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ORIGINAL RESEARCH

CARIES PREVENTIVE EFFECTS OF THEOBROMINE ON THE ENAMEL TOOTH SURFACE (AN IN VITRO COMPARATIVE STUDY)

Maryam Issa Al Ajeel[1], Aseel Haidar M. J. Al Haidar[2], Serar Nassir Alnajjar[3], Nahed Hazim[4], Abdulrahman Zaid Al-Najjar[5]

1. College of Dentistry, College of Dentistry Al-Kunooze University, Basrah, Iraq.
2. Department of Pediatric and Preventive Dentistry, College of Dentistry, University of Baghdad, Baghdad
3. College of Dentistry Al-Bayan University, Baghdad, Iraq.
4. Department of Chemistry, College of Science, university of Basra. Basra/ Iraq.
5. Department of Dental Basic Science, College of Dentistry, Gilgamesh University, Baghdad, Iraq.

abdulrahman.zaid@gu.edu.iq

Corresponding Author: Abdulrahman Zaid Al-Najjar / Department of Dental Basic Science, College of Dentistry, Gilgamesh University, Baghdad, Iraq. abdulrahman.zaid@gu.edu.iq

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ABSTRACT

Background and objective: A requisite to adopt the remineralization systems as a part of the noninvasive treatment of the early carious lesion has become a must and has recently gained increasing attention. Studies on remineralization aimed to create a novel preventive agent that can be used with fluoride or separately from it. Thus, theobromine was a substance that had been suggested to have anti-cariogenic properties. Therefore, this study was conducted to evaluate the preventive theobromine potential against acid demineralization in comparison to sodium fluoride.

Materials Methods: For this in vitro study, 34 freshly extracted intact upper first premolars were collected, the teeth were randomly divided into three groups (n=11): (1), the theobromine with artificial saliva group. (2), the sodium fluoride with artificial saliva group, (3), artificial saliva group (as a negative control). One tooth was left sound to be studied under the scanning electron microscopy (SEM). The treatment solutions were applied within a pH-cycling model, then, all the samples were subjected to an acid challenge. Vickers microhardness, EDX and scanning electron microscope were performed to assess enamel structure quantitatively and qualitatively and the data were statistically analyzed.

Results: In the present study, in treatment stage and demineralization stage theobromine-treated teeth had a higher mean of microhardness than the fluoride-treated teeth, with a statistically significant difference **($p < 0.05$).**

Conclusions: The theobromine solution had a higher preventive capacity than that found in other groups. Therefore, theobromine was an efficient remineralizing material and thus it could be a good substitute for sodium fluoride.

***Keywords*:** Enamel remineralization, Microhardness, Sodium fluoride, Scanning electron microscopy, Theobromine.

INTRODUCTION

Dental caries has been considered **the most prevalent oral disease among children** [1,2] .

Fluoride is widely recognized as an effective remineralization agent; it increases enamel's resistance to acid attack by inhibiting loss of minerals from the surface of the enamel [3-5]**.** Nevertheless, fluoride intake from various sources and swallowing fluoridated toothpaste were associated with an increased incidence of dental and skeletal fluorosis [6-8].

Increasing numbers of researches are being conducted to determine the efficacy of natural products; consequently, theobromine and other biocompatible fluoride-free products are gaining prominence in modern dental products [9,10].

Cacao extracts (theobromine) had an effective dental benefit and the lowest side effects as compared to fluoride. It is a crystalline powder bitter taste alkaloid belonging to the methylxanthine classes of chemical compounds. Theobromine increases the microhardness of the enamel surface, thereby making teeth less susceptible to decay [11-14]**.** However, other studies claim that it had no remineralizing potential or cariostatic effect [15,16]. Given these conflicting results, further studies are needed to investigate theobromine's ability to remineralize the tooth structure. In addition, there has been no previous investigation on preventive impact of

theobromine on dental enamel. All previous studies assessed theobromine's effect by remineralizing the enamel surface after forming artificial caries lesions Therefore, the current study was designed to test the effect of theobromine on the intact enamel tooth surface.

