In-vitro evaluation of effect of theobromine gel on surface hardness of demineralizedenamel, at different time exposures

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Abstract

Context: Remineralizing agents are significant in reducing the progression of early carious lesions and clinical treatment of the disease since they are non-invasive techniques to treating the lesions. One of the tooth remineralization agents that has been proven in preventing caries is fluoride. However, due to its detrimental effects which may occur due to overexposure to fluoride, a number of firms create tooth health products without fluoride. Cacao, or chocolate, is a substitute that is employed.Theobromine is one of the alkaloid compounds that can be found in cacao (Theobroma cacao). It is said that theobromine can prevent enamel demineralization and can restore the enamel's lost hardness.

Aim: The aim of this in vitro study was to evaluate the effect of Theobromine gel on surface hardness of demineralized enamel at different time exposures.

Materials & Methods: Thirty freshly extracted teeth were decoronated and divided mesiodistally into two halves to obtain a total of sixty samples. Samples were randomly divided into four groups as follows.

Group A (n = 15): No treatment

Group B (n = 15): Exposure to Theobromine gel for 1 month (for 4 minutes once in a week)

Group C (n = 15): Exposure to Theobromine gel for 3 months (for 4 minutes once in a week)

Group D (n = 15): Exposure to Theobromine gel for 6 months (for 4 minutes once in a week) The samples were subjected to pre and post- treatment surface hardness measurement using Knoop Microhardness Tester. The values obtained were then subjected to statistical analysis.
Statistical Analysis: Data was analyzed using ANOVA and repeated measures ANOVA. $P<0.05$
Results: The results showed a significant decrease, of microhardness values after demineralization with 1% citric acid. There was also a significant increase in hardness ($p<0.05$) after exposure of the demineralized specimens to theobromine gel for 16 minutes (32.3%), 48 minutes (39.8%), and 96 minutes (43.7%). It can be concluded that exposure to 200 mg/L theobromine gel for 16, 48, and 96 minutes increased enamel microhardness.
Conclusion- The application of Theobromine gel significantly increased the microhardness of demineralized enamel.

Keywords- Theobromine Gel, Enamel Microhardness, Remineralizing Agent, Theobroma Cacao

Introduction

Though the prevalence of dental caries has decreased over the past 30 years, it is still one of the most prevalent oral disorders.[1] Caries is a multifactorial disease that is influenced by the unique combination of pathogenic risk factors and protective variables in each patient. It occurs as a result of the interaction of four key factors: time, tooth, host, and bacteria. Microorganisms will break down substrate in the form of carbohydrates, and the metabolic process produces an organic acid. The subsequent production of the organic acid results in a drop in pH and a rise in H+. The transfer of hydroxyl ions from the enamel will weaken the enamel's structural integrity if this process continues. This process is called demineralization. Eventually, continuous demineralization will cause porosities in the enamel surface and lead to caries.[2] Remineralization is a natural process that replaces lost minerals in their ionic forms in the hydroxyapatite (HAP) crystal lattice.[3] This occurs when the pH is neutral and tooth minerals are redeposited into the carious site in ionic form, resulting in the deposition of freshly created apatite crystals. The newly produced crystals have a bigger size and are more soluble when attacked by acid.[4] One of the tooth remineralization agents that has been proven in preventing caries is fluoride. Fluoride can be delivered topically and systemically. Water fluoridation or taking dietary fluoride supplements in the form of tablets, drops, or lozenges are two ways to provide fluorideto the system. The development of fluorosis, which may occur as a result of fluoride consumption during tooth growth, is a potential risk of fluoride use.[5] Fluorosis can show as anything from white pitting to white striations on enamel surface, tooth damage, tooth loss and brittle bones in children.[6] Due to the potential risks of fluorosis resulting from fluoride consumption, a number of firms create tooth health products without fluoride. Cacao, or chocolate, is a substitute that is employed. A food product made from cacao seeds is chocolate (Theobroma cacao). Many individuals believe that chocolate plays a major role in the development of dental caries because of its sweet flavour. In truth, the high sugar content of the chocolate product is probablyto blame for the tooth damage that develops.[7] Therefore, this study sets out to test use of a topical theobromine gel for 16 minutes, 48 minutes,and 96 minutes on a demineralized enamel surface to examine its effect on enamel hardness as well as its suitability as one method of increasing oral hygiene and protection against caries.

