3 (negative control); tooth paste containing neither fluoride nor theobromine (Sensodyne Original®).

Each specimen was brushed using an Oral B electric toothbrushes with soft-rounded bristles for nine minutes and twenty seconds at a load of 200 gram. Tooth brushing was executed on 3 days using a slurry with a ratio of 1:3 toothpaste

to artificial saliva following pH cycling model described by **Amaechi (2019)** [9].

At end, specimens were rinsed with distilled water. All specimens were examined under SEM before demineralization, post demineralization, and post treatment with each toothpaste.

Statistical analysis:

One-way ANOVA succeeded by Tukey post-hoc test was employed to compare between more than two groups in non- correlated specimens. Repetitive measure ANOVA was utilized for comparison between more than two groups in correlated specimens. Paired sample t-test was used to compare between two groups in interrelated samples. The significance level was set at $p<0.05$. Statistical analysis was carried out with IBM® SPSS® Statistics Version 20 for Windows.

RESULTS

Scanning of enamel surface before demineralization showed normal pattern of enamel topography while after demineralization enamel surface become rough with increased width of pores. After remineralization by theobromine, enamel surface became smoother and nearly normal.

No statistically significant difference was found between the three groups before demineralization where ($p=0.567$) and after demineralization where ($p=0.321$). However, after remineralization the difference was statistically significant between them ($p<0.001$).

Moreover, there was a statistically significant difference between calcium levels before-demineralization, after-demineralization and after-remineralization in each tested group ($p<0.001$) (**Tables 1&2**).

Theobromine and fluoride showed a statistically significant difference when compared with the negative control group. Theobromine reported higher remineralization potential than fluoride, however, difference wasn't statistically significant ($p=0.558$).

Percentage of change from the baseline of calcium and phosphate described in table **(2)** was high after-demineralization due to mineral loss and lower after-remineralization due to mineral gain that did not reach the baseline values for each group.

The same findings were found for phosphates and summarized in tables **(3&4)**.

TABLE (1): Mean and standard deviation (SD) values of Calcium for the groups

Variables	Baseline				Post-demineralization				After remineralization				p-value
	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	
Group I	56.97	2.20	53.56	60.33	27.85	2.53	24.67	32.63	53.03	1.96	50.99	55.82	**<0.001***
Group II	57.87	2.04	54.98	61.33	29.00	2.73	24.67	33.66	51.99	2.02	49.98	54.99	**<0.001***
Group III	57.07	1.90	54.54	60.76	27.22	2.60	23.39	32.00	29.47	2.64	26.88	34.60	**<0.001***
p-value	**0.567ns**				**0.321ns**				**<0.001***				

significant (p<0.05)

TABLE (2): Mean and standard deviation (SD) values for percentage of change in calcium level in the groups

Groups	Post-demineralization				After remineralization				p-value
	Mean	SD	Min	Max	Mean	SD	Min	Max	
Group I	51.19	2.75	45.91	53.94	6.92	1.24	4.80	9.22	**<0.001***
Group II	49.93	3.67	44.07	55.13	10.15	2.21	6.92	14.76	**<0.001***
Group III	52.37	3.14	47.33	57.11	48.44	2.99	43.05	51.73	**<0.001***
p-value	**0.254ns**				**<0.001***				

Significant (p<0.05)

TABLE (3): Mean and standard deviation (SD) values of phosphate of the groups.

Variables	Baseline				Post-demineralization				After remineralization				p-value
	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	
Group I	15.18	1.60	12.85	16.98	11.62	0.99	10.01	12.68	14.84	1.55	12.56	16.88	**<0.001***
Group II	15.20	1.83	12.01	16.99	11.62	0.99	10.01	12.68	14.62	1.46	12.76	16.74	**<0.001***
Group III	15.59	1.53	12.95	17.44	11.62	0.99	10.01	12.68	13.44	1.42	11.70	16.47	**<0.001***
p-value	**0.826ns**				**0.998ns**				**0.092ns**				

**** Significant (p<0.05)***

TABLE (4): Mean and standard deviation (SD) values for percentage of change in phosphate level in the groups

