The Effects of Fluoride, Strontium, Theobromine and their Combinations on Caries Lesion Rehardening and Fluoridation

Running Title: F, Sr and theobromine effects on lesions

Frank Lippert

Indiana University School of Dentistry, Department of Cariology, Operative Dentistry and Dental Public Health, Oral Health Research Institute, Indianapolis, IN, USA

Corresponding author:

Frank Lippert

Indiana University School of Dentistry

Department of Cariology, Operative Dentistry and Dental Public Health

Oral Health Research Institute

415 Lansing Street

Indianapolis, IN 46202 (USA)

Tel. +1 317 274 3983, Fax +1 317 274 5425, E-Mail flippert@iu.edu

Highlights

- Strontium enhances caries lesion rehardening, but only in the presence of fluoride.
- Theobromine does not provide anti-caries benefits under the chosen conditions.
- No synergistic effects were found between strontium and theobromine.

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Abstract

Objective: The aim was to investigate the effects of fluoride, strontium, theobromine and their combinations on caries lesion rehardening and fluoridation (EFU) under pH cycling conditions.

Design: Human enamel specimens were demineralized at 37°C for 24h using a pH 5.0 solution containing 50mM lactic acid and 0.2% Carbopol 907 which was 50% saturated with respect to hydroxyapatite. Lesions were assigned to nine treatment groups (n=16) based on Knoop surface microhardness indentation length. Treatment aqueous solutions were: placebo, 11.9mM sodium fluoride (F), 23.8mM sodium fluoride (2×F), 1.1mM strontium chloride hexahydrate (Sr), 1.1mM F theobromine, Sr+theobromine, F+Sr, F+theobromine, F+Sr+theobromine. Lesions were pH cycled for 5d (daily protocol: 3×1min-treatment; 2×60min-demineralization; 4×60min & overnight-artificial saliva). Knoop indentation length was measured again and %surface microhardness recovery (%SMHr) calculated. EFU was determined using the acid-etch technique. Data were analysed using ANOVA.

Results: Model showed fluoride dose-response for both variables (2×F>F>placebo). For %SMHr, F+Sr+/-theobromine resulted in more rehardening than F, however less than 2×F. F+theobromine was similar to F. For EFU, F+Sr was inferior to F, F+theobromine and F+Sr+theobromine which were similar and inferior to 2×F. In absence of fluoride, Sr, theobromine or Sr+theobromine were virtually indistinguishable from placebo and inferior to F.

Conclusions: It can be concluded that a) strontium aids rehardening but not EFU and only in presence of fluoride; b) theobromine does not appear to offer any anti-caries benefits in this model; c) there are no synergistic effects between strontium and theobromine in the presence or absence of fluoride.

Keywords: caries; enamel; strontium; theobromine; fluoride

1.   Introduction

The anti-caries action of topical fluorides is now universally accepted and has been the subject of several reviews and meta-analyses of clinical data (Lenzi, Montagner, Soares, & de Oliveira Rocha, 2016; Marinho, Higgins, Logan, & Sheiham, 2003). The primary mode of action of fluoride is to enhance the remineralization of decalcified mineralized tissues (enamel and dentin carious lesions) while rendering them less susceptible to a subsequent acid attack through incorporation of fluoride (Featherstone, 2008). There is, however, a limitation to the caries prevention that can be achieved with fluorides as evidenced recently (Anderson et al., 2016). Likewise, alternative caries-preventing strategies must be considered for populations at risk to fluorosis.

A vast range of non-fluoride agents have been proposed and trialed over the years – antimicrobials (e.g. chlorhexidine, triclosan, iodine), polyols (e.g. xylitol) and calcium derivatives (e.g. CPP-ACP) (Cochrane, Cai, Huq, Burrow, & Reynolds, 2010; Rethman et al., 2011). Furthermore, metal ions apart from calcium have attracted some interest in the past, and in particular strontium due to its similarity with calcium (Bowen, 2001; Lippert & Hara, 2012). More recently, theobromine, an alkaloid found in cocoa beans, has been shown to provide anti-caries benefits under laboratory conditions (Kargul et al., 2012) and similar to that of a conventional fluoride toothpaste (Amaechi et al., 2013). These studies were based on earlier experiments which demonstrated efficacy for cocoa in preventing animal caries (Strålfors, 1966a-c & 1967).

