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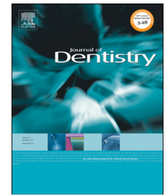

The effect of theobromine on the in vitro de- and remineralization of enamel carious lesions☆

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ABSTRACT

Objectives: This in vitro study investigated the effect of theobromine on the de- and remineralization of enamel carious lesions under plaque fluid-like conditions.

Methods: Early carious lesions were created in 272 bovine enamel specimens and assigned to sixteen groups (n = 17) based on Knoop surface microhardness (SMH). Lesions were demineralized again under plaque fluid-like conditions in the presence of fluoride (0.2 or 1 ppm) and theobromine (0; 10; 100 or 200 ppm) at different pH values (5.5 or 7.0) in a factorial design. SMH was determined again and percent SMH recovery (%SMHr) calculated. Three-way ANOVA was used for the fixed effects of fluoride, theobromine and pH levels to compare the differences between each level.

Results: The three-way interaction was not significant ($p = 0.712$). The two-way interaction between fluoride and pH was significant ($p = 0.030$), whereas those between fluoride and theobromine as well as that for pH and theobromine were not ($p = 0.478$ and $p = 0.998$, respectively). Theobromine did not affect %SMHr at any of the tested concentrations. There were trends for the higher fluoride concentration and the higher pH resulting in more rehardening with the lesions exposed to 0.2 ppm fluoride at pH 5.5 displaying significantly less rehardening than those exposed to 0.2 ppm fluoride at pH of 7.0 and lesions exposed to 1 ppm fluoride at pH of 5.5.

Conclusion: Theobromine, when continuously present in a plaque fluid-like medium at various concentrations and at different pH values, does not affect de- or remineralization of enamel carious lesions under the presently studied conditions.

Clinical significance: Based on the presently available evidence, theobromine cannot be recommended as an anticaries agent.

1. Introduction

Dental caries is one of the most common oral diseases, although its prevalence has declined over the last 30 years [1]. Caries is a multifactorial disease, which depends on the patient's own unique make-up of pathogenic risk factors and protective factors. Suitable primary and/or secondary preventive treatments include sealants, resin infiltration, fluorides, antibacterial mouth rinses, enhancing factors favorable for remineralization such as calcium and phosphate with the aid of remineralizing products, diet review and improvements thereof, and, in special populations, the use of saliva substitutes [2].

Fluoride's mechanism of action is three-fold: it enhances remineralization of demineralized hydroxyapatite-like minerals present in enamel and dentine, it prevents demineralization of such tooth minerals when present in the dissolution medium (plaque fluid), and lastly, fluoride has also been credited with the capability to inhibit bacterial enzymes [3]. It has been postulated that once the demineralized lesion (white spot) is clinically present, fluoride is not able to replenish the internal porous enamel with minerals. However, fluoride will impair the process, arresting the caries lesion and, consequently, the white spot's appearance will turn from opaque to shiny [4]. Likewise, the limitations of the efficacy of fluoride alone in relation to caries lesion progression were shown in a clinical study [5]: the use of a higher concentration of fluoride 1000 ppm was more effective in retarding the initiation of caries compared to a lower dose 250 ppm fluoride. However, no benefit was found on the progression of radiographically detected interproximal caries lesions for the higher fluoride concentration. More recently, a laboratory study highlighted that the effect of fluoride in preventing further demineralization diminishes with increasing lesion depths, and that its effect also depends on lesion baseline mineral distribution [6].

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As fluoride cannot provide complete caries protection, considerable research on non-fluoride agents has been conducted. Very recently, a range of fluoride-free toothpastes containing theobromine has become commercially available, claiming to naturally enhance caries lesion remineralization. Theobromine is an alkaloid, which belongs to the group of the methylxanthines. It is a natural ingredient found principally in cocoa beans. Studies in rats indicated that theobromine enhances crystallinity and dissolution resistance of enamel apatite [7,8], suggesting a potential mode of action of theobromine. Moreover, a laboratory study showed that topical application of theobromine can lead to the formation of precipitates on the human enamel surface, indicative of greater protection [9]. A similar study demonstrated that theobromine, when present in an apatite-forming medium, can enhance the potential of the medium to remineralize carious lesions comparable to that of fluoride under pH cycling conditions [10]. Most recently, however, a further pH cycling study demonstrated that theobromine does not appear to offer any anti-caries benefits in the presence or absence of fluoride [11].

In the presence of such divergent findings, more research is needed to investigate the potential role of theobromine in caries prevention. Thus, the present laboratory study was concerned with investigating the ability of theobromine alone and in combination with fluoride to enhance caries lesion remineralization and prevent further lesion demineralization. The novel approach undertaken presently was that the driving forces present in plaque fluid were mimicked. Prior research studied the effects of theobromine as a topical treatment and under pH cycling conditions. The present study, however, was the first that focused on how theobromine and/or fluoride interact with caries lesions under conditions that can be found in biofilms on active caries lesions with the intent to clarify if theobromine affects de- and remineralization of enamel carious lesions. The null hypothesis was that theobromine neither affects remineralization or prevention of demineralization of enamel carious lesions.

