

EVALUATION OF REMINERALIZATION POTENTIAL OF THEOBROMINE ON HUMAN ENAMEL SURFACES- AN *IN VITRO* STUDY

Dental Science

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ABSTRACT

Aim: The study was done to evaluate the remineralization potential of theobromine on human enamel surfaces.

Materials and methods: Thirty two freshly extracted human third molars and premolars collected and embedded in polymethylmethacrylate. The enamel specimens are subjected to baseline microhardness testing. The specimens were then demineralised using Mc Innes bleaching solution and then subjected to microhardness testing. The enamel specimens were randomly divided into 4 groups : Group A theobromine 100mg/l; Group B theobromine 200mg/l; Group C with GC tooth mousse plus containing casein phosphopeptide amorphous calcium phosphate fluoride -0.2 % w/w (900 ppm) for 5 minutes respectively. Enamel specimens in the control group (Group D) was kept in deionised water. Post remineralization enamel microhardness was determined by Vicker's microhardness test. Statistical significance of microhardness values of each specimen was analysed using Anova.

Results: Post remineralization microhardness values shows increase in enamel surface microhardness. Remineralization was indicated by increase in enamel hardness. There was an increase in enamel microhardness of about 27.4% in Group A; 36 % in Group B and 22 % in Group C. There was no alteration in enamel hardness in Group D (control). The difference noted in microhardness values from demineralization to remineralization in all the three test groups were found to be statistically highly significant. ($p < 0.001$).

Conclusion: Theobromine 200mg/l has a superlative remineralizing capacity than CCP-ACPF. Theobromine can be added to various dental products to remineralize enamel and prevent caries. It would be a cost effective alternative to fluoride, without the negative side effects of fluorosis.

KEYWORDS

INTRODUCTION

Dental caries is a multifactorial disease caused by the interaction of dietary sugars, dental biofilm and the host's dental tissue within the oral environment. It is the cumulative result of consecutive cycles of demineralization and remineralization at the interface between the biofilm and the tooth surface. Oral bacteria excrete acid after consuming sugar, leading to demineralization. Upon this acid challenge, the hydroxyapatite crystals will dissolve from the subsurface. Remineralization is the natural repair process for non cavitated lesions. Dental caries in its early stage of formation can be remineralized; and this remineralization can be facilitated by such agents as fluoride delivered via either mouth rinse or dentifrice. Fluoride is the most potent and effective substance yet developed for the prevention and treatment of caries. Fluoride is a double edged sword. Fluoride at optimal level, decreases the incidence of dental caries but at the same time at higher levels it can cause adverse effects like dental fluorosis and skeletal fluorosis. Few studies have shown that, a raw material from cocoa beans called theobromine, strengthens tooth enamel and exhibits better remineralization than fluoride without adverse health effects.¹

Theobromine is an alkaloid belonging to the methylxanthines. It is mainly found in cocoa and chocolate. Theobromine levels are highest in dark chocolate. It is also present in tea and cola nuts. In chocolate, theobromine exists in doses that are safe for humans to consume in large quantities. It was first discovered by Alexandra Voskresky in the year 1842. It has a similar effect like caffeine, which possess stimulant properties. It lowers blood pressure by dilating the blood vessels. Its relaxes bronchi and acts on vagus nerves and has anti tussive action. It is a potent diuretic, hence it can be useful in the treatment of cardiac failure, edema, syphilitic angina attacks, and degenerative angina. In the human body, theobromine levels are halved between 6–10 hours after consumption.

Sadeghpour (2012) measured the microhardness of human teeth enamel treated either with artificial saliva or artificial saliva with theobromine and found that theobromine stimulated the formation of new enamel.² “The mineral that makes up teeth, hydroxylapatite, under normal conditions is 0.5 μ . Theobromine allows for calcium and phosphorus from saliva to come together in a larger unit crystal of 2 μ which is four times the size of hydroxylapatite. Sadeghpour and Carey found that theobromine protected teeth from decay better than fluoride. “The amount of theobromine in a one ounce dark chocolate bar has a better effect on tooth hardness than a 1.1% prescription sodium fluoride treatment,” Carey showed that at a concentration 142 times less than that of fluoride, the theobromine active had twice the protective effect on teeth.³

According to study conducted by Amaechi et al (2012), theobromine found in chocolate is better than fluoride in remineralizing and hardening tooth enamel.¹ Theobromine belongs to methylxanthines. Theobromine was found to be readily absorbed by the gut, metabolized by humans. Hence, it can be safely ingested, obviating the “do not swallow” warning on fluoride toothpastes and mouthwash. This study was done to evaluate the remineralization potential of theobromine on human enamel surfaces and to compare it with CCP ACPF (Casein Phosphopeptide Amorphous Calcium Phosphate Fluoride. (fluoride – 0.2% w/w 900 ppm)

The objectives of this *in vitro* study were

- To assess the remineralizing effect of theobromine 200mg/l and 100mg/l on human enamel surfaces.
- To compare remineralization potential of theobromine with Casein Phosphopeptide Amorphous Calcium Phosphate Fluoride. (fluoride – 0.2% w/w 900 ppm)

MATERIALS AND METHODS

An *in vitro* study was conducted to evaluate the remineralization potential of theobromine on human enamel surfaces.