MATERIAL AND METHODS

This study was approved by the Ethical Committee of the College of Dentistry/University of Baghdad (Ref. 549322 on 17- April 2022), in compliance with the Helsinki Declaration and its guiding principles [17].

The Sample

A total sample of 34 extracted permanent human maxillary first premolars between the ages of 11 and 14 years for orthodontic purposes was collected [18]. Teeth with apparent caries, white spots, stains, or restorations were excluded [19]. One sound sample was used for SEM, while the other33 teeth were randomly allocated into 3 groups of 11 teeth each. One representative sample from each group was used for SEM while the others were used for microhardness and EDX test.

Sample preparation

All extracted teeth were cleaned polished with non-fluoridated pumice and washed with deionized water. The chosen teeth were stored in a 0.1% thymol solution (M Dent, Bangkok ,Thailand) to prevent dryness and growth of germs and fungus on the tooth surface until used [19,20].

After that, the root was cut 2 mm lower than the cemento-enamel junctions and placed (with the buccal surface directed upward) in the center of a ring with cold-cured acrylic resin (Akrodent, Ankara, Turkey). Then, the buccal surfaces were flattened and polished with grit paper (400; Whatman 1003110-DS U.S. A) 10 times in one direction [20]. The tooth was painted with nail varnish (Rimmel London, UK)) after adhering a tape measuring 4 mm x 2 mm to the middle third of the crown. After the dryness of the varnish, the tape was taken off to reveal the enamel [19-20].

Experimental Grouping

Group 1: 200 mg/L of theobromine-infused artificial saliva (Sigma-Aldrich, Hamburg, Germany) [22,23].

Group 2: sodium fluoride solution (0.05% sodium fluoride powder was dissolved in artificial saliva) [22,23].

Group 3: artificial saliva (as a negative control group) [22,23].

The experimental design of the study is presented in Figure 1.

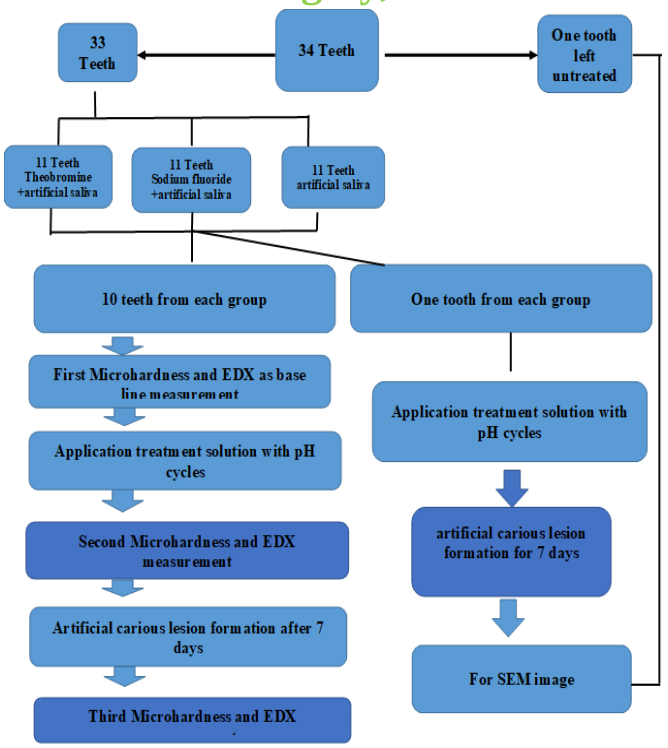

Figure 1. Experimental design of this study.

Treatment procedure

The treatment solution was applied with a demineralization-remineralization pH cycle to mimic the oral environment [22,24,25]. Once the specimens were treated with study solution, they were rinsed in flowing deionized water, dried with a paper towel, and then submerged in the next solution. For 28 days, the daily schedule was repeated, as follows:

1. Fluoride or theobromine therapy was applied for 3 min; three times a day followed by 1-hour demineralization period each time.
2. Soaked in the remineralizing fluid until the next cycle starts [22].

Acid challenge

Demineralization solution (pH 5.0) was prepared according to Ten Cate 1982 and was used to create caries-like lesions on all 33 teeth [25,26].

