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Remineralization of Artificial Enamel Lesions by Theobromine

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Key Words
Caries prevention · Remineralization · Theobromine

Abstract
Objective: This study investigated the remineralization potential of theobromine in comparison to a standard NaF dentifrice. ***Methods:*** Three tooth blocks were produced from each of 30 teeth. Caries-like lesion was created on each block using acidified gel. A smaller block was cut from each block for baseline scanning electron microscopy imaging and electron-dispersive spectroscopy (EDS) analysis for surface Ca level. A tooth slice was cut from each lesion-bearing block for transverse microradiography (TMR) quantification of baseline mineral loss (Δz) and lesion depth (LD). Then baseline surface microhardness (SMH) of each lesion was measured. The three blocks from each tooth were assigned to three remineralizing agents: (1) artificial saliva; (2) artificial saliva with theobromine (0.0011 mol/l), and (3) NaF toothpaste slurry (0.0789 mol/l F). Remineralization was conducted using a pH cycling model with storage in artificial saliva. After a 28-day cycle, samples were analyzed using EDS, TMR, and SMH. Intragroup comparison of pre- and posttest data was performed using t tests ($p < 0.05$). Intergroup comparisons were performed by post hoc multistep comparisons (Tukey). ***Results:*** SMH indicated significant ($p < 0.01$) remineralization only with theobromine (38 ± 32%) and toothpaste (29 ± 16%). With TMR (Δz/lD), theobromine and toothpaste exhibited significantly ($p < 0.01$) higher mineral gain relative to artificial saliva. With SMH and TMR, remineralization produced by theobromine and toothpaste was not significantly different. With EDS, calcium deposition was significant in all groups, but not significantly different among the groups (theobromine 13 ± 8%, toothpaste 10 ± 5%, and artificial saliva 6 ± 8%). ***Conclusion:*** The present study demonstrated that theobromine in an apatite-forming medium can enhance the remineralization potential of the medium.

Dental caries in its early stage of formation (noncavitated) can be remineralized [ten Cate and Featherstone, 1991; Featherstone, 2008], and this remineralization can be facilitated by such agents as fluoride delivered via either mouth rinse or dentifrice [Schafer, 1989; Wefel et al., 1995]. However, extensive use of fluoride mainly in the form of dentifrice has contributed to a rising incidence of dental fluorosis, particularly in preschool children, due to chronic ingestion of these products [Pendrys and Stamm, 1990]. Furthermore, the ability of fluoride to promote

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remineralization and inhibit caries formation in the oral environment is limited by the availability of calcium and phosphate in saliva, and ultimately in plaque fluid; thus in salivary dysfunction conditions, remineralization rate is not sufficient enough to prevent caries process with consequent rampant caries [Mandel, 1989].

The consumption of chocolate, as a sugar-containing confectionery, has been associated with dental caries [Gustafsson et al., 1954]. However, series of studies suggested that cocoa powder and chocolate might contain some caries-inhibitory substances [Strålfors, 1966a, b, c, 1967]. Studies in hamsters showed that pure cocoa powder inhibited dental caries by 84, 75, 60, and 42% when the percentage in the hamster diet was 20, 15, 10 and 2%, respectively, with defatted cocoa exhibiting higher significant anticaries effect than the fat-containing cocoa [Strålfors, 1966a]. These reports suggest that cocoa extract, the main component of chocolate, could contain an agent with anticaries potential.