With the exception of calcium & fluoride and chlorhexidine & fluoride, potential synergistic effects between actives have not been the subject of research studies. It is likely that effects are potentiated when compatible actives are co-present. When strontium and fluoride were co-present, an increase in crystallinity of carbonated apatites, a surrogate for dental enamel, was noted (Featherstone, Shields, Khademazad, & Oldershaw, 1983). Similar effects were proposed for theobromine (Nakamoto, Simmons, & Falster, 1999 & 2001). Hence, the present laboratory study was concerned with investigating potential additive or synergistic anti-caries effects between fluoride, strontium, theobromine and their combinations.

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2.   Materials and methods

2.1.   Study design

Early caries lesions were created in human enamel specimens. The lesions (n=16 per group) were assigned to nine treatment groups based on their surface microhardness. The nine groups were placebo, fluoride (tested at two concentrations), strontium, theobromine (both tested at one concentration) and their combinations. Lesions were pH cycled for 5 d. Then, surface microhardness and enamel fluoride content were determined.

2.2.   Specimen preparation

Enamel specimens were obtained from human permanent (predominantly molars and premolars, only buccal and/or lingual surfaces were used). Human teeth were extracted mainly for orthodontic reasons and were obtained from dental offices located in the State of Indiana, USA (water fluoridation at approx. 1 ppm F). IRB approval was obtained prior to tooth collection (NS0911-07). Tooth crowns were cut into 4×4 mm specimens using a Buehler Isomet low-speed saw. The teeth were stored in deionized water containing thymol during the sample preparation process. Specimens were ground and polished to create flat, planar parallel dentin and enamel surfaces using a Struers Rotopol 31/Rotoforce 4 polishing unit (Struers Inc., Cleveland, Pa., USA). The dentin side of the specimens was ground flat to a uniform thickness with 500-grit silicon carbide grinding paper. The enamel side of the specimen was serially ground using 1,200, 2,400 and 4,000 grit paper. The specimens were then polished using a 1 µm diamond polishing suspension on a polishing cloth until the enamel surface had a minimum of a 3×4 mm highly polished facet across the specimen. Resulting specimens had a thickness range of $1.7 - 2.2$ mm. This polishing procedure ensured the removal of surface enamel (approx. $200 - 300$ µm, depending on the natural curvature of the enamel surface) which may contain relatively high concentrations of artificially introduced trace elements (e.g. fluoride, strontium) that could otherwise compromise the comparison between treatments. The specimens were assessed under a Nikon SMZ 1500 stereomicroscope at 20× magnification for cracks, hypomineralized (white spots) areas or other flaws in the enamel surface that would exclude them from use in the study. Prepared specimens were stored at 100% relative humidity at 4 °C until further use.

2.3. Artificial caries lesion creation

Early, artificial caries lesions were formed in the specimens by a 24-h exposure at 37°C to a solution containing 50 mM lactic acid and 0.2% Carbopol 907 which was 50% saturated with respect to hydroxyapatite at pH 5.0 (White, 1987). This protocol will result in the formation of lesions with an average depth of 30 µm with a pre-mature surface zone formed in approx. 80% of the specimens (Lippert, & Lynch, 2014). After lesion creation, specimens were rinsed with deionized water. Specimens were stored at 100% relative humidity at 4 °C until further use.

2.4. Treatment solutions

The treatment solutions and their composition can be found in Table 1. All solutions were prepared using deionized water.