Materials and Methods

Thirty non-carious premolars freshly extracted for orthodontic reason were collected and cleaned of soft tissue debris. Utmost care was taken while handling the extracted teeth by disinfecting them with 5% NaOCl for 5 minutes and rinsed with water. The teeth were then stored in artificial saliva till further use. The teeth were derooted at the level of cementoenamel junction using diamond disc. The only crowns thus obtained were vertically sectioned in two equal halves mesio-distally. The buccal and lingual halves of each crown were mounted in an acrylic ring and a total of sixty samples were obtained. The initial hardness of each sample was measured using Knoop Microhardness Tester. All the samples were then immersed in 1% citric acid with a pH of 4 for 2.5 minutes for demineralization. The hardness of each sample was again measured using Knoop Microhardness Tester. The readings were recorded for further analysis. The samples were then randomly divided into four groups as follows:

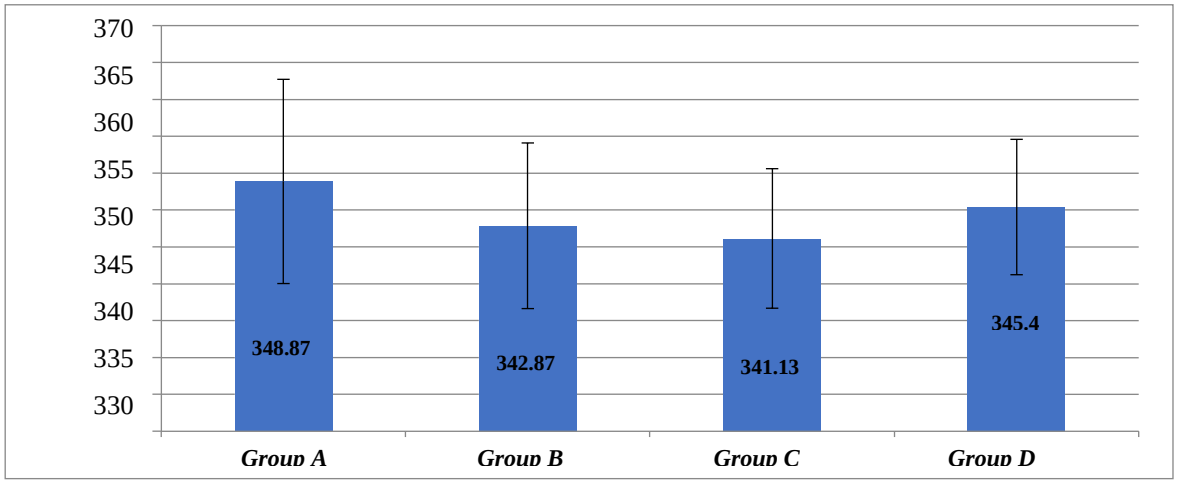
Group A (n = 15): No treatment
Group B (n = 15): Exposure to Theobromine gel for 1 month (for 4 minutes once in a week)
Group C (n = 15): Exposure to Theobromine gel for 3 months (for 4 minutes once in a week)
Group D (n = 15): Exposure to Theobromine gel for 6 months (for 4 minutes once in a week)

