Effect of phytate and zinc ions on fluoride toothpaste efficacy using an *in situ* caries model

Short title: **Effect of phytate and zinc ions on fluoride efficacy**

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Abstract

Objectives: To compare and explore the dose-response of phytate-containing 1150 ppm fluoride toothpastes on model caries lesions and to determine the impact of zinc ions.

Methods: This was a single-centre, randomised, blinded (examiner/laboratory analyst), six-treatment, four-period crossover, *in situ* study in adults with a removable bilateral maxillary partial denture. Study treatments were toothpastes containing: 0.425% phytate/F; 0.85% phytate/F; 0.85% phytate/Zn/F; F-only; Zn/F and a 0% F placebo. Where present, F was 1150 ppm as NaF; Zn was 0.3% as $ZnCl_2$. Human enamel specimens containing early-stage, surface-softened (A-lesions) or more advanced, subsurface (B-lesions) caries lesions were placed into the buccal flanges of participants' modified partial denture (one of each lesion type per side). A-lesions were removed after 14 days of twice-daily treatment use; B-lesions were removed after a further 14 days. A-lesions were analysed for surface microhardness recovery. Both lesion types were analysed by transverse microradiography and for enamel fluoride uptake, with B-lesions additionally analysed by quantitative light-induced fluorescence. Comparison was carried out using an analysis of covariance model.

Results: Statistically significant differences between 1150 ppm F and the placebo toothpastes ($p<0.05$) were shown for all measures, validating the model. No differences between fluoride toothpastes were observed for any measure with little evidence of a dose-response for phytate. Study treatments were generally well tolerated.

Conclusions: Results suggest phytate has little impact on fluoride's ability to promote early-stage lesion remineralisation or prevent more advanced lesion demineralisation in this *in situ* caries model. Similarly, results suggest zinc ions do not impair fluoride efficacy.

Clinical significance: Toothpastes may contain therapeutic or cosmetic agents that could interfere with fluoride's caries prevention efficacy. The present *in situ* caries study has demonstrated that phytate, added to provide enhanced extrinsic stain removal/prevention, and zinc, added to inhibit malodour, do not impair fluoride efficacy.

Introduction

Modern toothpastes are multifunctional, delivering therapeutic benefits to combat a variety of oral conditions (e.g., caries, gingivitis, dentine hypersensitivity, erosive tooth wear) while also offering cosmetic benefits such as control of extrinsic enamel staining or inhibition of oral malodour. The incorporation of 'cosmetic' ingredients allows toothpastes to be tailored to an individual's requirements; however, consideration must be given to ensure that the positive actions of therapeutic ingredients are not negated by the addition of such ingredients.

An important cosmetic function of a toothpaste is to control extrinsic dental stain. Common approaches include the use of physical abrasives, such as particulate silica or alumina, or soluble chemical agents with affinity for calcium ions, such as condensed phosphates (e.g., pyrophosphate, tripolyphosphate or hexametaphosphate) that interfere with attachment of stain molecules to enamel surfaces [1]. Chemical cleaning agents are an attractive addition to a conventional toothpaste to augment the abrasive-based stain control system without increasing toothpaste abrasivity [2]. However, condensed polyphosphate agents have been reported to interfere with the bioactivity of fluoride by inhibiting the exchange of minerals at the enamel surface, thereby interfering with the ability of fluoride to remineralise enamel [1, 3-5]. Few long-term clinical studies exist to clarify whether condensed phosphates impair the anticaries properties of fluoride, those that do, have not indicated an effect [6, 7]. However, such observations may not necessarily be extrapolated to all phosphate classes and a polyphosphate that provides the tooth whitening benefits typical of the class, without impact on fluoride-promoted remineralisation, is therefore of great interest.

Phytate is an organic polyphosphate ion naturally found in cereals and seeds where it is the principal storage molecule of phosphorous in plant tissues [8]. Chemically, phytate is similar to condensed phosphates by virtue of multiple phosphate groups, but in contrast to condensed (linear) phosphates, it is cyclic in nature with six phosphates groups bound to a cyclohexane ring (and no direct phosphate-phosphate bonds). Recently, a 12-week study has indicated enhanced stain removal ability for a toothpaste containing 0.85% w/w sodium phytate compared to a regular toothpaste [2]. Phytate may also offer caries protective benefits of its own. *In vitro*, phytate has been shown to modify the transport of ions across enamel and dentine surfaces, with potential benefits in terms of caries progression [9, 10] In animal caries studies, phytate has been shown to exhibit anticaries effects in the absence of fluoride [11].