While studying the effect of caffeine on the mineralization of the teeth, it was found that caffeine decreases the crystal size on enamel when exposed early at the critical period of growth [Falster et al., 1993]. In the subsequent study, it was demonstrated that these phenomena of decreased crystallinity lead to increased dental caries in rats, which was demonstrated with the significantly higher caries score in the caffeine group compared to the noncaffeine control group [Nakamoto et al., 1993], as the smaller crystal size increased dissolution of the minerals [Falster et al., 1992]. However, during this caffeine study, it was serendipitously discovered that theobromine, which is the same xanthine family, showed entirely opposite properties, i.e. theobromine enhances crystallinity [Nakamoto et al., 1999, 2001]. The newborn rats exposed to theobromine while growing up showed that less calcium, phosphorus and magnesium were dissolved from apatite of the enamel of rats exposed to theobromine compared to the control rats when the teeth were exposed to the weak acid solution [Nakamoto et al., 1999, 2001]. Theobromine (3,7-dimethylxanthine), a white crystalline powder, is an alkaloid readily available in cocoa (240 mg/cup) and chocolate (1.89%). Its levels are higher in dark chocolates (approximately 10 g/kg) than in milk chocolates (1–5 g/kg). Higher-quality chocolate tends to contain more theobromine than lower-quality chocolate. The mean theobromine content of cocoa beans is approximately 20.3 mg/g [Craig and Nguyen, 1984].

In view of the above animal studies [Nakamoto et al., 1999, 2001] that demonstrated less 'net' dissolution of the various minerals from the enamel surface exposed to theobromine compared to the nontheobromine group, further studies using human teeth were conducted to investigate how theobromine exposure may alter the enamel surface in vitro [Sadeghpour and Nakamoto, 2011; Kargul et al., 2012]. The results indicated that the microhardness of the enamel surface of the theobromine group was greatly enhanced compared to the fluoride group. The present study investigated the remineralizing potential of theobromine in comparison with a standard sodium fluoride dentifrice by determining the enamel lesion remineralization in an established in vitro remineralization/demineralization pH cycling model [White, 1987]. Our null hypotheses were that (a) each of the three treatments promotes enamel lesion remineralization, as determined by surface microhardness (SMH), transverse microradiography (TMR) and Ca deposit analyses, which is significantly greater than zero, and (b) remineralization of enamel lesions would not differ between artificial saliva with theobromine and fluoride dentifrice.

Materials and Methods

Production of Artificial Enamel Lesions

Following consent from the donors, freshly extracted human molar teeth were collected from various clinics of the Dental School of the University of Texas Health Science Center at San Antonio. The teeth were cleaned of debris/stains, and examined with transilluminator. Teeth without caries or enamel malformations were selected and cleaned with pumice to remove the remnants of pellicle from the buccal surface. Using a water-cooled diamond wire saw, 3 tooth blocks were produced from each of 50 teeth initially selected, with each block measuring approximately 4 mm in length × 2 mm in width × 1.5 mm in thickness. Then using plain back diamond lapping film (1 μm) in a MultiPrep™ Precision Polishing machine (Allied High Tech, USA), the enamel surface of each block was polished to achieve a flat surface. Following this, all surfaces of each block were painted with two coats of acid-resistant nail varnish except the buccal surface. Then an enamel lesion was created on this exposed buccal surface on each block by 7 days' demineralization in an acidified gel system [Amaechi et al., 1998]. The gel was prepared by adding 100 mmol/l sodium hydroxide to 100 mmol/l lactic acid to give a final pH value of 4.5. To this solution, 6% w/v hydroxyethyl cellulose was added whilst vigorously stirring. The final consistency of gel achieved had a viscosity in the region of 100 cP. Following lesion formation, the nail varnish was carefully and totally removed with acetone. So a total of 3 lesion-bearing blocks were obtained from each tooth.

Baseline TMR Data Analysis

Following lesion formation, a tooth slice (~150 μm thick) was cut from each lesion-bearing tooth block using a water-cooled diamond wire saw. This slice served as baseline used to determine the pretreatment TMR parameters (mineral loss, Δz, and lesion depth, LD) of the lesion before remineralization (pretest param-