2.5. pH cycling phase

A 5-day pH cycling model with a daily regimen consisting of three 1-min treatments sandwiched around two blocks of 60 min remineralization, 60 min demineralization and 60 min remineralization with remineralization overnight was employed in the present study. Artificial saliva (1.5 mM $CaCl_2 \times 2\ H_2O$; 0.9 mM KH_2PO_4; 130 mM KCl; 20 mM HEPES; 3.08 mM NaN_3; pH 7.0) was used during the remineralization phases (Lynch, & ten Cate, 2006). An acetic acid solution (50 mM acetic acid; 2.2 mM $CaCl_2 \times 2\ H_2O$; 2.2 mM KH_2PO_4; 3.08 mM NaN_3; pH 5.0) served as demineralization medium (modified after Lynch, & ten Cate, 2006).

2.6. Surface microhardness analysis

The surface microhardness (SMH) test was used to assess changes in the mineral status of the partially demineralized enamel specimens. SMH was measured using a designated microhardness tester (2100 HT; Wilson Instruments, Norwood, MA, USA). Each enamel specimen was secured on a one-inch square acrylic block with sticky wax and then placed on the microhardness tester. Five baseline indentations spaced 100 µm apart were placed with a Knoop

diamond under a 50 g load in the center of a flattened, polished sound enamel specimen. SMH was determined by measuring the length of the indentations 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 5 baseline indentation lengths had to be 43 ± 3 µm with a standard deviation of < 3. After in vitro demineralization, the enamel specimens were again SMH tested by placing five indentations 100 µm to the left of the baseline indentations. After lesion creation, specimens with a mean (n = 5) indentation length of 105 ± 15 µm with a standard deviation of < 10 were selected for the present study. After 5 d of pH cycling the enamel specimens were again SMH-tested by placing five indentations 100 µm to the right of the baseline indentations. The extent of rehardening (%SMHr) was calculated (Gelhard, ten Cate, & Arends, 1979): %SMHr = (D1-R)/(D1-B) × 100 (B = indentation length (µm) of sound enamel specimen at baseline; D1 = indentation length (µm) after first in vitro demineralization; R = indentation length (µm) after pH cycling).

2.7. Enamel fluoride uptake (EFU)

After completion of the SMH measurements, the fluoride content of each enamel specimen was determined using the acid-etch technique. Each enamel specimen was demineralized in 0.5 ml of 1 M $HClO_4$ for 15 s. Throughout this period the demineralization solution was continuously agitated with an up and down motion of the specimens. Immediately after demineralization, the specimens were rinsed thoroughly with deionized water. A sample of each solution was then buffered with TISAB II (0.25 ml sample, 0.5 ml TISAB II and 0.25 ml 1N NaOH) and the fluoride content determined by comparison to a similarly prepared standard curve (1 ml standard + 1 ml TISAB II). In order to calculate the amount of enamel removed by the 15 s demineralization procedure, the calcium content of the demineralization solution was determined by atomic absorption (0.05 ml sample, 1 ml $LaCl_3$ and 3.95 ml deionized water). From these fluoride and calcium data, the fluoride level of each specimen after the pH cycling period was calculated and expressed as ppm.

2.8. Statistical analysis

Treatment effects on %SMHr and EFU were evaluated using one-way ANOVA. Where significant differences were indicated, the individual means were analyzed using Student Newman Keuls test to control the overall significance level at 5%.

3.   Results

There were no statistically significant differences in sound enamel (range: 44.2-44.7 µm, $p = 0.949$) and lesion mean indentation lengths (range: 100.2-100.9 µm; $p = 1.0$) between treatment groups. Figure 1 shows the %SMHr and EFU data as well as the results of the statistical analysis. The model showed a fluoride dose-response for both variables with increasing fluoride concentrations resulting in greater surface rehardening and lesion fluoride contents (all $p < 0.001$).

Surface microhardness recovery

In the absence of fluoride, strontium, theobromine and their combination were not different from placebo ($p = 0.279$, $p = 0.586$, $p = 0.091$, respectively) and inferior to fluoride (group F; all $p < 0.001$). Strontium in addition to fluoride (groups F+Sr and F+Sr+theobromine) resulted in more rehardening than F alone ($p = 0.014$ and $p = 0.035$, respectively); however, less rehardening was observed in comparison to 2×F (both $p < 0.001$). Theobromine did not enhance lesion rehardening when co-present with fluoride ($p = 0.520$).