2. Materials and methods

2.1. Study design

Early carious lesions were created in bovine enamel specimens and assigned to 16 treatment groups (n = 17 per group). Lesions were then exposed to a plaque fluid-like solution varying in pH (5.5 or 7.0); fluoride (0.2 or 1.0 ppm) and theobromine concentration (0; 10; 100 or 200 ppm) for 24 h in a $2 \times 2 \times 4$ factorial design. The resulting changes in mineralization status of the specimens were determined using Knoop surface microhardness (SMH) after specimen preparation (sound enamel), lesion preparation, and exposure to plaque fluid-like solutions. Percent SMH recovery (%SMHr) was calculated from these data and served as the sole outcome variable.

2.2. Specimen preparation

Bovine tooth crowns were cut into 5×5 mm specimens using a Buehler Isomet low-speed saw. Teeth were stored in deionized water containing thymol during the sample preparation procedure. Then, they were ground and polished in order to create a flat and smooth enamel surface using a Struers Rotopol 31/Rotoforce Struers Inc., Cleveland, Ohio, USA. The enamel surface of the specimen was ground serially using 1,200-, 2,400- and 4000 grit paper, whereas the dentin part of the specimen was ground flat to a uniform thickness using 500-grit silicon carbide grinding paper. Then, specimens were polished using a polishing cloth containing 1-μm diamond polishing suspension until enamel surface presented at least a 4.5×4.5 mm highly polished facet across the specimen; this polishing procedure ensured the removal of surface enamel approx. 200−300 μm) which may contain relatively considerable concentrations of artificially introduced trace elements (e.g., fluoride) that could compromise the comparison between treatments. The specimens were assessed under a Nikon SMZ 1500 stereomicroscope at 20× magnifications for cracks, hypomineralized areas or other flaws in the enamel surface that would exclude them from use in the study. All surfaces of the specimens, except the enamel surface were covered with acid-resistant nail varnish. Three hundred and ten specimens were prepared to account for specimens failing inclusion criteria at sound baseline and after lesion creation, with 272 specimens (16 groups of 17 specimens each) being needed. Finally, prepared specimens were stored at 100 % relative humidity at 4 °C until further use.

2.3. Artificial caries lesion creation

Early carious lesions were formed in the specimens by a 24 h exposure at 37 °C to a solution containing 100 mM lactic acid, 4.1 mM $CaCl_2$, 8.0 mM KH_2PO_4, and 0.2 % Carbopol 907 (BF Goodrich), pH adjusted to pH 5.0 with KOH (modified after [12]). The specimens were stored at 100 % relative humidity at 4 °C until further use.

2.4. Secondary de- and remineralization

A plaque fluid-like solution, varying in fluoride, theobromine concentrations and pH as per Table 1, was used to de/remineralize all lesions for 24 h at 37 °C under static conditions. The plaque fluid-like solution had the following composition: 0.2 % Carbopol 907, 50 mM acetic acid, 4.1 mM $CaCl_2$, 8.0 mM KH_2PO_4, and 63 mM KCl (modified after [13]).

2.5. Knoop surface microhardness analysis

SMH analyses were performed on sound specimens, after lesion creation and after completion of secondary demineralization. SMH was measured using a designated microhardness tester (2100HT: Wilson Instruments, Norwood, Mass., USA). Five sound enamel indentations were placed with a Knoop diamond, spaced 100 μm apart and under a 50 g load in the center of each specimen. Indentation lengths were measured using Clemex CMT HD version 6.0.011 image analysis software. For enamel specimens to be acceptable for use in the study, the mean of the five baseline indentation lengths (B) had to be 43 ± 3 μm with a standard deviation (SD) of < 3. After lesion creation, the enamel specimens were again SMH tested by placing five indentations 100 μm to the left of the sound enamel indentations. To qualify for the study, the enamel indentation length (D) of a specimen needed to be within 20 μm of the mean indentation length of all specimens with a SD of < 15. Then, specimens were assigned to treatment groups to ensure there were no statistically significant differences in mean "D" between groups. After completion of the treatment phase, the specimens were again SMH tested by placing five indentations (T) 100 μm to the right of the sound enamel indentations. The sole variable, %SMHr, was calculated as follows [14]: $\%SMHr = (D-T)/(D-B) \times 100$. %SMHr

Table 1
Study treatment groups.