eters). Also the parameters of the control slices were used to select the lesions/blocks that were suitable for the remineralization study. The baseline slices were processed for TMR assessment as follows. First, both sides of the slice were polished using adhesive back lapping film in a MultiPrep Precision Polishing machine (Allied High Tech) to achieve planoparallel surfaces as well as reduce the thickness of the slice to 100 μm (the appropriate thickness for TMR). Following this, the slices were microradiographed on a type lA high-resolution glass X-ray plate (Microchrome Technology, Calif., USA) with a Phillips X-ray generator system using a Cu(Kα) X-ray source (Philips B.V., Eindhoven, The Netherlands). The plates were exposed for 10 min at an anode voltage of 20 kV and a tube current of 10 mA at a focus-specimen distance of 30 cm, and then processed. Processing consisted of a 5-min development in Kodak HR developer and 15-min fixation in Kodak Rapid fixer before a final 30-min wash period. After drying, the microradiographs were subjected to visualization and image analysis using a computer program (TMR2006 version 3.0.0.6). The hardware was a Leica DMR optical microscope linked via a Sony model XC-75CE CCTV camera to a Personal Computer. The enhanced image of the microradiograph was analyzed under standard conditions of light intensity and magnification and processed, along with data from the image of the step wedge, by the TMR program. By this method, the parameters of integrated mineral loss (Δz, vol% · μm) and LD were quantified for each enamel lesion.

Baseline Scanning Electron Microscopic Examination of Specimens

Based on the parameters of the control slices, 90 lesion-bearing blocks from 30 of the initially selected and processed 50 teeth (3 blocks from each tooth) were selected for the remineralization study. Following this selection, a smaller block measuring approximately 1.5 mm in length × 2 mm in width × 1.5 mm in thickness was cut from each block. This smaller block was used for a baseline scanning electron microscopy (SEM) and electron-dispersive spectroscopy (EDS) examination of the lesions. Before SEM examination, the blocks were placed in a Petri dish and air-dried under a laboratory hood for 24 h. Following drying, the blocks were sputter-coated with gold palladium (108 Auto Sputter Coater with the MTM-20 High Resolution Thickness Controller, Ted Pella) less than 50 nm in thickness. Then the samples were placed in a scanning electron microscope (JEOL Scanning Electron Microscope, JEOL USA Inc., model JSM-6610LV) vacuum chamber and then visualized and analyzed at an accelerated voltage of 15 kV. The image was captured and saved at a magnification of ×1,500. Each image bore both the magnification and sample Identification number. EDS spectrum analysis for calcium content was performed after SEM analysis. All examinations were performed at the center of each block.

Baseline SMH Testing

Prior to the microhardness assessment, specimens were stored in a moist environment. SMH was tested on each block surface using a Vickers diamond indenter (Tukon 2100; Wilson-Instron, Norwood, Mass., USA) with a load of 200 gf applied for 15 s. Three indentations were made at the middle, upper and lower ends of the enamel surface (preserving a reasonable sound area between the indentations), and the Vickers numbers were calculated and averaged for each block.

Table 1. pH cycling treatment sequence for the experiment

Daily events	Treatment
Day 1 is all-day storage in artificial saliva; subsequent days' treatments are as follows:	
1 h (start at 8.00 a.m.)	acid challenge – all groups
1 h	storage in artificial saliva
2 min	group 1: no treatment group 2: theobromine treatment group 3: toothpaste treatment
1 h	storage in artificial saliva
1 h (11.00 a.m.)	acid challenge – all groups
1 h	storage in artificial saliva
2 min	group 1: no treatment group 2: theobromine treatment group 3: toothpaste treatment
1 h	storage in artificial saliva
1 h (2.00 p.m.)	acid challenge – all groups
1 h	storage in artificial saliva
2 min	group 1: no treatment group 2: theobromine treatment group 3: toothpaste treatment
Till 8.00 a.m. next day	storage in artificial saliva

Treatment Procedure

The 3 blocks from each tooth were assigned randomly to three treatment groups (n = 30) as described below. The 30 blocks for each treatment group were distributed into three subgroups of 10 blocks. Using dental sticky wax, the 10 blocks for each subgroup were attached onto a cylindrical rod that is attached to the cover of a 100-ml color-coded treatment tube, giving a total of three tubes for each dentifrice group.