Enamel lesion fluoride content

Lesions not treated with any fluoride-containing solution showed considerably lower fluoride content than those treated with fluoride (all $p < 0.001$). Theobromine and fluoride, either in absence or presence of strontium, did not enhance lesion fluoride content to fluoride alone ($p = 0.084$, $p = 0.196$, respectively). Strontium did reduce lesion fluoridation when co-present with fluoride ($p = 0.002$).

4.   Discussion

The present study was designed to obtain a better understanding of potential additive or synergistic anti-caries effects of strontium, theobromine and fluoride. A conventional hard tissue

caries pH cycling model was employed which have been shown to be suitable tools in investigating mechanistic aspects of the caries process (e.g. Lippert, & Juthani, 2015) and in the evaluation of potentially anti-caries compounds (e.g. White, 1987). The theobromine concentration was based on that tested recently (Amaechi et al., 2013) with strontium being tested at an equimolar concentration to that of theobromine. The present findings relating to theobromine are in disagreement with a recent study where equivalence to fluoride in net remineralization of caries lesions was observed (Amaechi et al., 2013). Inherent differences in the employed cycling models and between baseline lesion characteristics (assumed based on chosen lesion creation protocols) can be considered when providing an explanation for the different results. As it has been demonstrated repeatedly that model parameters impact outcome measures (e.g. Lippert, & Juthani, 2015; Zhang et al., 2015), this discrepancy is not surprising. This also highlights the dilemma that the majority of in vitro caries models were designed to demonstrate a fluoride effect and may therefore be somewhat inappropriate for non-fluoride agents. Furthermore, the present study did not aim to replicate prior observations; it was designed with investigating theobromine's proposed efficacy under slightly different conditions instead. This, of course, begs the question as to the true efficacy of theobromine in caries prevention. A series of randomized caries clinical trials could potentially provide conclusive evidence either way, although their cost-prohibitive nature would be self-limiting. Hence, a range of laboratory models should be used first to not only study potential anti-caries benefits of novel agents, such as theobromine, but also to justify putting human subjects at risk later on, no matter how small the risk may be.

While not strictly related to the purpose of the present study, it is important nonetheless to consider shortcomings and opportunities of in vitro research. As pointed out above, virtually all in vitro caries models were designed with fluoride in mind as its mode of action (enhancement of remineralization, reduction of demineralization and some antimicrobial efficacy) is now well understood. Non-fluoride anti-caries agents, however, often require a different approach. An in vitro study on antimicrobial non-fluoride agents, such as chlorhexidine, would require models involving biofilms or at least single species of bacteria. A model such as the present one would therefore be inappropriate. However, agents promoting mineralization similar to that of fluoride also benefit from a more (micro)biological approach as the study by Zhang et al. (2015) has shown. Biofilms are important intra-oral reservoirs. For anti-caries agents to exert their activity,

they need to be retained intra-orally and ideally in close vicinity to the hard tissue biofilm interface. Fluoride, for example, depends on the co-presence of calcium to be retained in biofilms (Whitford, Wasdin, Schafer, & Adair, 2002). This highlights the need for further studies on theobromine using intra-oral and microbial caries models.

