COMPARISON OF FLUORIDE AND THE NOVEL ANTI-CARIES AGENT THEOBROMINE ON INITIAL ENAMEL CARIES: AN *IN VITRO* STUDY

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ABSTRACT: Fluoride has been used as a remineralization agent for many years. In recent years, the possible side effects of fluoride have led researchers to explore new remineralization agents. Theobromine, derived from cocoa beans, is one of the current remineralization agents. The aim of this *in vitro* study was to assess the remineralization efficacy of different concentrations of theobromine and fluoride in the treatment of initial caries lesions. For this purpose, 5 experimental groups were created with each consisting of 20 prepared bovine enamel samples: 200 mg/L theobromine group (Group T1), 500 mg/L theobromine group (Group T2), 500 ppm fluoride group (Group F1), 1,450 ppm fluoride group (Group F2), and a control group (Group C). All samples were incubated with demineralization solution for 32 hours to create artificial caries lesions. Surface microhardness values (SMH) (n=10 per group) calculated with a Knoop microhardness device and quantities of calcium (Ca), phosphorus (P), and Ca/P ratios (n=10 per group) were analyzed with scanning electron microscopy and energy dispersive X-ray spectrometry (SEM-EDS). Remineralization agents were applied to the groups in an experimental pH cycle for 8 days. Microhardness and mineral deposition measurements of the samples were repeated after treatment. According to the results of our study, the values of SMH, Ca, and P deposition after treatment in all groups except group C showed a statistically significant increase compared with post-demineralization values. There was a statistically significant increase in the F2 and T2 groups in terms of SMH and Ca values after treatment compared to all other groups. The results show that 500 mg/L theobromine increased the surface hardness and Ca and P deposition at a level close to the levels obtained with 1,450 ppm fluoride.

Key Words: Fluoride; SEM-EDS, Surface microhardness; Theobromine.

INTRODUCTION

Dental caries is still a challenge for people, especially for children and teenagers. First, the disease is considered to be an irreversible, progressive demineralization characterized by destruction in the hard tissues of the teeth.[1-4] Modern evaluation methods and early diagnosis enable the monitoring of the clinical stages of caries[5,6] and, if parameters in the oral ecosystem are changed positively, the progress of initial caries lesions can be stopped and even remineralized by preventive applications.[7] In current dentistry practices, operative treatment options are avoided as much as possible to minimize tissue loss and the patient's discomfort.[8] Nowadays, the best strategy for caries management is to focus on the recovery process with agents that might cause a remineralization effect so that mineral loss can be regained.[9-11] For this purpose, several remineralization agents and techniques—including fluoride, xylitol, amorphous calcium phosphate, casein derivatives, tri-calcium phosphate, calcium sodium phosphosilicate, nano-hydroxyapatite, laser, and ozone—have been widely used in preventive dentistry.[12]

Fluoride has been the most commonly used remineralization agent. The anti-caries effects of topical fluorides are globally accepted now and have been the subject of

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several clinical studies and meta-analyses.[13] However, the risk of swallowing of fluoride-containing dentifrices,[14] dental fluorosis due to excessive fluoride intake,[15] and some other systematic side effects reported in previous studies[16-21] have led to some doubts about the use of fluoride.[22] Therefore, during recent years, an increasing interest in the natural biologically active compounds that may potentially have a therapeutic effect in the treatment of initial caries lesions has been observed. Of these, theobromine found in cacao bean is an important natural product gaining popularity for the remineralization of initial enamel caries.[23,24]

Theobromine is the main alkaloid of cacao seeds and is responsible for the bitter taste. It is a white (or colorless) crystalline powder with the chemical structure 3,7-dimetylxantin.[25] Despite some previous studies that hypothesized a positive correlation between caries reduction and cacao–chocolate consumption, the effective chemical structure in cacao had not been defined.[26-31] The caries-preventive molecule (theobromine) in cacao beans was first found in the study of Nakamoto et al.[32-34] Researchers planned a simple *in vitro* study to evaluate the effects of xanthine compounds other than caffeine on the formation of hydroxyapatite (HA) crystals and they found that theobromine enlarges HA crystals up to 4 times and increases resistance of teeth against dissolution in acid environment. The relationship between theobromine and remineralization is a new field of study.

The present study aims to compare the remineralization potential of this new natural agent, theobromine, with fluoride by using a remineralization/demineralization pH-cycling model on artificial caries lesions. Our null hypothesis was that remineralization of demineralized enamel lesions would not show differences between the two different concentrations of fluoride and theobromine.