The remineralization was conducted using a pH cycling (demineralization/remineralization) model, simulating the activities within the oral environment as closely as possible. Artificial saliva [$MgCl_2 \cdot 6 H_2O$ (0.148 mmol/l), K_2HPO_4 (4.59 mmol/l), KH_2PO_4 (2.38 mmol/l), KCl (8.39 mmol/l), calcium lactate (1.76 mmol/l), fluoride (0.05 ppm), sodium carboxymethyl cellulose (2.25 mmol/l), methyl-4-hydroxybenzoate (13.14 mmol/l), pH adjusted to 7.2 using KOH] [Amaechi and Higham, 2001] was used as the remineralization medium in all treatment regimens, while an acidified buffer [2.2 mmol/l KH_2PO_4, 2.2 mmol/l (1,000 mmol/l) $CaCl_2$, 50 mmol/l acetic acid, 0.5 μg F/l (NaF), pH raised to 4.5 with KOH] [Amaechi et al., 1998] was used as the acidic challenge medium. The three treatment groups were produced as follows: group 1: artificial saliva; group 2: artificial saliva with theobromine (1.1 mmol/l); group 3: slurry of standard fluoride toothpaste with 0.24% NaF prepared by mixing 1 part dentifrice and 3 parts artificial saliva (9 g:27 ml) using a laboratory stand mixer until homogeneous. Fresh medium for each group was prepared just prior to each treatment episode. The cyclic treatment regimen for each day consisted of three 1-hour acid challenges, three 2-min test media treatment periods, and then storage in artificial saliva for the rest of the time (table 1). For treatment, 60 ml of the treatment medium

Table 2. Comparison of pre- and posttreatment data

Treatments	SMH, VHN			Ca deposit			Δz, vol% · μm			LD, μm		
	before treatment	after treatment	p value	before treatment	after treatment	p value	before treatment	after treatment	p value	before treatment	after treatment	p value
Artificial saliva	138±31	145±35	0.216	23±5	24±1	0.045*	737±136	683±144	0.096	40±10	37±9	0.12
Theobromine	119±27	157±30	0.001*	22±1	24±1	0.001*	815±316	584±183	0.001*	46±14	32±11	0.001*
1,100 ppm F dentifrice	114±26	146±32	0.017*	22±2	24±1	0.004*	680±163	493±163	0.01*	38±6	27±8	0.001*

Absolute data for SMH, Δz and Ca deposit comparing the pre- and posttreatment data expressed as mean ± SD. VHN = Vickers hardness number.
* Significantly different.

(artificial saliva, artificial saliva with theobromine, slurry of standard fluoride toothpaste or acidified buffer) was placed into each color-coded 100-ml treatment vial. All treatments were stirred at 350 rpm and were carried out in an incubator at 37°C. The pH of each medium was measured once daily before treatment. After treatment with one medium the specimens were rinsed with running deionized water, and dried with a paper towel before immersion into the next agent. The daily regimen was repeated for 28 days.

Posttreatment SEM

Following treatment of the blocks with the products, a smaller block measuring approximately 1.5 mm in length × 2 mm in width × 1.5 mm in thickness was cut from each block. This smaller block was used for a postexposure SEM and EDS examination of the lesions as described above. This process provided the EDS data of the lesions.

Posttreatment SMH

The posttest SMH testing was performed on the remaining portion of each block by three indentations on the free (unindented) surface of the block, and the average value calculated for each block. At this point the pretest (SMH_1) and posttest (SMH_2) SMH values of the lesions were available.

Posttreatment TMR Data Analysis

Following SMH testing, a tooth slice (~150 μm thick) was cut from each block and processed for microradiography as described above for the control specimens. Although the control sections had been microradiographed and analyzed for selection of the appropriate lesions, they were remicroradiographed and analyzed with the posttest sections to eliminate variation due to different processing conditions. This process yielded the following information: the pretest (Δz_1, LD_1) and posttest (Δz_2, LD_2) values for Δz and LD.