Theobromine has been shown to enhance crystallinity of apatites without affecting remineralization in animal caries studies (Nakamoto, Simmons, & Falster, 1999 & 2001), although no hypothesis on its mode of action has been provided yet. Theobromine (primarily found in cocoa and cocoa products) belongs to the group of methylated xanthines and is structurally similar to caffeine (coffee, tea) and theophylline (tea, cocoa), two other naturally occurring alkaloids. While prior caries-related research on theobromine per se is sparse, several animal caries studies on cocoa (Strålfors, 1966a-c & 1967) demonstrated efficacy for primarily its water extract which in addition to theobromine also contains a range of minerals and polyphenols (Holland, Welch, Unwin, Buss, & Paul, 2015). Cocoa polyphenols were reported to exhibit inhibitory effects on plaque accumulation due to their ability to prevent extracellular polysaccharide formation from sucrose (Kashket, Paolino, Lewis, & van Houte, 1985; Paolino & Kashket, 1985). Therefore, it is likely that the observations by Strålfors (1966a-c & 1967) were likely due to the co-presence of theobromine and polyphenols. Contrary to the findings on cocoa, caffeine (Nakamoto, Cheuk, Yoshino, Falster, & Simmons, 1993) and theophylline (Ruenis, Rosalen, Volpato, & Groppo, 2000) have both been shown to lead to a higher incidence of rat caries, although studies on caffeine were contradictory (Ruenis, Rosalen, Volpato, & Groppo, 2000). Coffee and tea, however, have been shown to potentially exhibit anti-caries properties; however, further in vivo research was proposed to provide more meaningful evidence as to their efficacy (Ferrazzano, Amato, Ingenito, de Natale, & Pollio, 2009).

The present observations for strontium in combination with fluoride (Fig. 1) have provided further evidence to its potential anti-caries effect. Previously, strontium was shown to promote in vitro remineralization when continuously co-present with fluoride in the remineralization medium in a mechanistic study (Thuy et al., 2008). Here, strontium was tested as a topical agent and at a considerably higher concentration (100 vs. 10 ppm), yet similar benefits were observed. However, the presently tested strontium concentration is considerably lower than those tested in rinse (450 ppm) and toothpaste (2200 ppm) formats under in situ conditions in the presence of not only fluoride (225 and 1100 ppm, respectively) but also a poly-acrylic acid polymer which

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supposedly aids fluoride retention via strontium bridging (Bowman et al., 1988a&b). Interestingly, strontium reduced lesion fluoridation in the present study compared to fluoride alone, although this effect was negated when theobromine was also present (Fig. 1). Strontium can substitute for calcium in the apatite crystal lattice due to their similar ionic radii (Elliott, 1973), thereby expanding the lattice. This can lead to preferential incorporation of fluoride over carbonate (i.e. a smaller for a larger ion) which was shown previously (Featherstone, Shields, Khademazad, & Oldershaw, 1983). These findings could not be replicated presently, although conditions were markedly different between studies. Strontium also increases the number of nucleation sites as it stabilizes apatite precursor phases (Drouet, Carayon, Combes, & Rey, 2008; Matsunaga & Murata, 2009), which would provide further explanation for the present observations. However, it must also be mentioned here that strontium and caries is still a controversial topic as the evidence from several epidemiological studies has been largely equivocal (Lippert & Hara, 2012).

No additive or synergistic effects of theobromine and strontium on lesion remineralization or fluoridation were observed presently. Given both agents were proposed to increase apatite crystallinity, it was surprising that no effect on lesion remineralization was observed – at least none that could be detected. Laboratory studies are useful screening tools as not every agent or combination of agents can be tested under clinical conditions. However, in vitro research has limitations. While there is a multitude of factors involved in the caries process (Selwitz, Ismail, & Pitts, 2007), only very few can be included in laboratory models. Effects of strontium, theobromine and fluoride on oral biofilms and their ability to prevent demineralization of both enamel and dentin are worthy of further research.

5.   Conclusions

Theobromine does not provide anti-caries benefits under the chosen conditions. Strontium was shown to aid caries lesion rehardening but not fluoridation and only in the presence of fluoride. No synergistic effects between strontium and theobromine in the presence or absence of fluoride were observed.