MATERIALS AND METHODS

Specimen preparation: Ethical approval of this study was approved by the University of Gaziantep Animal Experiments Local Ethics Committee on June 6, 2016 through decision number 2016/8. Fifty bovine mandibular incisor teeth were used in the study. The teeth were cleaned of debris; examined with a transilluminator for unusual demineralizations, enamel cracks, and malformations; and stored in deionized water containing 0.1% thymol at room temperature until they were used. The roots of the teeth were separated from the crowns by using a diamond disc and micromotor under water-cooling. The tooth crowns were then cut in the bucco-lingual direction using a Buehler Isomet low-speed saw, and 2 samples were obtained from each of the 50 teeth selected initially. One hundred samples were assigned randomly to 2 groups for surface microhardness (SMH) and scanning electron microscopy and energy dispersive X-ray spectrometry (SEM-EDS) testing.

The samples for the SMH and SEM-EDS analyses were prepared in different ways. For the microhardness measurements, 50 samples were embedded in epoxy resin by using 3 cm diameter prefabricated polytetrafluoroethylene molds. Samples were ground with a Struers LaboPol-5 polishing unit (Struers Inc. Cleveland, Ohio., USA) and serially 2,400–4,000 grid paper to create flat and smooth surfaces. Enamel surfaces were examined under a Nikon SMZ 1500 stereomicroscope (SMZ 1500, Nikon Instruments Europe B.V., Germany), and if the required smooth surface could not be obtained the process was repeated. Then specimens were polished with a 1 micrometer diamond polishing suspension and felt. A 4×4 mm adhesive strip was

adhered to 50 samples, and 2 layers of acid-resistant nail varnish were applied to the remaining surfaces.

For the SEM-EDS analysis, no preliminary preparation was made on the sample surfaces. Only a 4×4 mm adhesive strip was adhered to 50 samples, and 2 layers of acid-resistant nail varnish were applied to the remaining surfaces. At each stage of the study, samples were stored in deionized water to prevent drying. The samples were dried only to take measurements and they were then placed in storage containers.

Baseline SMH and SEM-EDS measurement: The baseline SMH of each of the 50 enamel samples was measured with a microhardness device (Future-Tech FM 700e/ Japan) and a Knoop indenter at a load of 50 g for 10 seconds before creating artificial caries lesions. Three initial indentations were made on the surface of enamel left open. Because the measurements on the microhardness device are dependent on the individual eye sensitivity, more accurate measurements were made using an inverted microscope (Nikon Eclipse MA100/Japan). Then Knoop hardness numbers were calculated for each specimen by measuring the length of the indentations using Imagej image analysis software (NIH, USA). Baseline elemental analysis of 50 enamel samples for Ca and P content was measured with a SEM (JEOL JSM-639OLV Scanning Electron Microscopy/USA) and an EDS instrument (IXRF Systems Model 550i). Examinations were performed at the center of each block.

Artificial caries lesion preparation: All samples were placed in the demineralization solution for 32 hours, which is sufficient time to create measurable caries-like subsurface lesions on enamel without surface erosion and to evaluate mineral gain or loss by surface test methods.[35] This solution was used both for the formation of artificial caries lesions and for the pH-cycling model on bovine enamel samples. A 0.05 mol/L acetate buffer with pH 5.0 and containing 1.28 mmol/l Ca, 0.74 mmol/L P and 0.03 mg F/mL was prepared from the salts $Ca(NO_3)_2.4H_2O$, KH_2PO_4, and NaF. After lesion creation, specimens were rinsed with deionized water.

The specimens were remeasured by using SMH and SEM-EDS analysis after artificial caries preparation.

Treatment procedure: SMH (n = 50) and SEM-EDS (n = 50) testing groups were randomly divided into 5 treatment subgroups (n = 10): 200 mg/L theobromine group (T1) (200 mg theobromine dissolved in 1 liter of deionized water),[36] 500 mg/L theobromine group (T2) (500 mg theobromine dissolved in 1 liter of deionized water),[36] 500 ppm fluoride group (F1) (0.552 g NaF dissolved in 500 g of deionized water),[37] 1,450 ppm fluoride group (F2) (1.602 g NaF dissolved in 500 g of deionized water),[37] and control group-deionized water (C). The pH-cycling model was applied for 8 days, the samples were left in the remineralization solution (0.1 mol/L Tris buffer containing 1.5 mmol/L Ca, 0.9 mmol/L P, 150 mmol/L KCl, and 0.05 mg F/mL (pH 7.0) prepared using tris buffer $Ca(NO_3)_2.4H_2O$, KH_2PO_4, NaF, and KCl salts) for 22 hours, and left in demineralization solution for 2 hours a day at 37ºC. The samples were kept in the treatment solutions 3 times a day for 5 minutes under agitation (9:00, 14:00, and 17:00). The samples were washed with deionized water before and after this process. The solutions were refreshed every 2 days.

