Evaluation of Human Enamel Surfaces Treated with Theobromine: A Pilot Study

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Purpose: The objectives of this in-vitro study were to investigate the effect of theobromine, which is the principle xanthine species in *Theobroma cacao*, at two concentrations on the surface hardness and topography of human enamel.

Materials and Methods: Twenty-four freshly extracted human third molars were collected and stored in distilled water with 0.1% thymol solution at room temperature prior to the experiments. The enamel specimens were treated with one coat of theobromine at two concentrations (100 mg/l or 200 mg/l in distilled water) for 5 min. Enamel surfaces in the control group received no theobromine. They were then kept in distilled water for 1 week and subjected to SEM analysis. The specimens were demineralised by storing them in acidic hydroxyethylcellulose for three days. After baseline microhardness measurements, they were incubated either in 100 or 200 mg/l theobromine for 5 min. The control group was kept in distilled water. After washing the specimens under distilled water, they were kept in a remineralising solution for 18 h. Microhardness of the enamel surface was initially determined for each specimen before artificial demineralisation. After demineralisation, the experimental groups were incubated in 100 mg or 200 mg theobromine and control-group specimens were placed in remineralising solution.

Results: Enamel surfaces of the untreated control group presented a generally smooth and slightly hummocky surface with small lines of pits. Specimens treated with theobromine showed differences between the two concentrations. The group treated with 200 mg/l solution for 5 min showed a greater quantity of globules on enamel than did specimens treated with 100 mg/l solution.

Conclusion: As shown by the microhardness values, a consistent and remarkable protection of the enamel surface was found with the application of theobromine.

Key words: enamel, fluoride, SEM, *Theobroma cacao*, theobromine

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In spite of the progress of in situ and in vivo research in cariology, laboratory tests are still widely used to evaluate dental caries and the effect of fluoride (F) on the inhibition of enamel-dentin demineralisation and enhancement of remineralisation (Queiroz et al, 2008). It is safe to assume that in vitro experiments are the most commonly applied methods in dental research. In vitro remineralisation models include experiments directed toward determining fundamental factors important to caries lesion repair and the efficacy of treatment modalities in potentiating lesion consolidation (White, 1995).

Feagin et al (1969) combined microhardness studies with chemical analyses of the changes in calcium (Ca) and phosphorus (P) loss and uptake. Under carefully controlled conditions, a stoichiometric dissolution and redeposition of hydroxylapatite was found in enamel demineralisation and remineralisation.

The first report on the remineralisation of dental enamel in vitro was published by Head in 1910, who observed a rehardening of acid-softened enamel after exposure to saliva. In the past 50 years, many investigations have focused on the relation-

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ship of fluoride to enamel solubility and remineralisation. Typically, such studies measure the enamel surface microhardness initially, after demineralisation and after 2, 6 and 10 days of the remineralising treatment, using a Kentron microhardness tester equipped with a diamond Knoop indenter and a 500-g load. Mineral changes in superficial enamel layers are directly related to microhardness alterations, i.e. remineralisation of enamel carious lesions is associated with an increase of enamel surface microhardness (Ten Cate and Arends, 1978; Finke et al, 2000). Although microhardness change is an indirect method for measuring mineral deposition within lesions, it seems to be the most sensitive criterion, as reflected by the levels of statistical significance of the data at the different stages of the remineralising treatment. The fact that microhardness recovery is greater than that shown in abrasion biopsies suggests that in reconstitution of the lesion, loose particles binding high amounts of fluoride are deposited in porosities left after dissolution of the well-crystallised mineral of sound enamel. Apparently, such particles within the lesion present a disproportionately higher resistance to indenter penetration than indicated by the density of the tissue (Jima and Koulourides, 1988). Remineralisation of incipient carious lesions occurs naturally in the oral environment when the cariogenic challenge is minimised. In vitro experimentation shows that remineralisation can be attained by exposure of demineralised enamel to solutions containing calcium and phosphate ions. The remineralisation rate appears to be proportional to the degree of supersaturation of the solutions with respect to hydroxylapatite, and it is enhanced by fluoride at low concentrations. These observations are significant because they are very similar to those reported in relation to crystal growth rates in supersaturated solutions seeded with hydroxylapatite crystals. In spite of the considerable volume of literature dealing with remineralisation in vitro, there are not enough clinical studies to assess the possible benefits of synthetic remineralising solutions. Furthermore, enamel demineralisation under oral conditions is generally thought to result from acid conditions produced by the metabolism of cariogenic microorganisms in dental plaque. However, the formation of dental caries is more complex than a simple dissolution process of the hard tissue. The striking histological feature of the incipient lesion is the presence of a relatively sound surface layer of enamel overlying the demineralised zone, i.e. the bulk of mineral loss in the early stages of demineralisation occurs at some distance from the enamel surface. For this reason, the term 'subsurface lesion' is used to describe this stage of the process (Moreno and Zahradnik, 1979).