Group No.	F [ppm]	Theobromine [ppm]	pH
1	0.2	0	5.5
2	0.2	10	5.5
3	0.2	100	5.5
4	0.2	200	5.5
5	0.2	0	7.0
6	0.2	10	7.0
7	0.2	100	7.0
8	0.2	200	7.0
9	1.0	0	5.5
10	1.0	10	5.5
11	1.0	100	5.5
12	1.0	200	5.5
13	1.0	0	7.0
14	1.0	10	7.0
15	1.0	100	7.0
16	1.0	200	7.0

values greater than zero were indicative of lesion remineralization after secondary demineralization, whereas %SMHr values smaller than zero did indicate further demineralization.

2.6. Power calculation

Based on a pilot study, the within-group %SMHr SD were estimated to be 20. With a sample size of 17 per theobromine-fluoride-pH combination, the study had 80 % power to detect %SMHr differences of 20 between any two groups, assuming two-sided tests each conducted at a 5% significance level.

2.7. Statistical analysis

Data were summarized for overall %SMHr and %SMHr by fluoride, theobromine and pH levels. Three-way ANOVA was used for the fixed effects of fluoride, theobromine and pH levels to compare the differences between each level. Interaction terms were added for all three different groups. Pairwise comparisons were made between different levels of fluoride and pH. The Fisher's Protected Least Significant Differences method was used to control the overall significance level of the pair-wise comparisons. A 5% significance level was used for the tests.

3. Results

No surface loss was detected on any of the specimens as the sound enamel baseline indentations were clearly visible after completion of the experimental procedures.

The three-way interaction was not significant ($p = 0.712$). The two-way interaction between fluoride and pH was significant ($p = 0.030$), whereas those between fluoride and theobromine as well as that for pH and theobromine were not ($p = 0.478$ and $p = 0.998$, respectively). Fig. 1 shows the %SMHr data for all treatment groups as a function of theobromine concentration and pH. Theobromine did not affect %SMHr at any of the tested concentrations. There were trends for the higher fluoride concentration and the higher pH resulting in more rehardening with the lesions exposed to 0.2 ppm fluoride at pH 5.5 displaying significantly less rehardening than those exposed to 0.2 ppm fluoride at pH of 7.0 ($p = 0.018$) and lesions exposed to 1 ppm fluoride at pH of 5.5 ($p = 0.008$).

4. Discussion

The present in vitro study aimed to investigate the effects of theobromine on enamel carious lesion de- and remineralization under conditions typically found in plaque fluid. Plaque fluid is the fluid phase in the biofilm covering the teeth. It is considered more important in the process of understanding caries formation than whole plaque or saliva fluoride concentrations because its composition is influenced by saliva, crevicular fluid and the bacterial metabolism [15]. In the present study, the chosen fluoride concentrations were representative of resting plaque fluid (0.2 ppm F) and those found in plaque fluid after brushing with fluoride toothpaste (1.0 ppm F) [16]. Likewise, the chosen pH values of 5.5 and 7.0 are the extremes in the range of pH values found in plaque fluid [17]. A wide range of theobromine concentrations (0–200 ppm) was studied for two reasons. Firstly, there is a lack of pharmacokinetic studies on intra-oral retention of theobromine that could have informed the present study design. Secondly, prior studies utilized concentrations of up to 200 ppm theobromine as a topical treatment [9–11], which served as the upper range in the present study. Lastly, lesions were analyzed using Knoop surface microhardness as this is one of the most sensitive techniques in studying changes in the mineral status of the surface of early carious lesions [18] and as previous studies on theobromine also employed surface microhardness techniques [10–12].

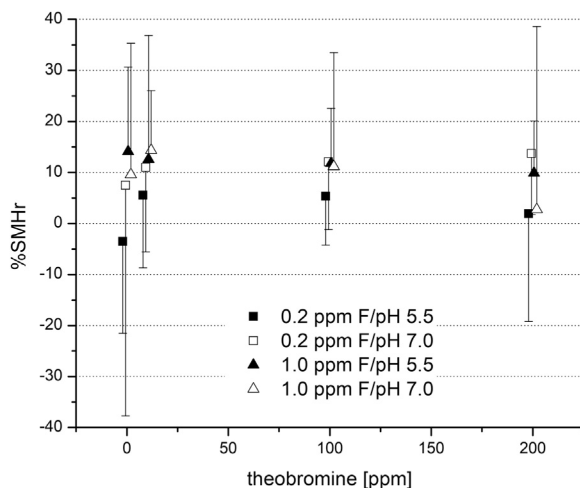

Fig. 1. %SMHr (mean ± standard deviation) as a function of theobromine and fluoride concentrations.