Microhardness examination

Digital Vickers microhardness tester (Jinan Kason Testing Equipment Co., Ltd., China) was used with a load and time of 300 gram and 15 seconds, respectively. The same calibrated equipment and examiner were used. The hardness value for each specimen was determined by calculating the mean of three indentations [3,20,21].

Surface morphology evaluation using Scan Electron Microscope (SEM)

One sound and one representative specimen from each study group were chosen to examine the morphological changes of the enamel surface. A sputter coater was used to coat each tooth in gold and palladium in a vacuum examination. magnifications of 10Kx and 15kx was applied [20,27,28].

Dispersive X-ray Analysis (EDXA)

Enamel samples were chemically analyzed for their calcium (Ca) and phosphorus (P) ratio using energy dispersive x-ray spectroscopy (EDXA). The Ca/P ratios in all samples were calculated to indicate the changes in mineral content during remineralization and demineralization [29].

Statistical analysis

Data description and analysis were performed using Statistical Package for Social Science (SPSS version -22, Chicago, Illinois, USA). Shapiro-Wilks, two-way ANOVA and Tukey's HSD were used to evaluate if the groups had statistically significant differences. Statistical significance was described as a *p*-value of ≤ 0.05

RESULTS

Microhardness

The microhardness values at baseline did not vary significantly across the research groups. After treatment, there was a statistically significant difference concerning the microhardness values ($p = 0.000$) between the

groups. After demineralization, the artificial saliva group had a lesser microhardness than the other two groups. Meanwhile, the theobromine group had the highest microhardness, Table 1.

Table 1. Microhardness comparison across phases among groups.

GROUPS		Baseline	After treatment	After demineralization	F	*p*-value
Theobromine with saliva	**Mean**	334.85	317.15	171.55	105.896	0.000**
	SD	26.862	25.269	25.471		
Sodium fluoride with saliva	**Mean**	353.885	296.516	142.784	48.628	0.000**
	SD	30.814	31.120	30.227		
Artificial saliva	**Mean**	352.043	215.934	82.406	33.518	0.71
	SD	28.963	19.929	6.899		
F		1.324	28.628	70.510		
***p*-value**		0.28	0.000**	0 .000**		

***Using ANOVA **=significant at $p \leq 0.0$**

Table 2 shows that there was a statistical significance difference among phases for all groups.

Table 2. Multiple pairwise comparison of Microhardness among stages within group using Tukey HSD

Groups	(I) Stages	(J) Stages			
		Treatment		Demin.	
		Mean Difference	p value	Mean Difference	p value
Theobromine+saliva	Baseline	17.709	0.000	162.904	0.000
	treatment			145.195	0.000
Sodium fluoride+Saliva	Baseline	57.369	0.000	211.101	0.000
	Treatment			153.732	0.000
Control	Baseline	136.109	0.000	297.427	0.000
	Treatment			161.318	0.000

Table 3 shows that after treatment, theobromine differ from sodium fluoride but no statical difference between them, in demineralization phase, statistically significant differences were found between all the groups, in treatment and demineralization. The higher mean difference belongs to theobromine group. *p*-value < 0.05.

Table 3. Comparisons of Microhardness between the groups after the treatment and the demineralization phase

Stages	(I) Groups	(J) Groups	Mean Difference	p value
Treatment	Control	Theobromine+saliva	-101.216	0.000
		Sodium fluoride+Saliva	-80.582	0.000
	Theobromine+saliva	Sodium fluoride+Saliva	20.634	0.630
Demin.	Control	Theobromine+saliva	-117.339	0.000
		Sodium fluoride +Saliva	-88.168	0.000
	Theobromine+saliva	Sodium fluoride +Saliva	29.171	0.045

***Significant at $p \leq 0.05$**

EDX-SEM

In Table 4, The Ca/P ratios values at baseline did not vary significantly across the research groups. While, after treatment and demineralization, a statistically significant difference was observed among the groups. After demineralization, the theobromine group had the highest Ca/P ratios (1.64), followed by the sodium fluoride group with a mean of 1.28. However, the artificial saliva group lost more minerals than the other two groups, and it had the lowest mean. The same table shows a statistically significant difference between all the phases within the theobromine group and the sodium fluoride group.