Theobromine is a bitter alkaloid of the methylxanthine family, which also includes the similar compounds of theophylline and caffeine (William, 1943). Present in the *Theobroma cacao* tree, it is the primary alkaloid found in cocoa and chocolate. In chocolate, theobromine exists in doses that are safe for humans to consume in large quantities. In vivo results from experiments on rats indicate that theobromine enhances crystallinity and dissolution resistance of the apatite in enamel (Nakamoto et al, 1999; Nakamoto et al, 2001). The amount of theobromine that rats consumed has an equivalent human consumption in proportion to body weight and pharmacokinetic differences. For the most accurate estimate, the extrapolation should be adjusted either for the pharmacokinetics or theobromine metabolism. In humans, the half life of theobromine in plasma is about 6 h. Assuming that the theobromine content of a 1-oz. bar of milk chocolate is 45 to 105 mg, the intake of theobromine used in the animal study (Nakamoto et al, 1999; Nakamoto et al, 2001) is approximately 3 to 7 1-oz. bars of milk chocolate, when extrapolated using the pharmacokinetics of theobromine metabolism. Theobromine is the active ingredient in chocolate.

Cocoa beans, which are the main constituent of chocolate, contain some polyphenols which exhibit anti-glucosyltransferase activity. A study by Ooshima et al (2000) reported that the sucrose-dependent cell adherence of mutans streptococci was significantly depressed by cocoa mass extract. It was also shown that cocoa mass extract in a 40% sucrose diet and drinking water reduced plaque and caries development in *Streptococcus sobrinus*-6715-infected rats, but not significantly. The result of this experiment showed that although cocoa mass extract did contain a certain anti-caries potential, it was not enough to significantly reduce the cariogenic strength of sucrose.

Chocolate has been suspected to be less cariogenic than would be expected from its high sucrose content; a group of patients in the Vipeholm study who received chocolate daily between meals were found to have more caries than the control group, but less than other groups to whom sucrose was provided in caramels or toffees (Gustaffson et al, 1954). Although some promising results exist in the literature, different concentrations of theobromine have not been studied and their effect on

enamel surfaces is not known. Remineralising agents and agents inhibiting the dissolution of apatite can prevent caries formation or arrest the progress of the caries lesions, for instance, by adding or increasing calcium phosphate levels in plaque and saliva (Zahradnik et al, 1979). Because de- and remineralisation processes are difficult to detect at early stages of formation on enamel by visual inspection, many diagnostic techniques exist for detecting the demineralisation which occurs as a result of the caries process (Lippert et al, 2004). Better knowledge of demineralisation/remineralisation cycles has led to the development of several new approaches to determining enamel microhardness. A major drawback of previous microhardness studies was that the remineralised enamel was not subject to subsequent demineralisation. The objective of this in vitro study was therefore to investigate the effect of two different concentrations of theobromine on the surface hardness and topography of human enamel; the null hypothesis was that theobromine treatment had no effect on the surface hardness and topography of enamel.

MATERIALS AND METHODS

Specimen preparation

Twenty-four freshly extracted human third molars were collected and stored in deionized water for up to 3 months until they were used in the experiments. Soft tissue remnants were gently removed using hand instruments and scalers. The roots were then sectioned under cooling and the crowns were mounted in metal rings using polymethylmethacrylate (Palapress Vario, Heraeus Kulzer; Wehrheim, Germany). A total of 24 enamel blocks of 3 x 2 mm were sectioned from the buccal surfaces of the teeth with a slow-speed saw (Isomet, Buehler; Lake Bluff, IL, USA) under water cooling.