Throughout the experiment, all the samples were stored in artificial saliva. The samples in Group A did not receive Theobromine gel treatment and were used for descriptive comparison, while the samples in the remaining three groups were exposed to Theobromine gel, 200 mg/L, for different duration specifically, 16 minutes (n = 15), 48 minutes (n = 15), and 96 minutes (n = 15). After each handling, the hardness of the specimens was measured. The three durations of gel exposure were used as an analogy for exposures of 1 month, 3 months, and 6 months, with the calculation of each application being 4 minutes every week. After the application of theobromine gel to all the samples, the samples were subjected for post- treatment surface hardness measurement. The values obtained were then subjected to statistical analysis. Descriptive and inferential statistical analyses were carried out in the present study. Results on continuous measurements were presented on Mean ± SD. Level of significance was fixed at p=0.05 and any value less than or equal to 0.05 was considered to be statistically significant. Analysis of variance (ANOVA) was used to find the significance of study parameters between the groups (Inter group analysis). Further post hoc analysis was carried out if the values of ANOVA test were significant. Repeated measures Analysis of variance (RM-ANOVA) was used to find the significance of study parameters within the group at different time intervals (Intra group analysis). Further Bonferroni's post hoc analysis was carried out if the values of RM-ANOVA test were significant. The Statistical software IBM SPSS statistics 20.0 (IBM Corporation, Armonk, NY, USA) was used for the analyses of the data and Microsoft word and Excel were used to generate graphs, tables etc**.**

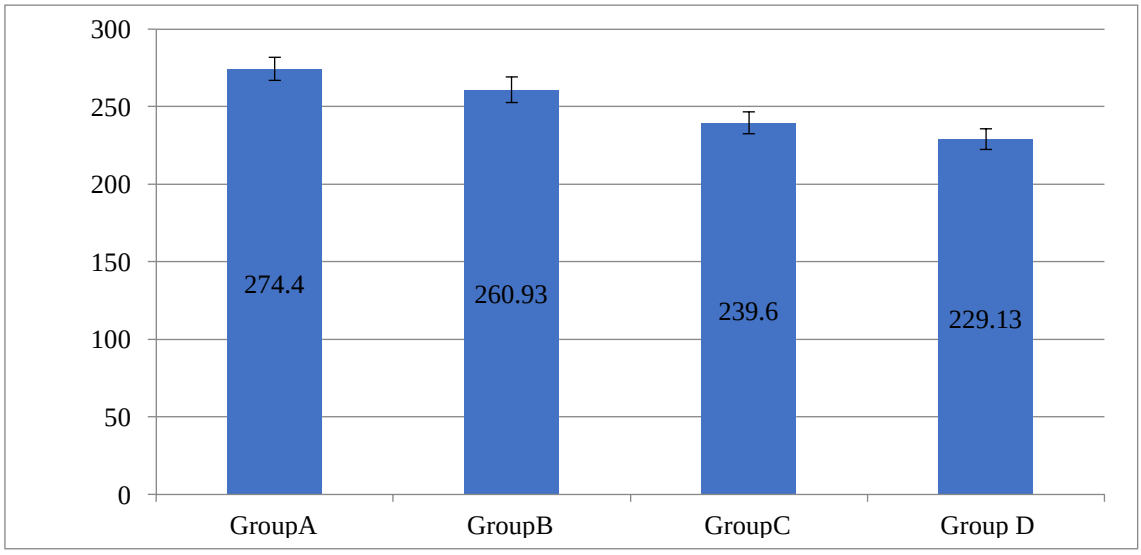
Results

GROUPS	Time interval	N	Mean	Std. Deviation	Wilk's Lambda value	P value
GROUP A	At Baseline	15	348.87	13.851	188.458	<0.001**
	After demineralization	15	274.40	7.443		
	After remineralization	15	295.33	6.020		
GROUP B	At Baseline	15	342.87	11.224	305.047	<0.001**
	After demineralization	15	260.93	8.276		
	After remineralization	15	333.73	11.847		
GROUP C	At Baseline	15	341.13	9.463	482.054	<0.001**
	After demineralization	15	239.60	7.049		
	After remineralization	15	353.47	9.906		
GROUP D	At Baseline	15	345.40	9.179	1867.232	<0.001**
	After demineralization	15	229.13	6.653		
	After remineralization	15	368.73	9.153		
	(p < 0.05 - Significant*, p < 0.001 - Highly significant**)					