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. Our power analysis and sample size calculation were performed using nQuery Advisor software (Statistical Solutions, Cork, Ireland) and were based on the results of our previous studies [Amaechi et al., 1998; Amaechi and Higham, 2001], in which the mean value (±standard deviation, SD) of pretreatment %Δz was 28.5 ± 31.2. For our null hypothesis that enamel lesion remineralization will be significantly greater than zero, the proposed sample size of n = 30 will have power greater than 0.95 with a one-sided significance level of 0.05 to detect a difference between a null hypothesis mean of zero and a sample mean %Δz equal to or greater than 10%. The mean and standard error of the SMH, TMR and Ca level parameters were calculated for the pre- and posttest treatment data. All data were examined for normality and homogeneity of variance. Since each selected tooth yielded 3 blocks, this means that not all observations are independent, so the data were analyzed with consideration of the lack of independence of the blocks. The pretest and posttest parameters within each group were compared using t tests at the 95% confidence level. Intergroup comparisons were performed with the percent change in SMH, Δz, LD and Ca level using one-way analysis of variance ($p < 0.05$), followed by post hoc multistep comparisons (Tukey WSD). Percentage change for each measurement (SMH, Δz, LD and Ca level) after treatment was calculated for each specimen as: (pretest – posttest)/pretest and expressed as a percentage.

Results

There were no statistically significant differences in the mean values for the baseline data between the experimental groups for SMH, Δz and Ca deposit, although the mean values were numerically different between the groups. With SMH all three groups experienced some degree of remineralization; however, only the theobromine and toothpaste groups exhibited significant remineralization (table 2), with the pretest and posttest SMH data being significantly different ($p < 0.01$). When the groups were compared based on their relative percent change in SMH (fig. 1), the theobromine (38 ± 32%) and toothpaste (29 ± 16%) groups showed significantly ($p < 0.01$) higher percent gain in hardness (remineralization) relative to the artificial saliva group (7 ± 20%). The theobromine and toothpaste groups were not significantly different from each other, although there was a greater hardness gain

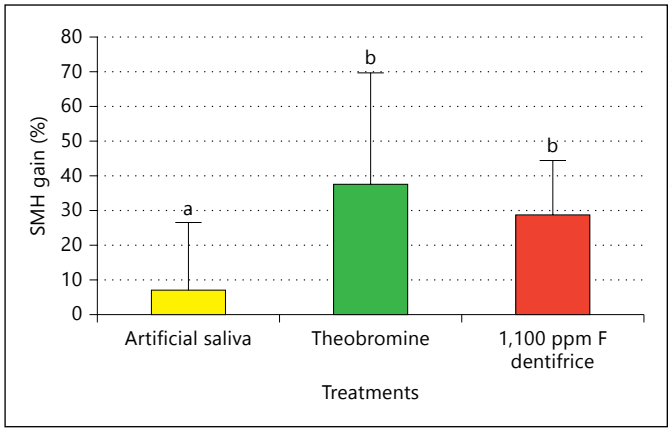

Fig. 1. Percent change in hardness (% remineralization) following pH cycling. Different letters are significantly ($p < 0.01$) different, the same letters are not.

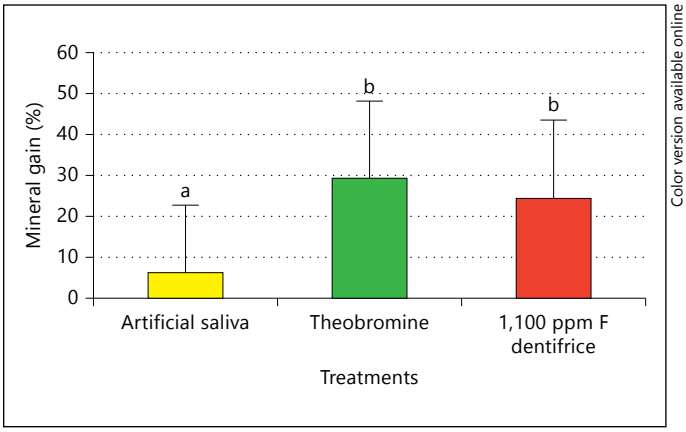

Fig. 2. Percent change in mineral loss (% remineralization) following pH cycling. Different letters are significantly ($p < 0.01$) different, the same letters are not.