Study design

The 24 specimens were randomly divided into 3 groups: two in which theobromine (100 mg/l or 200 mg/l of distilled water) was applied and the control group (without theobromine). Each specimen was treated with theobromine for 5 min using a clean brush prior to SEM analysis.

Microhardness tests

The 24 enamel specimens (3 x 2 mm) were embedded in epoxy resin. The enamel surfaces were ground using 1200-grit waterproof silicon carbide paper under water cooling, removing about 200 µm enamel. The enamel samples were then stored in tap water prior to testing. To produce demineralised lesions, the samples were stored in acidic hydroxyethylcellulose (HEC, pH 4.8) for three days, in accordance with the method of Amaechi et al (1998). Immediately afterwards, the enamel samples were carefully washed using tap water to remove any excess acid. After demineralisation, 8 specimens each were incubated in 100 or 200 mg theobromine dissolved in one liter of distilled water for 5 min. For the control group, 8 specimens were kept in remineralising solutions (1.5 mM calcium, 0.9 mM phosphate, 150 mM of KCl in 0.1 M tris buffer, pH 7.0) for 18 h.

Hardness was determined by the Vickers hardness test (VMT-7, Buehler) under a load of 25 g. In a preliminary investigation, loads in the range of 10–100 N were studied. Impressions were performed at 10 regions in each one of the specimens. Vickers hardness values were calculated using equation 1, where 'P' was the applied load (N), 'd' was the average of the diagonal length (m) and α the angle between the opposite faces of the indenter (136 degrees) (Harper et al, 1978).

$$H_V = \frac{\alpha . P}{d^2} \qquad \text{(Eq. 1)}$$

Scanning electron microscopy (SEM)

Cold field emission SEM (JSM-5500, JEOL Instruments; Tokyo, Japan) images were taken at 25 kV at a magnification of 1000X. The enamel surfaces were first sputter coated with a 3-nm-thick layer of gold (80%) / palladium (20%) prior to examinations.

Statistical analysis

Statistical analysis was performed using the SPSS 14.0 software for Windows (SPSS; Chicago, IL, USA). The means of each group were analysed by 2-way ANOVA with the microhardness values as the dependent variable and the theobromine concentration as the independent variable. Due to the sig-

Table 1 Median, minimum and maximum microhardness values for the enamel surfaces before and after demineralisation and after theobromine 100 mg/l application

	Median	Min	Max	*P*-value
Baseline	302.80	198.50	338.30	$P < 0.05$, baseline vs after demineralisation
After demineralisation	255.10	35.10	285.40	$P > 0.05$, after demineralisation vs theobromine 100 mg/l
After theobromine 100 mg/l	224.00	23.60	321.40	$P < 0.01$, baseline vs theobromine 100 mg/l

Table 2 Median, minimum and maximum microhardness values for the enamel surfaces at baseline, after demineralisation and after theobromine 200 mg/l

	Median	Min	Max	*P*-value
Baseline	269.10	207.70	307.10	$P > 0.05$, baseline vs after demineralisation
After demineralisation	255.40	51.90	319.90	$P < 0.05$, after demineralisation vs theobromine 200 mg/l
After theobromine 200 mg/l	165.00	22.80	279.00	$P < 0.01$, baseline vs theobromine 200 mg/l

Table 3 Median, minimum and maximum microhardness values for the enamel surfaces after remineralisation and after theobromine application (100 mg/l and 200 mg/l)

	Median	Min	Max	*P*-value
After theobromine 100 mg/l	224.00	23.60	321.40	$P < 0.05$, after theobromine 100 mg/l vs after theobromine 200 mg/l
After theobromine 200 mg/l	165.00	22.80	279.00	$P < 0.01$, after theobromine 200 mg/l vs after remineralisaion
After remineralisation	246.70	193.70	321.50	$P < 0.05$, after theobromine 100 mg/l vs after remineralisation

nificant difference between groups ($P = 0.005$), Bonferroni's post-hoc test was performed. *P*-values less than 0.05 were considered to be statistically significant in all tests.