Table 4. Calcium to phosphate ratio (Ca/P ratios) data were compared across stages and groups*.

GROUPS		**Baseline**	**After treatment**	**After demineralization**	**F**	***p*-value**
Theobromine + saliva	Mean	2.11	1.81	1.64	97.596	0.00**
	SD	0.195	0.056	0.117		
Sodium fluoride saliva	Mean	2.03	1.5	1.28	32.720	0.00**
	SD	0.328	0.196	0.103		
Artificial saliva	Mean	2.028	1.3	1.05	1.890	0.13
	SD	0.022	0.216	0.065		
F		1.784	25.148	93.060		
***p*-value**		0.19***	0.00**	0 .00**		

*Using ANOVA test. ** Significant at $p \leq 0.05$

Table 5 shows that there was a statistical significance difference among phases for all groups.

Table 5. Multiple pairwise comparison of (Ca/P ratios) among stages with in usingTukey HSD

Groups	(I) Stages	(J) Stages	
		Demin.	
		Mean Difference	p value
Theobromine+saliva	Baseline	0.179	0.000
	Treatment	0.193	0.000
Sodium fluoride +Saliva	Baseline	0.165	0.000
	Treatment	0.203	0.000
Control	Baseline	0.351	0.000
	Treatment	0.322	0.000

Table 6 shows that after treatment, a statistically significant difference was found only between the theobromine group and the artificial saliva group. Whereas after demineralization, statistically significant differences were found between all the groups.

Table 6. Comparisons of (Ca/P ratios) between the groups after the treatment and the demineralization phase*.

Stages	(I) group	(J) Group	Mean difference (I–J)	SD	*p*-value
After treatment stage	Theobromine +Artificial saliva	Sodium fluoride+ Artificial saliva	0.30966	0.724	0.01
		Artificial saliva	0.51	0.724	0.000**
	Sodium fluoride +Artificial saliva	Artificial saliva	0.20	0.724	0.027
After demineralization stage	Theobromine +Artificial saliva	Sodium fluoride+ Artificial saliva	0.36	0.043	0.000**
		Artificial saliva	0.59	0.043	0.000**
	Sodium fluoride +Artificial saliva	Artificial saliva	0.23	0.043	0.000**

*Using Tukey's HSD multiple pairwise **significant at $p \leq 0.05$

Scanning electron microscopy (SEM)

1. Sound enamel surface reveals a flattened, smooth surface, and no surface deposits (Figure 2).

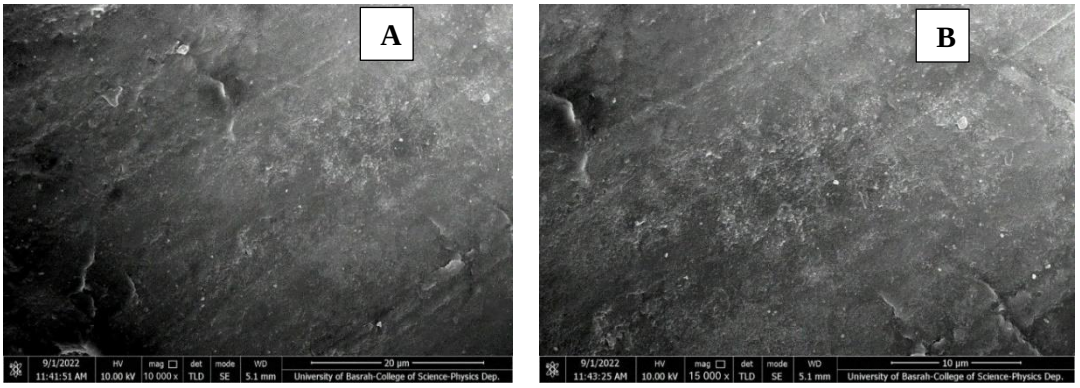

Figure 2. SEM image of the sound enamel surface. (A) Magnification (10kx) , (B) Magnification (15kx) .