Groups	Post-demineralization				After remineralization				p-value
	Mean	SD	Min	Max	Mean	SD	Min	Max	
Group I	23.25	3.16	17.51	27.47	2.20	0.92	0.59	3.34	**<0.001***
Group II	23.20	3.95	16.65	28.17	3.52	4.18	6.24	9.15	**<0.001***
Group III	25.27	3.79	18.15	33.40	13.68	5.39	5.56	20.97	**<0.001***
p-value	**0.362ns**				**<0.001***				

**** Significant (p<0.05)***

Results of EDX analysis

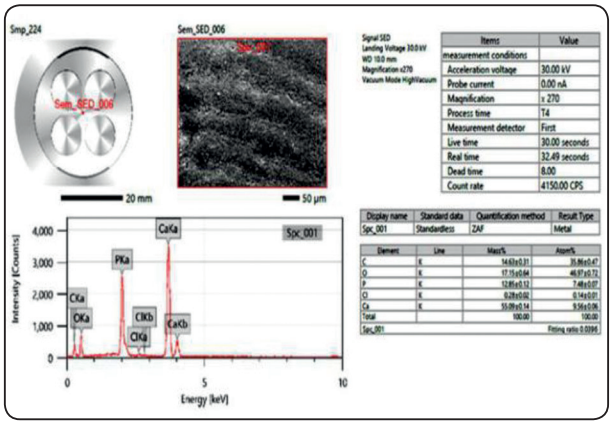

Fig. (1): Baseline specimen

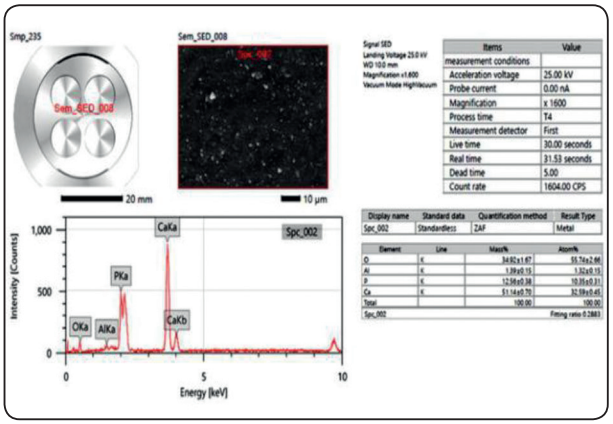

Fig. (2): Group 1 (Theobromine)

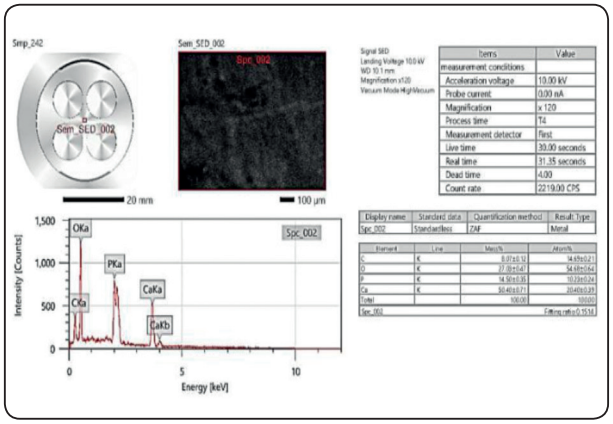

Fig. (3): group 2 (Fluoride)

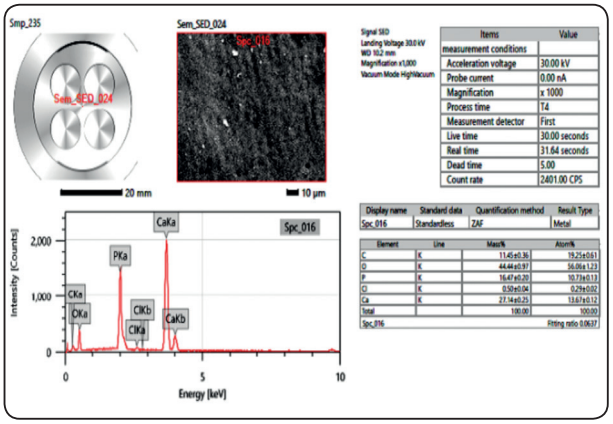

Fig. (4): Group 3 (control group)

DISCUSSION

Fluoride was the gold standard for prevention of dental caries for decades However; ingestion of excess fluoride can cause acute toxicity or chronic toxicity in form of dental and skeletal fluorosis.