RESULTS

Microhardness test

Surface microhardness (SMH) was measured at baseline, after demineralisation and after theobromine 100 mg/l, 200 mg/l or remineralisation of the control. SMH showed statistically significant differences between baseline, after demineralisation and after theobromine 100 mg/l ($P = 0.0023$) (Table 1). The differences between baseline, after demineralisation and after theobromine 200 mg/l ($P = 0.0016$) were also statistically significant (Table 2). Furthermore, there were also statistically significant differences between baseline, after demineralisation and after remineralisation ($P = 0.031$). SMH showed that 200 mg/l theobromine protected enamel specimens better than did 100 mg/l theobromine ($P < 0.05$; Table 3).

SEM analysis

Representative SEM micrographs at a magnification of 1000X show that enamel surfaces of the untreated control group were generally smooth and slightly hummocky with small lines of pits (Fig 1). In the samples treated with theobromine, the amount of deposits differed depending on concentration. The group that was treated with 200 mg/l solution for 5 min had the largest quantity of globules on enamel.

The enamel surfaces of the groups treated with theobromine were only slightly less smooth than those of the untreated controls, with the shallow lines or pits more pronounced than in the untreated control specimens, as shown in representative SEM micrographs (magnification 1000X). There was a variable amount of deposits observed for the two concentrations (100 mg/l, Fig 2; 200 mg/l, Fig 3). The group that was treated with the 200 mg/l theobromine solution for 5 min exhibited the largest quantity of globules (Fig 3), which indicated greater protection of the enamel surface.

DISCUSSION

With less severe demineralisation, chemical changes in enamel should reflect a more linear correlation with changes in microhardness. The difference in values between baseline and demineralization in Table 1 cannot be reasonably explained. However, the fact that the hardness after remineralisation was lower than at baseline may suggest that hydroxylapatite is not the whole product (Wei and Koulourides, 1972).

The limitations of in vitro test systems for both mechanistic and profile applications were summarised in a previous review (White, 1995). Overall, in vitro models are mechanistically limited in three key ways: inadequate simulation of biological aspects of caries, difficulty in matching solid/solution ratios occurring in vivo and artifacts associated with substrate choice/reaction conditions. The inability to match the breadth of relevant biological conditions limits the clinical relevance of in vitro test results and profile applications of these models. A second limitation of in vitro protocols is the difficulty in simulating the volume and composition of saliva as well as tooth surface area encountered in in vivo remineralisation. The proper simulation of in vivo remineralisation would require knowledge of fluid volume, coverage and transport over substrate surfaces accounting for dynamic variations under dental plaque. A third mechanistic limitation encountered by in vitro models (and by in situ models as well) involves artifacts associated with choice of substrate and test conditions, particularly the time periods (rates) of de- and remineralisation used in studies. With respect to time, it must be considered that in vitro test systems examining de- and remineralisation necessarily accelerate the mineral dynamics associated with the caries process and reversal, so much shorter time periods are involved than occur in vivo (White, 1995).

In vitro demineralisation and remineralisation studies remain important dental research tools for both fundamental and applied (profile) testing purposes. The main advantage of in vitro testing which drives its continued utilisation despite limitations is that it provides investigators with the capability of performing single-variable experiments under controlled conditions. Such experimentation permits mechanistic studies to be performed which are difficult if not impossible to carry out in vivo (White, 1995).

Cocoa beans, which form the main constituent of chocolate, contain some polyphenols which exhibit

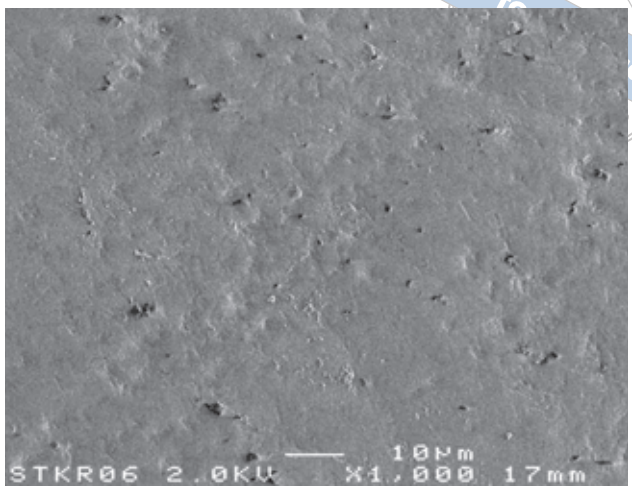

Fig 1 SEM micrograph showing enamel surface of the untreated control group (1000X).