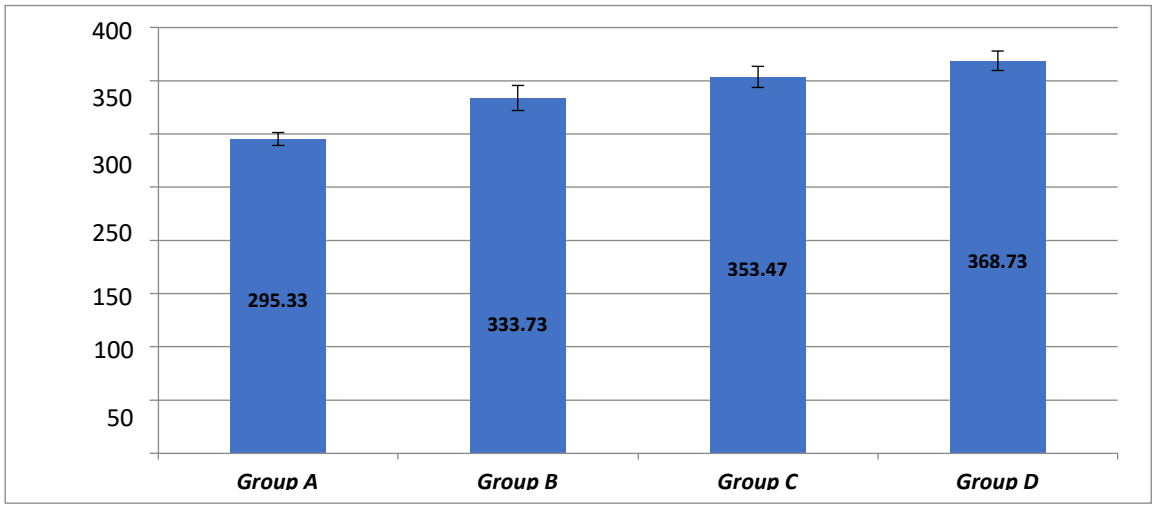
Table 1: Depicts the comparison of the micro hardness at different time intervals in terms of {Mean (SD)} in group A,B,C and D using Repeated measures ANOVA test. It is seen that there is statistically highly significant difference between the mean values of microhardness of the samples of all groups at the different time intervals (At baseline, after remineralization and after demineralization).



Graph 1: Depicts the comparison of the micro hardness at baseline in terms of {Mean (SD)} among all the groups using ANOVA test. It is seen that there is no statistically significant difference in microhardness amongst the 4 groups at baseline levels.



Graph 2: Depicts the comparison of the micro hardness after demineralization in terms of {Mean (SD)} among all the groups using ANOVA test. It is seen that there is statistically highly significant difference in microhardness amongst the 4 groups after demineralization.



Graph 3: Depicts the comparison of the micro hardness after remineralization in terms of {Mean (SD)} among all the groups using ANOVA test. It is seen that there is statistically highly significant difference in microhardness amongst the 4 groups after remineralization

Discussion

The dental caries is most prevalent problem with oral health. It is the most complex microbial disease that is accelerated in the oral environment by salivary changes, pH changes brought on by accumulated food debris, microorganisms and their by-products. Caries is not a continuous, unidirectional process; rather, it is a cyclical event with periods of remineralization and demineralization, demineralization causes a subsurface lesion in enamel and ultimately