It is not surprising that children below 3 years swallow 30 to 75% of the toothpaste on their brushes since it is not easy to teach young children to spit out toothpaste, especially if it is flavored [10]. Moreover, it has been reported that a relationship between fluoride ingestion and lower IQ scores for children [5,6]. Therefore, research on effective alternative remineralizing agent, such as theobromine was performed.

Theobromine is a natural alkaloid derived from the theobroma cocao. It is believed that theobromine molecule attracts calcium and phosphate ions, so that the deposition of calcium and phosphate occurs to form a new hydroxyapatite crystal called Theobromine apatite [7].

Toothpastes used in this study were Theodent-classic, which is correspondent to theobromine

solution at a concentration of 500 mg/L, Sensodyne f toothpaste containing 1450 ppm of Fluoride, and Senodyne original toothpaste that does not contain either fluoride or theobromine as a control group.

The study was designed as in vitro pH-cycling model as it allows for precise scientific control with minimal variability[11]. SEM was selected for assessment since it is one of the most sensitive in vitro methods to evaluate the demineralization and remineralization processes [12]. It gives detailed high resolution images of the specimens. Furthermore, EDX analysis was performed to afford elemental recognition and quantitative information about the composition.

Brushing duration used was the outcome of brushing two times daily for a month on one tooth surface; that was anticipated to get ten seconds of brushing in a cycle [13].

Based on the technical specification for wear test of tooth brushing [14], the brushing load utilized was 200 grams in order to mimic the in vivo tooth brushing.

The specimens were immersed in the demineralization solution for 4 days at 37°C creating a subsurface demineralization with an intact surface simulating an early enamel lesion. This demineralizing procedure was intended to produce a white spot lesion [15].

pH-cycling models involves a sequential exposure of specimens to combinations of demineralization and remineralization. These models simulate the in vivo caries dynamics of mineral loss and gain [16].

The results of EDX analysis of the present study indicated that theobromine was able to increase the minerals level more than fluoride but the difference was not statistically significant. Parallel results were reported by **Amaechi et al. (2013)** [17] and **Nakamoto et al. (2016)** [10].

SEM images showed that theobromine also gave smooth surface of the remineralized enamel which is close to the normal surface appearance before demineralization. The same finding was reported by **Taneja et al. (2019)** [18].

Irawan et al. (2017) [19]**; Sulistianingsih et al. (2017)** [20] and **Suryana et al. (2018)** [21] reported an increase in enamel micro hardness of enamel after application of theobromine gel. These findings are in consistent with our results where we found a significant rise in Calcium phosphate ratio after application.

However, results of the current study contradict those reported by **Thorn et al. (2020)** who evaluated the remineralizing ability of different concentrations of fluoride and theobromine at acidic and neutral conditions. Results revealed that various concentrations of theobromine existing in a plaque fluid-like medium did not enhance remineralization. The disagreement may be attributed to difference in of the demineralizing and remineralization agents. In addition to the, use of continuous exposure instead of pH cycling model used in the present study [22].

Lippert et al. (2017) compared the remineralization potential of fluoride, theobromine and their combinations on demineralized carious lesions. They concluded that theobromine did not provide remineralization under the selected conditions. These results are in disagreement with the current study showing that remineralization occurred in both theobromine and fluoride groups.

This disagreement may be as a result of using lower concentration of theobromine (200 ppm) solution prepared using deionized water while in the current study, the concentration of theobromine was higher (500 ppm) and it was delivered in toothpaste form, which contains calcium and phosphate that was mixed with artificial saliva before brushing[23].

CONCLUSIONS

1. Theobromine has comparable remineralization potential as fluoride.
2. Theobromine can improve the remineralization potential of medium rich in calcium and phosphate.
3. Theobromine concentration expressed in moles is 27 times lower than the fluoride concentration with equivalent remineralizing potential.
4. Theobromine applied to enamel produces smooth surface through remineralization.

RECOMMENDATIONS

1. Theobromine is a nontoxic, natural, and an effective remineralizing agent that could be suitable alternative to fluoride.
2. Theobromine can be a valuable substitute to fluoride in commercial dentifrices.

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