The present study failed to demonstrate an effect of theobromine in enhancing remineralization and/or to prevent further demineralization of early carious lesions created in enamel specimens. Based on these results, the null hypothesis was supported. These results are in agreement with a previous study [11]; however, they are in disagreement with the findings of other researchers [7–10]. The reasons for this discrepancy are manifold and most likely include the following. Firstly, there were inherent differences in the composition and properties of the de- and especially remineralization media between studies. For example, one study utilized artificial saliva also containing magnesium [10]. Magnesium has been shown to enhance the formation and stability of amorphous calcium phosphate [19], and there may have been synergistic effects due to the copresence of theobromine. Earlier studies [7,8] on the other hand were conducted at a much higher pH (9–9.5) and considerably lower calcium and phosphate concentrations (0.01 mol/L each) than those employed presently, making a comparison not straightforward either. Secondly, studies concerned with lesion remineralization employed lesions of different severities and mineral distributions at baseline. This has been shown to greatly impact the lesions' subsequent de- and remineralization behavior [20] as models can be tuned to net de- or remineralization by the choice of lesion alone. Thirdly, experimental approaches; i.e., pH cycling vs. crystal growth vs. continuous exposure studies such as the present one and durations thereof, were different. While different experimental approaches are undoubtedly needed to investigate novel compounds for their potential ability to interfere in the caries process, the contradictory findings shed light on the difficulty of mimicking the complexity of in vivo caries in vitro. Many, perhaps too many, modelling systems exist that address a limited number of aspects of in vivo caries. Thus, it is not surprising to see contradictory findings and only a series of randomized caries clinical trials would provide irrefutable evidence either way. However, these trials are not only time-consuming but also cost-prohibitive and would need to be based on substantial evidence derived from lower-tier caries models.

Theobromine was discovered in 1841 by a Russian scientist named Alejandro Voskresensky, who gave the name Theobroma which means “the food of the Greek Gods”. It is an alkaloid which belongs to the group of the methylxanthines. Theobromine has a similar structure and is as addictive as caffeine. Differing from caffeine, theobromine contains three methyl groups (one additional group than caffeine) which retards its elimination from the body, promoting a more prolonged action but it is approx. ten times weaker than caffeine. Also, theobromine does not affect the central nervous system as does caffeine, but acts on the Vagus nerve, dilating the cardiovascular system and relaxing the smooth muscle of the bronchi [21]. Theobromine is a natural ingredient found principally in cocoa beans, and thus in chocolate, which is its active ingredient. Theobromine is a white and bitter substance that is also encountered in tea, guarana, cola and mate leaves. It derives from the upper Amazon,

Orinoco regions of South America. It can only grow on hot and humid area as an understory plant. Cocoa beans contain 1–2 % of theobromine by weight (approx. 25 g/kg); with the added sugar and other ingredients theobromine is reduced to 2−5 g/kg in milk chocolate [22]. Theobromine could only be found in foods until a range of fluoride-free toothpastes containing theobromine (Theodent™) had been launched in the United States very recently. However, clinical research will be needed to determine any benefits these products provide in comparison to conventional fluoride toothpastes.

Several limitations need to be considered in the interpretation of the present findings. Firstly, a high variability in the post-treatment hardness data was encountered, thereby limiting the sensitivity of the model. Lesions had to fulfill certain criteria for inclusion in the study (see Materials and Methods), thus the source of variability was most likely introduced during the artificial plaque fluid exposure. While the sample size of n = 17 per group was higher than in previous studies concerned with theobromine (a post-hoc post-study power calculation revealed similar results to the above shown pre-study calculation), all groups had lesions that either de- or remineralized. Subtle differences in the baseline lesion severity and perhaps also the relatively short duration of artificial plaque fluid exposure could provide an explanation. While a longer treatment duration could have been desirable, this would have limited the clinical relevance of the study even further as, for example, pH fluctuations occur during a 24-h period and as both fluoride and theobromine concentrations are unlikely to remain constant. Future studies should include additional analytical techniques to shed more light on these early stages of de- and remineralization. Secondly, the present study did not incorporate a cariogenic biofilm. There is emerging evidence [23,24] which shows that theobromine can impair the growth of a cariogenic biofilm. Future studies need to explore this further by studying enamel and dentin de- and remineralization using microbial models. Lastly, the present study did not investigate theobromine in the absence of fluoride. Fluoride may have overcome any potential benefit of theobromine.

5. Conclusion

Considering the present findings, it can be concluded that theobromine cannot be recommended as an anticaries agent.

Ethical approval

No ethical approval was required prior to the conduct of the present study.

Declaration of Competing Interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Anna K. Thorn: Conceptualization, Data curation, Investigation, Methodology, Project administration, Visualization, Writing - original draft. **Wei-Shao Lin:** Methodology, Writing - review & editing. **John A. Levon:** Methodology, Writing - review & editing. **Dean Morton:** Methodology, Writing - review & editing. **George J. Eckert:** Formal analysis, Writing - review & editing. **Frank Lippert:** Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing - review & editing.

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