Following consent from the donors, freshly extracted human molar and premolar teeth were collected. Caries-free molars requiring extractions for impaction reasons and caries free premolars extracted for orthodontic reasons were taken. Thirty two freshly extracted human third molars collected and stored in de-ionized water were used in the experiments. Soft tissue remnants were gently removed using hand instruments and scalers. The enamel specimens were embedded in polymethylmethacrylate. The enamel surfaces were ground using 1200 grit waterproof silicon carbide paper under water cooling. The specimens were ground to provide a flat surface to measure microhardness with the hardness indenter. The enamel specimens were polished to achieve a flat surface. The enamel specimens were then stored in de ionized water prior to testing.

For microhardness testing, the specimens were placed on the stage of the tester and stabilized. Then the exposed areas were selected by focusing with 400x objective lens. The specimens were subjected to a load of 50g with a dwell time of fifteen seconds to record the indentations. To avoid any discrepancy in values, two indentations were made and an average value of both was obtained and tabulated. The values were recorded in terms of Vickers Hardness Number. The indentations formed were viewed carefully with the objective lens as seen clearly on the display monitor.

In this study, freshly prepared McInne's bleaching solution was chosen as the demineralizing agent that was indigenously prepared. Composition of McInne's bleaching solution:

- 1ml of 36% hydrochloric acid
- 1ml of 30% hydrogen peroxide
- 0.2 ml of anesthetic ethyl ether

The contents were carefully mixed in a proportion of 5:5:1.

The mixture was freshly prepared in a conical flask and used for each application.

Freshly prepared McInne's solution was applied onto the exposed enamel surface of all samples one by one using a cotton microapplicator and left for five minutes.

The specimens were then washed under de-ionized water, dab dried, subjected for microhardness testing and the Vickers Hardness number values were tabulated.

The theobromine at two concentrations were extracted by means of the following procedure. About 20 grams of cocoa bean husk was diluted with approximately 150 ml of deionised water in a heated circulating bath at 90° centigrade for 90 minutes. The solution was then cooled down to room temperature, it was centrifuged at 3000 rpm, and the supernatant was filtered through a Millipore filter of 45 µm of pore diameter. Subsequently, the solution was injected into the chromatographic system for the cleanup procedure and determination. The retention factor for theobromine was found at 0.7 and theobromine was spotted using thin layer chromatography plates. The solution containing theobromine is then boiled to get the powdered theobromine extract. From the theobromine extract obtained, 100mg of the powdered extract was dissolved in 1litre of de ionized water to prepare 100mg/l theobromine. Similarly, 200 mg of the extracted theobromine was dissolved in 1litre of de ionized water to prepare 200mg/l theobromine.

Thirty two enamel samples were divided into:

Test group: Group A – Theobromine 200mg/l; Group B – Theobromine 100mg/l; Group C – CCP-ACPF; Control group: Group D – De ionized water. After baseline, microhardness testing, the enamel samples were demineralised using McInne's bleaching solution. Post demineralization microhardness testing was done. The enamel samples are then subjected to remineralization using the respective remineralizing agents for 5 minutes. Enamel specimens in the control group were kept in de ionized water. Later, they were subjected to post remineralization microhardness testing

The results were analyzed using the statistical package for social science system (SPSS) version 17.0. For individual group comparison, one way ANOVA was employed. The significance level was set at $p <$

0.05. Tukey post hoc test was used for multiple comparisons.

RESULTS:

The study was conducted to evaluate the remineralization potential of theobromine on enamel surfaces. An *in vitro* study was done using theobromine at two different concentrations 100mg/l and 200mg/l and their effect was compared with fluoride containing CCP-ACPF. Vickers microhardness testing was done to evaluate the increase in enamel surface microhardness.

Mean microhardness values at baseline and post demineralization:

Baseline microhardness of all the samples were tested. After which, the samples were demineralised using McInne's bleaching solution and then subjected to microhardness testing. There was demineralization in all the samples indicated by decrease in enamel microhardness values. There was 30% demineralization in Group A; 31 % in Group B, 24 % in Group C and 23 % in Group D respectively. There was reduction in microhardness noted from baseline to demineralization in all the groups and this reduction was found to be statistically significant. (Table 1)

Mean microhardness values after remineralization using theobromine 100mg/l; theobromine 200mg/l; CCP-ACPF; and control group:

After demineralization, the enamel samples were treated with the study and test products respectively. Eight samples in Group A were treated with 100mg/l theobromine for 5 mins; Eight samples in Group B were treated with 200mg/l theobromine for 5 mins; Eight samples in Group C were coated with (GC tooth mousse plus containing casein phosphopeptide amorphous calcium phosphate fluoride -0.2 % w/w (900 ppm) respectively for 5 mins and Eight samples in Group D were kept as control and they were stored in de ionized water. They were then subjected to microhardness testing.