cavitation. Remineralization, the process of reintroducing minerals to partially demineralized enamel, canthen take place when the pH in the body returns to neutral and the concentration of soluble calcium and phosphate is supersaturated in comparison to that in the tooth. Therefore, dental caries lesions at the tooth surface and subsurface level originate from a dynamic process of tooth matter degradation (demineralization) and restoration (remineralization). Over the courseof a tooth's life, these events occur several times per day and are influenced by a variety of variables, such as the number and type of microbial flora in the biofilm, diet, oral hygiene, genetics, dental anatomy, the makeup of the dentin and enamel, the use of fluorides and otherchemotherapeutic agents, the composition of the saliva, salivary flow, and buffering capacity. These factors are highly individual and tooth specific, and will differ from person to person, tooth to tooth in the same individual, and site to site on a same tooth. Remineralizing agents are significant in reducing the progression of early carious lesions and clinical treatment of the disease since they are non-invasive techniques to treating the lesions. Fluoride has been the most frequently recognized, utilized, and incorporated in both in-office and at-home applications among the several remineralizing agents used in dentistry. Other substances that have been developed over the years include casein phosphopeptide (CPP), amorphous calcium phosphate (ACP), bioactive glass, tricalcium phosphate, xylitol, CPP- ACP, and casein phosphopeptide ACP fluoride (CPP-ACPF). Fluoride is the most commonly used remineralizing agent. Fluoride present in the microenvironment stops enamel disintegration as soon as the acid attacks the enamel surface, causing the pH to rise. Fluoride crystals that include fluorhydroxyapatite become larger as the pH rises, limiting enamel demineralization and promoting remineralization.[8] Fluoride can be delivered topically and systemically. However, it was known that very high levels of fluoride in water were detrimental. One manifestation of a larger set of developing enamel abnormalities is dental enamel fluorosis. Histologically, it is defined as hypomineralized subsurface enamel, and clinically, as evident enamel opacities.[9] The degree of fluoride ingestion and the time period of its occurrence determines the severity. The clinical appearance can range from white specks throughout the dentition to brown discolouration and surface pitting. Enamel loss occurs at very high fluorideexposure levels. Prolonged, excessive accumulation of fluoride can cause a debilitating bone and joint disease known as skeletal fluorosis. As an increased level of fluoride accumulates in the bones, it alters the bone metabolism, affecting the entire skeleton. Due to the skeletal changes, bones become weaker and more brittle, while the pain and stiffness in joints increase. In most severe cases, joint immobility, muscle wasting, and neurological problems can lead tocrippling. Fluoride use has been effective in preventing dental caries, but more innovative strategies and exciting new caries prevention products need to be developed and tested. Concerns have beenexpressed on the harmful effects of the long-term use of fluoride-containing dentifrices.[10] Additionally, the availability of calcium and phosphate ions in saliva and plaque fluid affects fluoride's capacity to prevent caries from forming. As a result, problems including abnormalities of salivary function alter the rate of remineralization.[11] Dental research has continuously developed more non-toxic, anti-cariogenic compounds for alternative use, even if fluoride remains the cornerstone of non-invasive caries care. A number of firms create tooth health products without fluoride. As the king of junk foods, many people believe that chocolate is primarily responsible for toothcavities. Truth be said, chocolate is hardly the most cariogenic