Post-treatment SMH and SEM-EDS analysis: After the pH-cycling model, SMH and SEM-EDS were again determined.

Statistical analysis: Statistical analysis of the data was conducted using SPSS statistical software (PASW Statistics 18.0), with the level of significance selected at 0.05. A power analysis and sample size calculation were performed using the GPower secondary software and were based on the results of the study by Amaechi et al.[38] All data was examined with the Kolmogorov–Simirnov test for normality and homogeneity. All the parameters within each group were compared using t tests at a 95% confidence level. Intergroup comparisons were performed with the percent change in SMH, Ca, P, and Ca/P ratio level using one-way ANOVA ($p<0.05$), followed by Tukey post hoc multistep comparisons.

RESULTS

The SMH data are shown in Table 1.

Table 1. Mean value and standard deviation of surface microhardness (SMH) in the theobromine and fluoride treatment groups and in the control group (n=10 per group; group T1=theobromine 200 mg/L; group T2= theobromine 500 mg/L; F1=fluoride 500 ppm (mg/L); group F2= fluoride 1,450 ppm (mg/L); group C=deionized water)

Group	SMH				
	Baseline*	After demineralization* (AD)	Difference (baseline – AD)	After treatment (AT)	Difference* (AD – AT)
Group T1	331.91 ±15.33 Aa	103.34 ±10.34 Ba	–228.57 ±61.52	214.38 ±3.19	111.04 ±39.47 Ca
Group T2	333.61 ±19.73 Aa	112.77 ±27.33 Ba	–220.84 ±97.87	242.61 ±6.33	129.84 ±91.10 Cb
Group F1	330.2 ±14.11 Aa	98.21 ±12.69 Ba	–231.98 ±62.20	213.66 ±4.56	115.45 ±40.66 Ca
Group F2	333.05 ±13.61 Aa	88.09 ±8.9 Ba	–244.96 ±53.96	272.14 ±8.99	184.05 ±35.20 Cc
Group C	332.96 ±16.62 Aa	121.95 ±26.38 Ba	–211.00 ±94.05	185.43 ±8.97	63.47 ±86.85 Bd

Different uppercase letters (in row) and different lowercase letters (in column) denote significantly different ($p<0.05$) data in the same parameter, while similar symbols means not significantly different.

There were no statistical differences in the mean values for the baseline and after demineralization data between the all groups for SMH, Ca, P and Ca/P ratio ($p>0.05$). Although, in intragroup comparison SMH, Ca, P data showed statistically decreased from baseline to after demineralization, Ca/P did not showed statistically differences between baseline and after demineralization. When the treatment groups (F1, F2, T1, T2) were compared with the control group based on mean SMH values, all treatment groups showed statistically significant higher value SMH gain compered the control group ($p<0.05$). Although the T2 and F2 group showed statistically significant different SMH value compared to other groups (T1 and F1), the F2 group showed the highest SMH value compared the others ($p<0.05$). T1 and F1 groups showed similar SMH values compared with each other ($p>0.05$). With in dose-dependent analysis of groups; T2 groups showed statistically differences compared T1 and F2 group showed statistically differences compared F1 ($p<0.05$).

The SEM-EDS (Ca, P, Ca/P ratio) are shown in Tables 2A and 2B.

Table 2A. Mean value and standard deviation of the Ca, as analyzed with scanning electron microscopy and energy dispersive X-ray spectrometry (SEM-EDS), in the theobromine and fluoride treatment groups and in the control group (n=10 per group; group T1= theobromine 200 mg/L; group T2= theobromine 500 mg/L; F1=fluoride 500 ppm (mg/L); group F2= fluoride 1,450 ppm (mg/L); group C=deionized water)

Group	Ca as analyzed with SEM-EDS				
	Baseline*	After demineralization* (AD)	Difference (baseline – AD)	After treatment (AT)	Difference* (AD – AT)
Group T1	23.37 ±4.4 Aa	18.64 ±4.94 Ba	–4.73 ±2.29	24.49 ±3.08	5.85 ±4.61 Cab
Group T2	27.26 ±4.68 Aa	21.46 ±4.66 Ba	–5.80 ±3.08	28.05 ±2.65	6.59 ±3.56 Cb
Group F1	25.55 ±4.81 Aa	21.18 ±3.56 Ba	–4.36 ±2.48	24.84 ±3.68	3.65 ±3.46 Cab
Group F2	27.58 ±2.89 Aa	20.81 ±3.18 Ba	–6.76 ±3.78	28.36 ±3.99	7.54 ±4.16 Cb
Group C	28.17 ±2.56 Aa	21.45 ±4.48 Ba	–6.71 ±2.94	20.79 ±3.26	–0.66 ±3.06 Cb

*Different uppercase letters (in row) and different lowercase letters (in column) denote significantly different ($p<0.05$) data in the same parameter, while similar symbols means not significantly different.