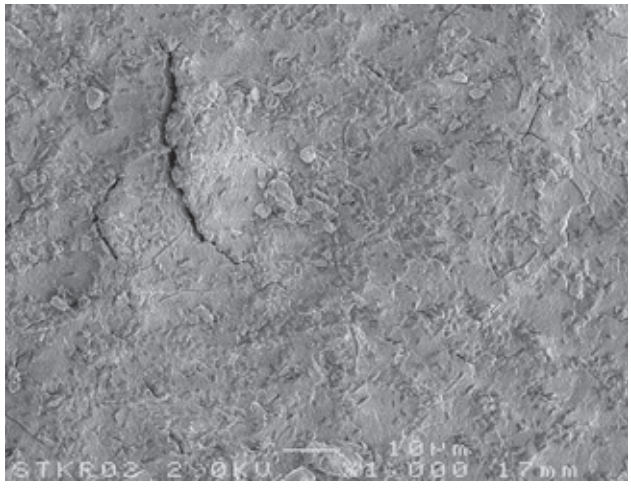

Fig 2 SEM micrograph showing enamel surface alteration after theobromine 100 mg/l application (1000X).

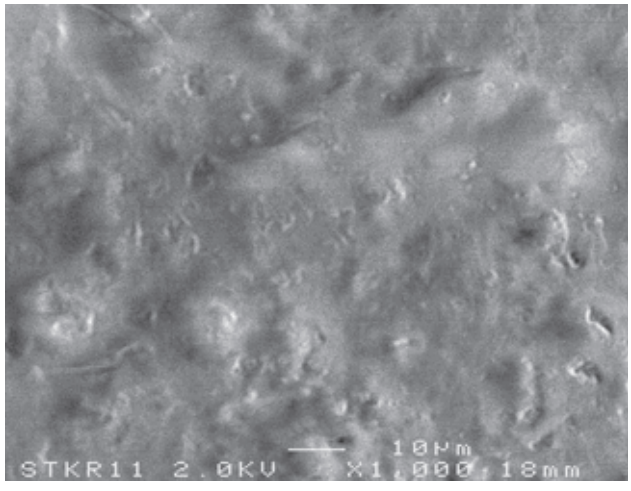

Fig 3 SEM micrograph showing enamel surface alteration after theobromine 200 mg/l application (1000X).

anti-glucosyltransferase activity. Cocoa-bean husk extract was examined for cariostatic activity in in vitro and animal experiments. It reduced the growth rate of mutans sterptococci, insoluble glucan synthesis by glucosyltransferase of mutans streptococci and caries induction in specific pathogen-free rats infected with human mutans streptococci. These results indicate that the cocoa extract possesses anticariogenic potential, but this anticariogenic effects itself is not enough to inhibit the caries (Ooshima et al, 2000). Cocoa mass extract, the main component of chocolate, showed weak antiglucosoyltransferase activity in vitro experiments and also exhibited a weak, but not significant, cariostatic activity in specific pathogen-free rats infected with *Streptococcus sobrinus* 6715.

Theobromine increases the crystal size of hydroxylapatite, as shown in previous studies in vitro (Nakamoto et al, 1999; Nakamoto et al, 2001), and the significant alteration of the enamel surface by theobromine is indicated by the increased hardness in the present study. Based on the results of this study, the null hypothesis was rejected. The mineral changes in superficial enamel layers are directly related to microhardness alterations, i.e. remineralisation of enamel carious lesions is associated with an increase of enamel surface microhardness (Ten Cate and Arends, 1978; Finke et al, 2000). We propose that theobromine may possess a unique property of preventing the dental caries due to the increase of the crystal size, which inhibits the dissolution of the apatite of the enamel surface. This would facilitate caries prevention. Theobromine has been observed to be much better than fluoride at increasing the hardness on the enamel surface (unpublished observations), which supports the idea that chocolate may be less cariogenic than would be expected from its high sucrose content (Ooshima et al,2000).