2.theobromine treated group exhibited improved surface properties, including smooth enamel that sometimes revealed depressions (Figure 3).

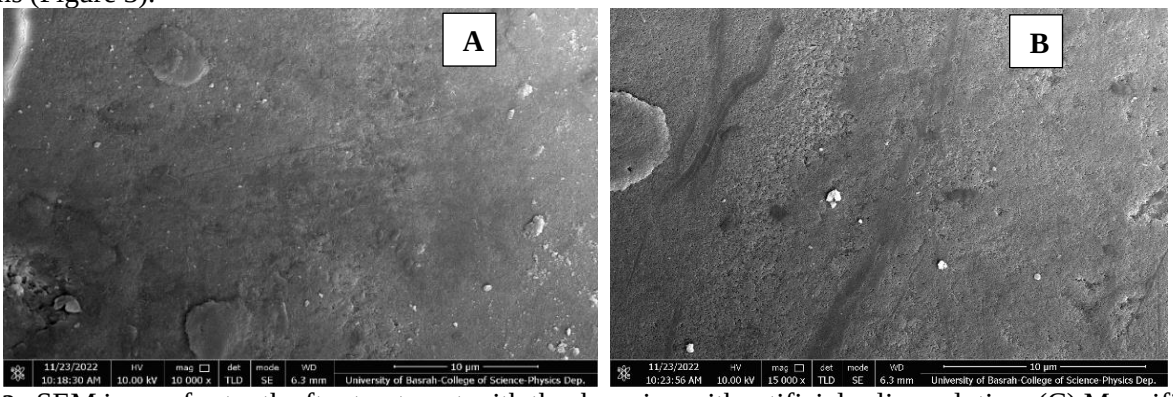

Figure 3. SEM image for tooth after treatment with theobromine with artificial saliva solution. (C) Magnification (10kx) , (D) Magnification (15kx) .

3. sodium fluoride treated group exhibited signs of improvement in the enamel state, with no well-observable flaws. (Figure 4).

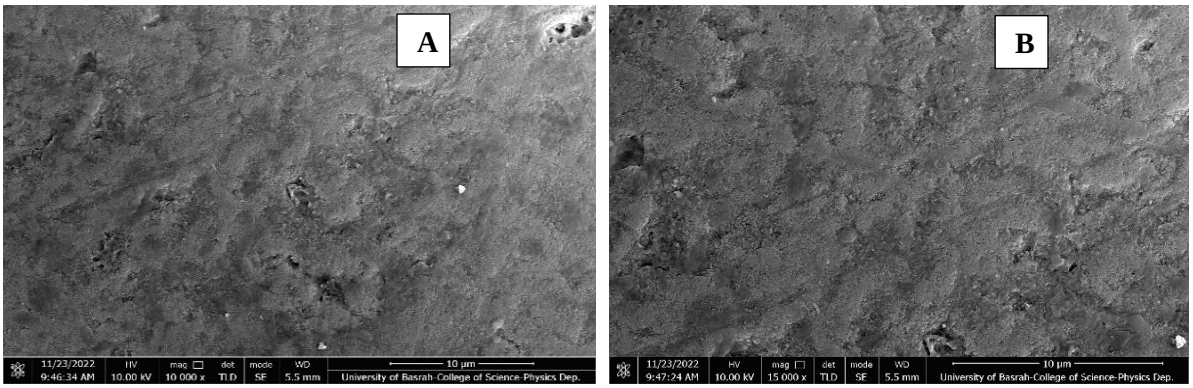

Figure 4. SEM image of tooth after treatment with sodium fluoride solution. (E) Magnification (10kx) , (F) Magnification (15kx) .