Table 2B. Mean value and standard deviation of the P and the Ca/P ratio, as analyzed with scanning electron microscopy and energy dispersive X-ray spectrometry (SEM-EDS), in the theobromine and fluoride treatment groups and in the control group (n=10 per group; group T1= theobromine 200 mg/L; group T2= theobromine 500 mg/L; F1=fluoride 500 ppm (mg/L); group F2= fluoride 1,450 ppm (mg/L); group C=deionized water)

Group	P as analyzed with SEM-EDS				
	Baseline*	After demineralization* (AD)	Difference (baseline – AD)	After treatment (AT)	Difference* (AD – AT)
Group T1	13.99 ±2.42 Aa	11.84 ±2.5 Ba	–2.15 ±0.48	13.96 ±2.47	2.12 ±0.74 Cab
Group T2	15.46 ±2.3 Aa	13.21 ±1.89 Ba	–2.25 ±1.02	15.53 ±1.51	2.32 ±0.86 Cb
Group F1	13.81 ±2.33 Aa	12.21 ±1.68 Ba	–1.59 ±1.2	13.57 ±1.59	1.36 ±0.56 Cab
Group F2	15.35 ±2.01 Aa	12.66 ±1.26 Ba	–2.69 ±1.27	15.87 ±1.14	3.2 ±1.07 Cb
Group C	13.59 ±2.4 Aa	11.43 ±2.21 Ba	–2.16 ±0.62	12.19 ±2.48	–0.75 ±0.85 Ba

Group	Ca/P ratio as analyzed with SEM-EDS				
	Baseline*	After demineralization* (AD)	Difference (baseline – AD)	After treatment (AT)	Difference* (AD – AT)
Group T1	1.7 ±0.35 Aa	1.62 ±0.47 Aa	–0.07 ±0.2	1.8 ±0.41	0.17 ±0.39 Aa
Group T2	1.79 ±0.37 Aa	1.65 ±0.4 Aa	–0.14 ±0.26	1.81 ±0.17	0.16 ±0.27 Ba
Group F1	1.86 ±0.28 Aa	1.73 ±0.19 Aa	–1.2 ±0.23	1.84 ±0.29	0.1 ±0.28 Aa
Group F2	1.83 ±0.34 Aa	1.66 ±0.32 Aa	–0.16 ±0.31	1.79 ±0.26	0.13 ±0.32 Aa
Group C	2.1 ±0.25 Aa	1.87 ±0.14 Aa	–0.23 ±0.31	1.73 ±0.2	0.13 ±0.27 Ba

*Different uppercase letters (in row) and different lowercase letters (in column) denote significantly different ($p<0.05$) data in the same parameter, while similar symbols means not significantly different.

With Ca and P deposition level in the after treatment time, all treatment groups showed increased level compared to control group, although only F2 group and T2 group showed statistically significant differences compared treatment groups each other, except the control. Regarding the Ca/P ratio, all groups showed an increased level compared the control, however they didn't show any difference between each other ($p<0.05$).

Regarding post-treatment SMH, and the Ca and P levels; all treatment groups except control group showed statistically significant remineralization in inter-group comparison. However, only T2 group showed statistically significant remineralization regarding Ca/P ratio data ($p<0.05$).

DISCUSSION

The present study was designed to compare the remineralization effect of different doses of theobromine and fluoride on initial caries lesions using an *in vitro* pH-cycling model.[35] Modern pH-cycling models are frequently used in *in vitro* studies to simulate the caries process and evaluate the anti-caries effects of remineralization agents.[39-41] The tooth surfaces that are used in studies evaluating the efficacy of remineralization agents are important for the reliability of the results of the study. Although human teeth are widely used in *in vitro* studies, bovine teeth have been histologically and morphologically proven to be similar to human teeth and are frequently preferred in dental research due to limited access to unaffected human teeth and ethical reasons.[39,42]