In a study by Stralfors (1967), cocoa reportedly reduced the occurrence of dental caries in hamsters when incorporated in their diet and given ad libitum, but no explanation was given as to why cocoa extract decreased the caries incidence in hamsters. We suggest that the possible caries-preventive effect of cocoa extract is due the effect theobromine has in forming large crystals (Nakamoto et al, 1999; Nakamoto et al, 2001) and higher values of microhardness on the enamel surface, as shown in the present study. Greenby (1974) demonstrated that young adults on a chocolate skim milk diet for 5 days accumulated less dental plaque than those on a normal diet. Caffeine intake in newborn rats has been shown to increase dissolution of Ca, P and Mg from the enamel surface (Nakamoto et al, 1993). This possibly indicates an impairment of amelogenesis (Falster et al, 1992); in fact, caffeine intake resulted in the formation of the smaller crystallites (Falster et al, 1993) and a higher caries score (Nakamoto et al, 1993). Thus, based on these results, we hypothesized that caffeine intake may lead to an increased susceptibility to dental caries, as the smaller crystal size is known to facilitate increased dissolution of the minerals. The widespread human consumption of caffeine during the critical period of tooth development could be a possible threat to the healthy development of teeth and may lead to the increase in caries in these offpring. Paolino and Kashket (1985) suggested that the inhibitory effects of cocoa on plaque accumulation and caries induction could be due to its inhibition of bacterial polysaccharide production, as extracts of defatted cocoa were found to inhibit the biosynthesis of extracellular polysaccharide. The cariogenic potential of plain chocolate is proportional to that of the same concentration of sucrose contained in the chocolate, when the cariogenic potential of food was examined in rats under strictly controlled conditions; the authors speculated that chocolate itself contains no cariogenic substances (Greenby and Mistry, 1995).

Theobromine shares the pharmacologic effects of the other methylxanthines, theophylline and caffeine. These effects include central nervous system stimulation, production of diuresis, stimulation of cardiac muscle. Theobromine is present in all cocoa products. In a study by Resman et al (1977), six nursing mothers ingested 113 gm of Hershey's milk chocolate containing 240 mg of theobromine. If a mother ate a 4-ounce chocolate bar every 6 h and the infant nursed when the theobromine concentration in milk was at its peak, the infant could ingest about 10 mg of theobromine per day. The amount of theobromine contained in four ounces of Hershey's milk chocolate is similar to the amount (240 mg) used as a single pharmacological dose (Resman et al, 1977). In the United States, theobromine is a constituent of such dietary staples as chocolate, coffee, tea and many colas, and as such may be consumed in pharmacologically relevant amounts (6 mg/kg is present in five cups of chocolate). Theophylline is frequently prescribed in the treatment of asthma to relax the smooth respiratory muscles.

In the SEM images, the enamel surfaces of the control group were generally smooth. The enamel

surfaces of 100 mg/l theobromine group were only slightly less smooth, showing shallow lines or pits. Mineral deposition takes place as crystal growth, rather than as mineralisation of the organic matrix in enamel. Furthermore, enamel remineralisation as surface precipitation has been suggested to be a seeded growth of hydroxylapatite-like material in which an amorphous precursor phase is formed that undergoes rapid transformation to crystalline hydroxylapatite. Greenby (1974) found that the exposure of surface-softened enamel samples to a remineralisation solution resulted in the deposition of a fairly rough, crystalline surface layer.

As far as we are aware, theobromine and fluoride are the only two chemicals to increase the crystal formation. Theobromine, which unlike fluoride is a nontoxic substance, should be considered in future studies for various applications in the oral field, including caries prevention. In addition, in vivo studies are necessary to confirm or disprove the hypothesis that cocoa has a possible cariostatic role via theobromine-induced large crystal formation and increased microhardness of the enamel surface.

CONCLUSIONS

Based on the results of this study, the following conclusions can be drawn:

- Surface microhardness values showed that 200 mg/l theobromine protected enamel specimens more than 100 mg/l theobromine did.
- A consistent and remarkable protection of the enamel surface was observed with the theobromine groups.

Thus, we propose theobromine, a nontoxic compound, as an alternative to fluoride, which is commonly used in toothpaste world-wide. We hypothesise that the possible cariostatic effect of cocoa extract is due to the physical alteration of the enamel surface by theobromine.

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