Post remineralization microhardness values shows increase in enamel surface microhardness. Remineralization was indicated by increase in enamel hardness. There was an increase in enamel microhardness of about 27.4% in Group A; 36 % in Group B and 22 % in Group C. There was no alteration in enamel hardness in Group D (control). The difference noted in microhardness values from demineralization to remineralization in all the three test groups were found to be statistically highly significant. (Table 2).

On comparing the post remineralization microhardness values, there was 27.4 % remineralization in theobromine 100mg/l and 36 % remineralization in theobromine 200mg/l. The remineralization potential of theobromine 200mg/l was higher compared to theobromine 100mg/l and the difference noted was found to be statistically significant. ($p=0.046$). (Table 3)

When comparing remineralizing ability, there was 27.4 % remineralization in theobromine 100mg/l and 22% in CCP-ACPF. Theobromine 100mg/l showed a higher remineralization potential when compared with CCP-ACPF and this difference noted was not statistically significant. ($p=0.515$). (Table 3)

Theobromine 100mg/l showed a better remineralizing effect compared to control group as there was zero percentage remineralization in control group and this difference noted was found to be statistically very highly significant. ($p=0.000$). (Table 3)

Remineralization potential was 36 % in samples treated with theobromine 200mg/l, and 22% remineralization in CCP-ACPF group. Theobromine 200mg/l has higher remineralization potential when compared with CCP-ACPF and the difference noted among these groups were found to be statistically highly significant. ($p=0.003$).

Remineralization potential of theobromine 200mg/l group was 36 % compared to zero percentage increase in control group. Theobromine 200mg/l had a greater remineralizing ability compared to control group and this difference noted was found to be statically very highly significant. ($p=0.000$). (Table 3)

The remineralization capacity of CCP-ACPF was 22% which when compared to control group, showed no change. CCP-ACPF had a

higher remineralization potential compared to control group and this difference noted was found to be statistically very highly significant. (p=0.000).

The overall results signifies that theobromine 200mg/l has higher remineralization potential, than theobromine 100mg/l and CCP-ACPF. The results shows that theobromine 200mg/l has a better remineralizing capacity when compared with CCP-ACPF.

Table 1: Mean microhardness values at baseline and post demineralization:

Groups	Mean baseline	Mean post demineralization	Percentage demineralization
Group A	322.98	222.94	30%*
Group B	362.70	249.64	31%*
Group C	314.94	238.78	24%*
Group D	323.14	248.06	23%*

** P<0.001 highly significant

Table 2: Mean microhardness values at post remineralization:

Groups	Mean post demineralization	Mean post remineralization	Percentage remineralization
Group A	222.94	293.56	27.4%**
Group B	249.64	340.64	36%**
Group C	238.78	292.34	22%**
Group D	248.06	248.06	0%

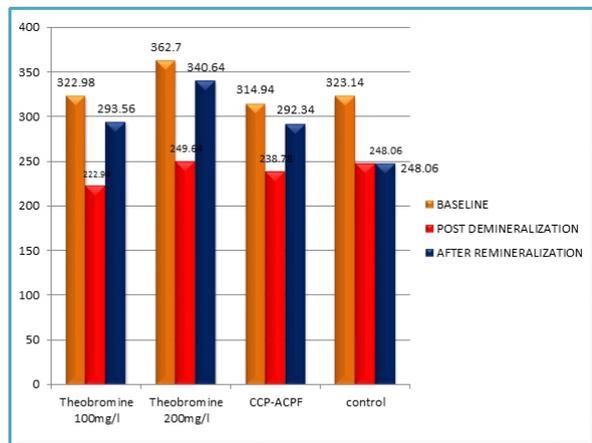
** P<0.001 highly significant

Table 3: Comparison of percentage of remineralization potential between the groups

Group	Percentage remineralization %	Groups	Percentage Remineralization	Significance
Group A	27.4%	Group B	36%	0.046*
		Group C	22%	0.515
		Group D	0%	0.000**
Group B	36%	Group A	27.4%	0.046*
		Group C	22%	0.003**
		Group D	0%	0.000**

** P<0.001 highly significant; * p<0.05 statistically significant
p<0.05 not significant

COMPARISON OF REMINERALIZATION POTENTIAL OF ALL THE GROUP



DISCUSSION

The present study investigated the caries preventive effect of theobromine by examining its ability to cause remineralization of enamel lesions. Theobromine, a natural product has shown enamel remineralizing property. This study was conducted using theobromine at two different concentrations (100mg/l; 200 mg/l) to evaluate its remineralization potential. The remineralization capacity of theobromine was compared with the proven remineralizing agent CCP-ACPF.