substance by any means. Furthermore, cocoa butter within the chocolate is said to protect the teeth, giving it a coat of buttery goodness that can resist damage from sugar. Second, there is a component of the cocoaplant that has the potential to stop tooth decay and dental caries.[12] The cocoa bean's husk is present when cocoa is in its natural state, despite the fact that we cannot see it. The cocoa beanitself includes anticariogenic compounds, which is another benefit of chocolate made from cocoa beans. The cocoa bean husk, a by- product of the chocolate business, is well recognizedfor being rich in dietary fibres like cellulose, pectin, and lenin as well as polyphenols.[13] Theobroma cacao's pre-roasted beans are separated from the cocoa bean husk. It has been discovered that the cocoa bean husk contains two different forms of cariostatic compounds, one of which has anti- glucosyltransferase (GTF) action while the other has antibacterial activity.[14] One of the main ingredients in the cocoa bean is theobromine. Theobromine, previously known as xantheose, a water-insoluble, crystalline bitter powder, is an alkaloid of the cacao plant with a chemical formula 3.7 dimethylxanthine, and is differentiated from caffeine by only one methyl group and is therefore found in chocolate along with teas and other foods. It is also found in the leaves of the tea plant and the kola (or cola) nut. Theobromine helps harden tooth enamel, reducing the risk of decay in teeth.[15] 1%–4% of theobromine is found naturally in cocoa beans. Theobromine concentrations in cocoa powder range from 1.2%to 2.4%, with dark chocolate having greater concentrations than milk chocolate.[16] It is claimedthat a higher molecular weight polyphenolic molecule found in cocoa bean husk extract has potent anti- glycosyltransferase properties. In addition, it has unsaturated free fatty acids including oleic and linoleic acids, which are antibacterial for S. mutans. These physiologically active components are responsible for the cocoa bean husk's cariostatic properties Knoop microhardness testing method was preferred in this study over the vicker’s method as with an identical test load, the Knoop diamond indenter penetrates the specimen only half as deep as the Vickers diamond indenter. Due to this shallow indentation depth, the Knoop method is particularly suitable for testing very thin layers (e.g., aluminium foil). Also, Due thelong and narrow (elongated) test indent, the Knoop method is best suited for use with small, longish test specimens, whilst the Vickers method is better for small, rounded specimens (square indentation). The Knoop method is particularly suitable for testing very hard and brittle materials (glass, ceramics), where the Vickers indentation would lead to cracking(measurement accuracy). For any given indentation depth, the Knoop diagonal (the longitudinal diagonal) is around three times as long as the arithmetic mean of the Vickers diagonals, which means that the Knoop method provides higher measurement precision, especially in conjunction with very low-test loads. Based on prior studies showing that citric acid accelerates erosion at low pH values, in our study the demineralization process was replicated using 1% citric acid, pH 4. Because of its strong affinity for calcium, citric acid is also twice as harmful to enamel than chloric or nitric acid.[17] The 150 second acid exposure was intended to mimic a circumstance where a patient would consume sugar-containing food and fail to clean dental plaque. The pH utilized in the study was enough to initiate the cariogenic process.[18] Citric acid has the potential to promote demineralization because of its chelating action.[19] According to the study by Barbour ME et al, citric acid at pH at 4 causes dissolution of enamel if the latter is immersed in it for 150 seconds. This is equivalent to 40 minutes exposure in vivo orange juice.[20] We also used citric