Competing interests

Funding

Ethical approval

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

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Figure legend

Fig. 1. Mean ± standard deviation for both lesion rehardening (%SMHr) and enamel lesion fluoride content (EFU) as a function of treatment during pH cycling period. Statistically significant differences are highlighted by different letters.
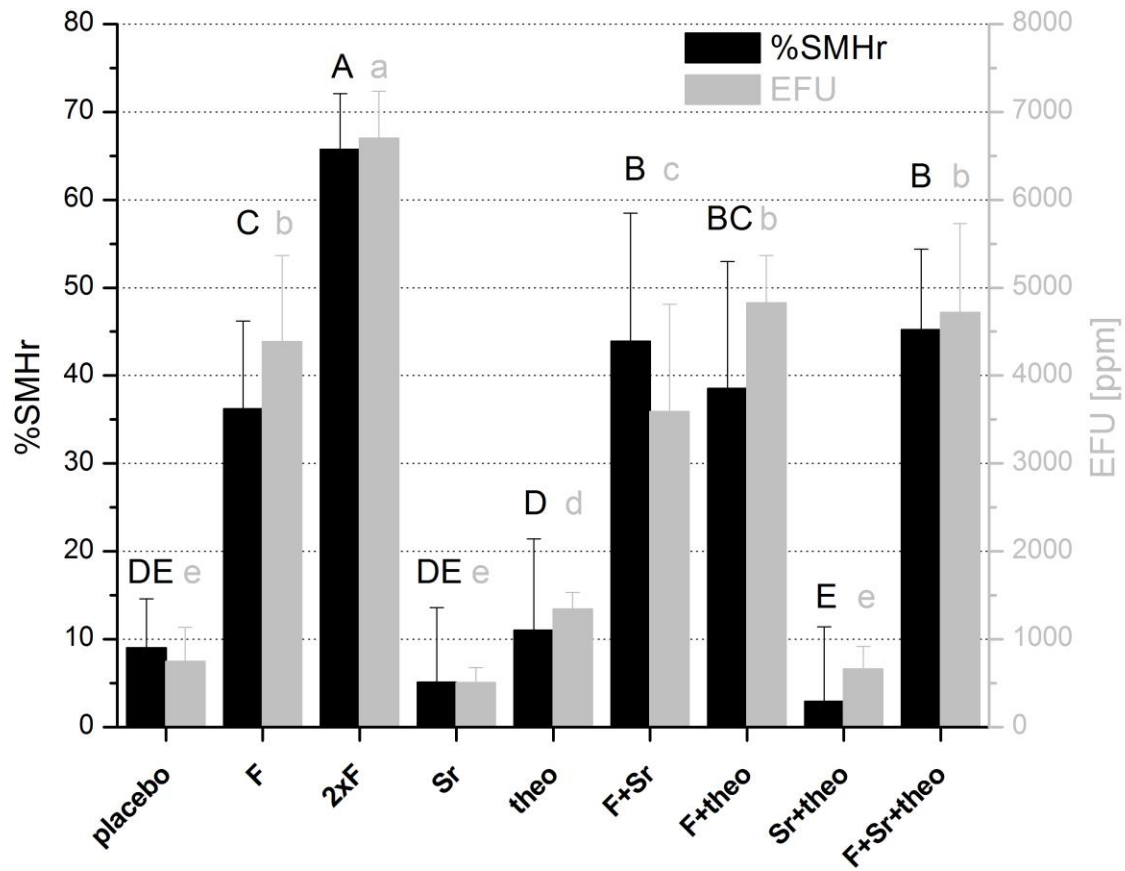
Table 1

Treatment solutions and their composition

Group Code	Composition	F [mmol]	Sr [mmol]	theobromine [mmol]
placebo	deionized water	-	-	-
F	226 ppm F as sodium fluoride	11.9	-	-
2×F	452 ppm F as sodium fluoride	23.8	-	-
Sr	100 ppm Sr as $SrCl_2 \times 6\ H_2O$	-	1.1	-
theo	200 ppm theobromine	-	-	1.1
F+Sr	226 ppm F + 100 ppm Sr	11.9	1.1	-
F+theo	226 ppm F + 200 ppm theobromine	11.9	-	1.1
Sr+theo	100 ppm Sr + 200 ppm theobromine	-	1.1	1.1
F+Sr+theo	226 ppm F + 100 ppm Sr + 200 ppm theobromine	11.9	1.1	1.1