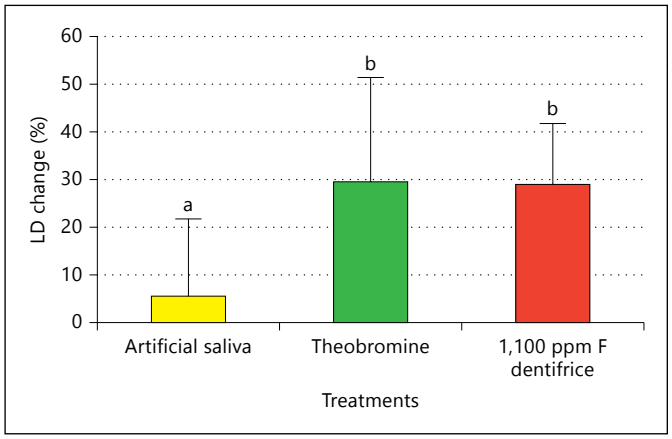

Fig. 3. Percent change in LD following pH cycling. Different letters are significantly ($p < 0.01$) different, the same letters are not.

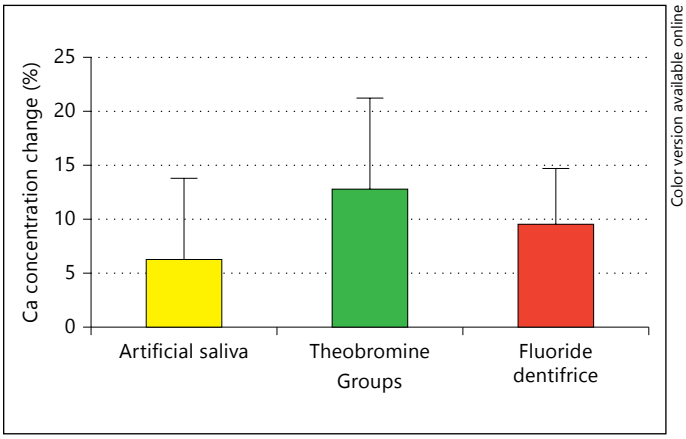

Fig. 4. Percent change in Ca level on lesion surface following pH cycling.

(remineralization) in the theobromine group, 31 and 9% larger relative to artificial saliva and toothpaste, respectively.

With both the Δz and LD measured with TMR, the same trend was found as in SMH, with the theobromine and toothpaste groups exhibiting significantly ($p < 0.01$) higher percent gain in mineral (remineralization) relative to the artificial saliva group in terms of percent change in Δz and LD (table 2, fig. 2, 3). The result of the quantification of the calcium level on the tooth surface using EDS on SEM images showed that after 28 days of treatment, each treatment produced statistically significant ($p < 0.05$) calcium deposition on the tooth surface (table 2). However, among the three groups, no statistical differences were found in the percent change of calcium deposited (fig. 4).

Discussion

The present study investigated the caries-preventive effect of theobromine by examining its ability to cause remineralization of enamel lesions and enhance the resistance of the enamel lesion to further acid attack. This was conducted using an established in vitro caries pH cycling model [White, 1987] that subjected the enamel lesions to

remineralization and demineralization cycles equivalent to the conditions in the oral environment. The three remineralization modalities examined in this study contained the same artificial saliva as in group 1, the difference in composition being the theobromine in group 2, and the sodium fluoride and other toothpaste ingredients in group 3. The three treatment modalities produced net remineralization of the enamel lesions, which was only significant and of comparable levels in theobromine and NaF dentifrice groups. However, it is pertinent to mention that the fact that both the demineralization and remineralization buffer contain fluoride could partially mask the beneficial effect of the treatments. Although the theobromine and NaF toothpaste exhibited comparable levels of remineralization, on a molar level, the current amount of theobromine (0.0011 mol/l) required to produce a cariostatic effect is 71 times less than that required of fluoride (0.0789 mol/l) in a dentifrice to produce a comparable effect. The present study is in agreement with previous studies that demonstrated the capability of the chosen artificial saliva [Adam et al., 2011] and sodium fluoride dentifrice [Walsh et al., 2010; Zero et al., 2010] to induce the remineralization of enamel lesions. The remineralization observed with theobromine confirmed the report of two previous studies [Sadeghpour and Nakamoto, 2011; Kargul et al., 2012], in which regular exposure of a demineralized enamel surface to theobromine induced surface recrystallization and increased the enamel SMH compared with sodium fluoride [Sadeghpour and Nakamoto, 2011]. In their extensive study of theobromine, Sadeghpour and Nakamoto [2011] subsequently subjected the remineralized surfaces to further acid challenge and observed that the theobromine-treated teeth had lost 8% less calcium than fluoride-treated teeth, when the amount of calcium dissolved from each group was measured.