Theobromine at a concentration of 200mg/l showed a greater remineralization potential when compared with CCP-ACPF and theobromine 100mg/l. The significant alteration of the enamel surface by theobromine is indicated by the increased hardness in the present study. A study conducted by Tencate et al (1978)¹⁴ and Finke et al (2000)¹⁵ stated that remineralization of enamel carious lesions is associated with an increase in enamel surface hardness. In vitro studies conducted by Nakamoto et al (1999) and Nakamoto et al (2001) has shown that theobromine increases the crystal size of hydroxylapatite.¹⁶ This unique property inhibits the dissolution of the apatite of the enamel surface thereby facilitating caries prevention.¹ According to Amaechi et al (2013) theobromine has found to be better than fluoride, at increasing the hardness on the enamel surface which is in accordance with the results of our present study.¹

A study conducted by B.T.Amaechi et al (2013) investigated the remineralization potential of theobromine in comparison to a standard NaF dentrifice. Theobromine and NaF toothpaste exhibited comparable levels of remineralization, on a molar level, the 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 dentrifice to produce a comparable effect. The present study is in agreement with previous studies that demonstrated the capability of the theobromine to induce the remineralization of enamel lesions.¹

The remineralization observed with theobromine was found to be similar to that observed in the report of studies by Sadeghpour and Nakamoto et al (2011)²; Kargul et al (2012)¹² in which regular exposure of a demineralised enamel surface to theobromine induced surface recrystallization and increased the enamel surface microhardness compared with sodium fluoride. A study conducted by Kargul et al, (2012), scanning electron microscopy shows that the enamel surfaces treated with 200mg/l theobromine had largest quantity of globules on enamel which indicated greater protection of the enamel surface. This could be the reason why in our present study theobromine 200mg/l shows a greater remineralizing potential.

Sadeghpour and Nakamoto (2011)² in their study of theobromine, 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. This can be attributed to a previous observation in a study done by Nakamoto et al., (1999, 2001)^{16,17}, which indicates that crystallite size was increased and crystallinity of teeth was improved by growing hydroxyapatite in an apatite-forming system containing an effective amount of theobromine. Among the various concentrations of theobromine 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. Similar results were observed in vivo study done by Stralfors (1966,1967)^{18,19} on analysis of the teeth of animals grown with theobromine added to the diet.

Study conducted by Eanes (1979)²⁰ had shown that in addition to enhancement of the crystallinity of the apatite, there was remarkable improvement in the acid dissolution resistance of the apatite. 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 hence a limited surface area for chemical reactivity, which dissolves slower than small crystals offering an enormous area for reactivity. Further research has to be done to provide sustenance that it offers resistance to demineralization.

The cariostatic effect of cocoa as shown in studies done by Stralfors, (1966,1967); may be due to the large crystal formation within the tooth tissue and increased microhardness of the enamel surface in the presence of theobromine as demonstrated in the present study and as well as studies done by Sadeghpour and Nakamoto, (2011); Kargul et al., (2012). However, the mechanism by which theobromine increases the crystal size need to be investigated.

The inadequate simulation of biological aspects of caries and in vitro model are potential limitations of the study. There is a clear need for further human clinical studies to exploit the benefit of theobromine in both oral hygiene and caries preventive products.

Fluoride being an effective remineralizing agent, it has adverse effects such as dental fluorosis, tooth discoloration and if ingested it might cause serious effects like gastric irritation, whereas theobromine can be safely ingested.

SUMMARY AND CONCLUSION

From the results of this study, it can be inferred that

- Theobromine offers considerable protection of enamel surface.
- Theobromine 200mg/l had a greater remineralizing potential followed by theobromine 100mg/l and then CCP-ACPF.
- It signifies that theobromine 200mg/l has a superlative remineralizing capacity than CCP-ACPF.

Theobromine is a naturally occurring substance found in high concentrations in cocoa and has demonstrated the capability to remineralize enamel. Theobromine increases the size of the hydroxyapatite crystals in enamel, which is correlated to a decrease in caries incidence. Theobromine can be added to various dental products to remineralize enamel and prevent caries. It would be a cost effective alternative to fluoride, without the negative side effects of fluorosis. Due to minimal side effects, theobromine would be acceptable to use on pediatric patients. Due to the availability of theobromine in chocolate and other food sources, it is becoming a superior alternative to fluoride. It is safe to ingest as it obviates the do not swallow on fluoride dentrifices. It can be incorporated into toothpastes and the pleasant chocolate flavour would make the kids relish it.

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