Even although the doses of agents used in this study have been tested in previous studies, this study aimed to test the effective concentration comparatively within the same study protocol. There is some evidence that fluoride concentration must be at least 1,000 ppm to control tooth caries in primary and permanent dentition, but this concentration raises concerns about the risk of fluorosis, especially in young children.[43-45] Two different solutions were prepared under laboratory conditions: a 500 ppm fluoride concentration that is often preferred in young children and a 1,450 ppm fluoride concentration that is mostly preferred across the world, especially for adult use. The most effective theobromine concentration that protects against caries is still unclear, so various doses have been tried in previous studies.[36,38,46] The highest theobromine dose (500 mg/L) corresponds to the average theobromine level in commercial cacao powder and represents the upper solution limit of theobromine in water. Solutions containing 200 mg/L and 500 mg/L theobromine were prepared and evaluated for their efficacy.

Mineral changes in the superficial enamel layer are directly related to the change in microhardness. As a result, minerals deposited on the enamel surface by remineralization lead to an increase of the hardness of the surface.[47] In a pilot study examining the effect of two different theobromine concentrations on the microhardness of the enamel, it was observed that 200 mg/L theobromine increased the surface hardness more than 100 mg/L theobromine. In addition, topographic improvement of the enamel surface was observed in both groups.[36] Our study is in agreement with this pilot study that theobromine increases the surface microhardness due to the dose increase.

Sadeghpour and Nakamoto studied the effect of theobromine on microhardness in a detailed laboratory study using a wide dose range (1–500 mg/L). They concluded that less theobromine than fluoride is needed to obtain harder enamel surfaces.[46] In another *in vitro* study, Amaechi et. al. compared the remineralization potential of a standard NaF-containing toothpaste with theobromine. A statistically significant increase in surface microhardness value was observed in both the fluoride and theobromine groups and they reported that theobromine may be an alternative to fluoride in commercial products.[38] The results of our study are similar to the previous studies in that remineralization occurred in all treatment groups compared with the control group, but the highest microhardness increase was observed in the 1,450 ppm fluoride group in our study.

Lippert et. al. compared the remineralization effects of fluoride, theobromine, strontium, and their combinations on artificial caries lesions and concluded that theobromine alone did not provide remineralization under the selected conditions and there was no synergistic effect between strontium and theobromine. Findings of this study relating to theobromine are in disagreement with our study that remineralization occurred in both theobromine groups, and theobromine had an improving effect close to that of fluoride in our study.[48] Current remineralization agents have to be tested in laboratory conditions to prove their reliability before they can be used in human experiments. However, *in vitro* studies have some limitations. While many factors affect the process of caries, few factors in laboratory models can be included in this process.[49] The differences in the protocols for forming artificial caries lesions, in lesion characteristics, and the variation of the pH-cycling models may explain the discrepant results that have occurred in previous studies. The discrepancy between the results of the studies is not surprising considering this situation.[39,48,50]

The remineralization process depends on the mineral changes in dental hard tissues. The levels of Ca, P, and the Ca/P ratio in enamel are considered to indicate a tooth's mineralization degree. A decrease in the Ca, P, and Ca/P ratio in the structure of HA crystals is seen as a decrease in the mechanical characteristics, crystallization of structure, hardness of structure, and reduction of the young modulus. Changes created by remineralization agents on the demineralized enamel were evaluated by using SEM-EDS in the present study.[51,52] In the study of Amaechi et al., Ca accumulation on lesions after treatment was evaluated using SEM-EDS. Even though there was no significant difference between groups, a higher mineral accumulation was observed in the theobromine and NaF groups. The results from the present study indicate that theobromine increases Ca accumulation in a manner similar to fluoride.[38] Similar to this study, in our study all treatment groups were compared with the control group, and an increase was seen in Ca accumulation but no statistically significant difference was observed between treatment groups. Furthermore, an increase was observed in Ca, P values, and Ca/P ratios on the enamel surface after the application of pH-cycling model and remineralization agents, compared with the demineralized state.

CONCLUSIONS

In conclusion, the data obtained with the micro-hardness and SEM-EDS assessments support each other, and these results show that the agents used have

remineralization potential. A remarkable remineralization of enamel surface was observed in all treatment groups. In addition, the lower theobromine worked to encourage remineralization and theobromine showed dose response remineralization process. Within the limits of the present study, however, it is suggested that theobromine increased the surface hardness and Ca and P deposition at a level close to fluoride but fluoride maintains its position in preventive dentistry. However, there is a clear need for further human clinical studies to exploit the benefit of theobromine in both oral hygiene and caries preventive products.

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