Observation of significant net remineralization, when the rate of demineralization is known to be greater than that of remineralization [ten Cate et al., 1988], confirmed that the treatments in this study, especially theobromine and NaF, enhanced the resistance of the remineralized surface to subsequent acid challenge. Thus the remineralization produced by the theobromine treatment signifies increased resistance to further acid attack by the enamel crystals repaired by the theobromine-containing medium. This is not surprising as it can be attributed to a previous observation that crystallite size was increased and crystallinity of teeth improved by growing hydroxyapatite in an apatite-forming system containing an effective amount of partially alkylated xanthine (theobromine) either in vitro such as in artificial saliva or in vivo like in growing rats feeding on theobromine-containing diet [Nakamoto et al., 1999, 2001]. Among the various concentrations of theobromine (0.0056, 0.0278, 0.0555, 0.139, 0.278, 0.555, 1.1, 2.78 mmol/l) investigated in these studies, the effective concentration capable of producing the desired increase in crystallite size was 1.1 mmol/l. A crystallite or a cluster of crystallites measuring over 2 μm was observed when grown in vitro in the presence of 1.1 mmol/l of theobromine, while a crystallite measuring 0.5 μm was obtained in the absence of theobromine [Nakamoto et al., 1999, 2001]. Similar results were observed in vivo, on analysis of the teeth of animals grown with theobromine added to the diet. In addition to enhancement of the crystallinity of the apatite, the acid dissolution resistance of the apatite was also found to be improved. Crystallite size is one of the major factors that control the rate of apatite dissolution. A large crystal has a small specific surface area and therefore presents a limited surface area for chemical reactivity, and hence dissolves slower than small crystals that offer an enormous area for reactivity [Eanes, 1979]. The cariostatic effect of cocoa as shown in animal studies [Strålfors, 1966a, b, c, 1967] may now be explained by the large crystal formation [Nakamoto et al., 1999, 2001] within the tooth tissue and increased microhardness [Sadeghpour and Nakamoto, 2011; Kargul et al., 2012] of the enamel surface in the presence of theobromine as demonstrated in the present study.

Although EDS quantification of the calcium level in mineral deposit on the surface of the lesions did not indicate any significant difference between the three treatment modalities, more mineral, as represented by the calcium content, was deposited with theobromine and NaF treatments. This increased calcium deposition may contribute to the increased crystal formation and crystal size in an apatite-forming system [Nakamoto et al., 1999, 2001] (such as artificial saliva) in the presence of theobromine or NaF, and may have contributed to the enhancement of lesion resistance to subsequent acid demineralization. However, the mechanism by which theobromine will induce increased mineral deposition and increased crystal growth still needs to be investigated, and it is the subject of the authors' current studies.

In conclusion, the present study demonstrated that theobromine in an apatite-forming medium can enhance the remineralization potential of the medium as determined using an established in vitro caries cycling model, and may be a viable alternative to fluoride additives in commercial dentifrices. In this model, theobromine, at a molar level 71 times less than that of fluoride, has a remineralization effect on enamel lesions comparable to that

of fluoride. 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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Disclosure Statement

There were no conflicts of interest on the part of any authors or investigators; however, Arman Sadeghpour and Tetsuo Nakamoto are employees of Theodent LLC, New Orleans, La., USA, the company that provided the funding to Dr. Bennett Amaechi.

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