4. In the artificial saliva group, the enamel had a rough surface, and various tiny holes and cracks (Fig. 5).

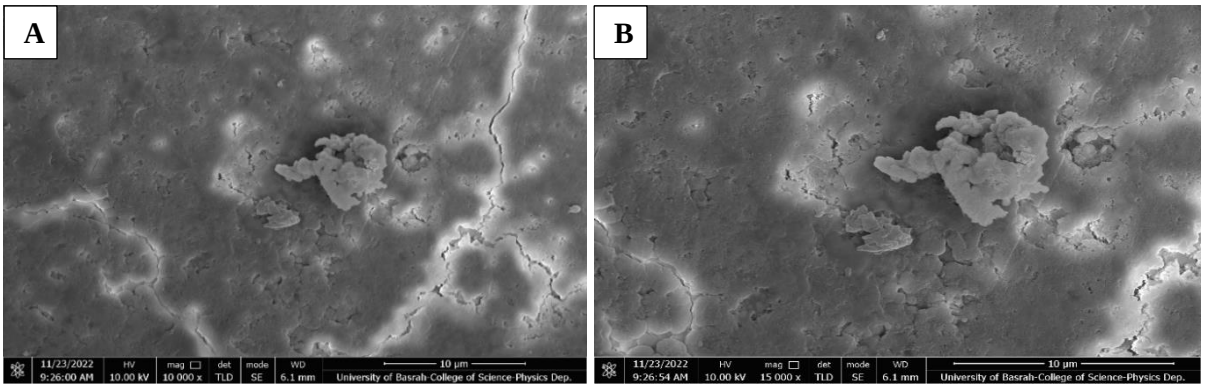

Figure 5. SEM image of tooth after treatment with artifcial saliva solution. (G) Magnification (10kx) , (H)Magnification (15kx) .

DISCUSSION

The findings of this investigation indicated that there was no significant statistical difference among the groups in terms of the microhardness value at baseline. Nevertheless, the treatment stage significantly improved the enamel's microhardness after treatment using theobromine with artificial saliva (group 1) and sodium fluoride with artificial saliva (group 2) with the highest score of microhardness value related to group 1. The theobromine revealed a stronger protective potential in the present experiment because it enhanced enamel resistance to acid attach in the demineralization phase. The artificial saliva group exhibited the lowest mean value. These findings were consistent with the results obtained from the EDS analysis conducted in this study. Results of the analysis demonstrated that during the treatment and demineralization phases, theobromine with artificial saliva group exhibited a higher mean Ca/P ratio subsequently followed by the group of sodium fluoride with artificial saliva as compared to the values observed in the artificial saliva group. The observed outcome can be ascribed to the ability of theobromine to infiltrate the micro spaces within the enamel and serve as a mold during precipitation. This, in turn, facilitates the attraction of a significant quantity of calcium and phosphorus from the remineralizing solution to the enamel surface, thereby promoting the desired remineralization result [11,14,22]. this finding is consistent with prior studies that had demonstrated the ability of theobromine to improve remineralization potential in calcium and phosphate-rich environments when combined with artificial saliva [30].

Several recent studies either disregarded theobromine's remineralization potential or assigned it an inferior rank as compared to fluoride [9,12,15,16]. This debate could be attributed to disparities regarding the pH cycle model, and the qualities of the demineralization and remineralization media.

The structural analysis was seen using a scanning electron microscope. The current study's SEM images of the sound specimen demonstrated that its surface was homogeneous and smooth. Both Huang et al. and El Moshy et al. found similar results [31,32]. SEM images of the tooth treated with theobromine with artificial saliva appear to have a smooth surface, these findings agreed with previous studies which reveal that theobromine treated enamel was closely like normal enamel [30,33]. Enamel treated with sodium fluoride with artificial saliva show sign of remineralization and recrystallized, but the surface remains rough, with more pores and depressions, which was consistent with previous research rough surfaces, with more pores and depressions which was consistent with previous research [34]. The SEM images of the tooth treated with artificial saliva shows tiny porosities and cracks, which may be due to the acidic challenge.

CONCLUSION

Treatment with theobromine encouraged special protection and increased resistance against further acid attacks, as observed by the microhardness and EDS values. Therefore, it can serve as a safe and effective alternative to fluoride.

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Data Availability

The data used to support the findings of this study are included in the article.

Conflicts of Interest

There are no conflicts of interest.

Financial support and sponsorship

Nil.

Competing interest

The authors declare that there are no competing interest.

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Ethical Approval

Not applicable

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