acid at pH 4. It was observed that following exposure to the citric acid, all four exposure groups (groups A, B, C and D) displayed a considerable decrease in mean microhardness score due to demineralization. This was due to erosive action of acid which resulted into dissolution of calcium and phosphate ions from enamel surface. In turn, the ion dissolution may have caused gapping between crystals, which is an irreversible process and may have caused porosity of the enamel resulting in the decreased hardness score. Nakamoto et al demonstrated that theobromine increases apatite crystal size, which is related to enamel surface hardness. Nakamoto also stated that theobromine is less toxic than fluoride, making it a safer substance.[21] Hence, in our study, theobromine was used as a remineralizationagent. The theobromine gel concentration of 200 mg/L was selected based on research by Sadeghpour on the effectiveness of theobromine at different concentrations, ranging from 1 mg/L to 500 mg/L.[7] His research demonstrated a significant increase in enamel surface hardness scores at concentrations of 100 mg/L to 500 mg/L. Kargul and Nakamato studied the effects of theobromine on enamel surface hardness using two different concentrations, 100 mg/L and 200 mg/L, and results showed 200 mg/L theobromine to be more effective at increasing enamel surface hardness.[7] The results of our study showed that the demineralized enamel surfaces of all three exposure groups (groups B,C and D) increased in hardness following exposure to 200 mg/L theobromine gel. The three theobromine gel exposure times selected in this study, 16 minutes, 48 minutes, and 96 minutes were based on representing gel exposure for 1 month, 3 months, and 6 months.One exposure lasts for four minutes per week, assuming use in a person with a minimal cariesrisk. The 16-minute exposure group (group B) increased 28.07%, the 48-minute group (groupC) increased 47.08%, and the 96-minute group (group D) increased 61.13%. The repeated measures analysis of variance test revealed a significant difference ($p < 0.05$) in all three groups, confirming this finding. The microhardness of all the samples in 3 exposure groups increased significantly after demineralization. This may be due to theobromine's ability to increase apatite crystal size, which Nakamoto confirmed by X-ray diffractometry, is consistent with the rise in enamel hardness.[21] Theobromine interstitial reaction in the crystal micro-tunnel is thought to be the cause of the increase in crystal size. It indirectly raises apatite crystal microstrain and crystal density. This results in increase in tensile strength between atoms making the apatite tougher to break down. Macroscopically, this can be seen as the increase in enamel surface hardness. Theobromine in the 16-minute group (group B) improved enamel surface hardness following demineralization but did not bring it back to its original hardness. However, in the 48 minute (group C) and 96 minute (group D) groups the enamel surface hardness increased beyond the initial hardness. This suggests that a demineralized enamel surface may remineralize in response to 200 mg/L theobromine gel. It is assumed that the 16-minute group (group B) wasn't restored to initial hardness because of less exposure time of the theobromine gel to the tooth enamel surface so that the change of crystal size was not optimized. The Tukey's post hoc analysis showed a significant difference in change of hardness between the groups. The 96- minute group (group D) showed the best results, perhaps as a result of the lengthier exposure time. In this case, the change of crystal size is likely to be maximized compared to exposures of 16 (group B) and 48 minutes (group C). In this study, the erosive properties of 1% citric acid caused demineralization thereby increasing the porosity of the enamel prism layer.[22] The opening of the enamel prism layer (porosity) will, in turn, increase contact with and penetration of

theobromine gel on the enamel surface, which causes elevation of apatite crystals so that the enamel will be hardened and can withstand acid. These results are in accordance with a study conducted by M I P Irawan et al (2017) in whicha statistically highly significant increase in surface hardness was seen in the group that was exposed to theobromine for longer duration as compared to the short duration exposure and thecontrol group.[23] In our study, Group A was the control group, which did not receive the theobromine treatment. The mean baseline hardness before demineralization was 349 KHN. After demineralization, it was 274 KHN. However, it was observed that the hardness of enamel of the samples had increased (295 KHN) even without application of the theobromine gel. This could be becauseof the storage of samples in artificial saliva during the study. The artificial saliva may have caused mineral deposition on the samples resulting in rehardening of softened enamel thus

increasing enamel hardness. This is in accordance with a study conducted by M Eisenburger et al (2001).[24] Based on this research, it can be concluded that 200 mg/L theobromine gel can increase the enamel surface hardness that being demineralized with citric acid 1% with the exposure duration 16 minutes, 48 minutes, and 96 minutes. These results cannot be extrapolated in vivo, as routine caries control measure. Further research is warranted to recommend theobromine asa remineralizing agent.

Conclusion

Within the limitations of this in vitro study, we observed that:

- All the samples in the 4 experimental groups showed significant reduction in enamel microhardness following demineralization using 1% citric acid for 2.5 minutes.
- Highly significant difference ($p < 0.001$) was observed in mean values of microhardness at baseline, after demineralization with citric acid and after remineralization with application of theobromine gel.
- Following application of theobromine for the different time exposures, the enamel microhardness significantly increased in all the 3 exposure groups. (Groups B, C and D).
- Statistically significant difference ($p < 0.05$) was observed between groups C (48 minutes) and D (96 minutes).
- Group D (96 minutes) showed the highest increase in microhardness which shows that theobromine gel showed better results with lengthier exposure time.

Therefore, we conclude that Theobromine gel can be used as a safe and natural remineralizing agent. However further research is warranted to recommend its use as a routine caries control measure.

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