A second important cosmetic function of a toothpaste is control of oral malodour. Zinc ions are commonly added to toothpaste formulations to reduce malodour by chemically binding to volatile odour molecules [12, 13] and by inhibiting malodour-generating plaque bacteria [14]. Zinc has an affinity for enamel surfaces via binding to surface phosphate groups and so also has the potential to interfere with fluoride's action against caries [15, 16]. Since a further benefit of use of zinc toothpastes is a reduction in calculus formation [17] via inhibition of formation of apatites and their precursors [18], it has the potential to interfere with enamel remineralisation. *In vitro* mechanistic studies have demonstrated the potential for this to occur in both a positive and negative fashion [15, 16]. Furthermore, like phytate, zinc may offer its own protection against demineralisation. Data from *in vitro* and *in situ* studies support this proposition [19, 20]. Overall, the effect of zinc on caries development is proposed to be neutral [6, 21].

It is important to understand the interaction of these 'cosmetic' agents on fluoride efficacy, separately and together, when formulated in a toothpaste. A program was undertaken to investigate the remineralisation/demineralisation effects of phytate and zinc on fluoride efficacy from toothpastes. Studies have already been performed to understand their effects on enamel remineralisation/demineralisation using a relatively simple, single-treatment 'erosion' *in situ* protocol [22, 23]. These studies showed significant inhibition of fluoride-induced remineralisation by phytate and by zinc. In the current study, a protocol based on an established longer-term *in situ* caries model [24] was employed to examine the effects of phytate (at two concentrations, the primary focus of the study) and zinc (the secondary focus) separately and together in a regular fluoride toothpaste on remineralisation and prevention of further demineralisation of enamel.

Materials and Methods

This was a single-centre, randomised, blinded (examiner and laboratory analyst), six-treatment, four-period crossover, *in situ* study conducted at the Oral Health Research Institute of the Indiana University School of Dentistry, Indianapolis, Indiana, USA. The protocol was approved by the Indiana University Institutional Review Board (IRB #1512088822) and the study was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. The study was registered at clinicaltrials.gov (NCT02751320). There were minor amendments to the protocol that did not affect study flow or outcomes.

Participants

All participants provided written informed consent prior to screening. Participants aged 18–85 years were eligible for inclusion if they had good oral health and a removable bilateral maxillary partial denture with sufficient room in the posterior buccal flange areas to accommodate two enamel specimens and an unstimulated and stimulated salivary flow rate of at least 0.2 and 0.8 mL/min, respectively. Exclusion criteria included: pregnancy; breastfeeding; taken antibiotics or had received professional fluoride application in the 2 weeks prior to screening; use of fluoride supplements or mouth rinse; dental restorations in poor state of repair; active caries or moderate/severe periodontal disease; using a denture adhesive to secure the denture other than non-zinc Poligrip® (GSK Consumer Healthcare, Weybridge, UK).

In situ remineralisation model

This study used the *in situ* caries remineralisation model developed by Zero et al. [25, 26]. Human permanent teeth were cut into 4 × 4 mm enamel specimens, which were polished to create flat surfaces as described previously. Surface-softened lesions (A-lesions) were created according to a modified method of White [27] by immersing enamel specimens in 40 mL 0.05 mol/dm^3 lactate 50% saturated with respect to hydroxyapatite and containing 0.2% (wt/vol) Carbopol® 907 (BF Goodrich, Cleveland, OH, USA) adjusted to pH 5.0 with KOH at 37°C for 24 h. Subsurface lesions (B-lesions) were prepared by demineralising enamel specimens at 37°C for 9 days in a solution containing 0.1 M lactate, 4.1 mM $CaCl_2$, 8.0 mM KH_2PO_4 and 0.2% w/v Carbopol® 907, pH adjusted to pH 5.0 using KOH [28]. Lesions were deemed acceptable if lesioned areas displayed uniform opacity and surface shine on exposure to overhead light. Specimens were stored in a moist environment to prevent dehydration and were sterilised by ethylene oxide gas prior to insertion into dentures.

At each treatment start visit, one A-lesion and one B-lesion enamel specimen was placed flush in each buccal flange area of the bilateral mandibular denture for a total of two of each specimen type per denture. Each enamel specimen was covered with Polyester Knit Fabric (Item P01628, Bard Peripheral Vascular, Tempe, AZ, USA) to encourage plaque formation [29, 30]. Specimens were mounted such that the surface was flush with the buccal flange surface and were luted in place using a light cured dental composite (Triad VLC material, Dentsply Int., York, PA, USA). Upon study completion, the denture was permanently repaired with acrylic.

Study design and treatments

At the screening visit, participants provided written informed consent and their demographic details and medical and current medication history was recorded. They underwent an oral soft tissue (OST) and oral hard tissue (OHT) examination and their stimulated and unstimulated salivary flow rate was assessed. An OST examination was also performed before and after each treatment period; an OHT examination was performed following dental prophylaxis and at the end of each treatment period. Prior to each treatment period, participants received a dental prophylaxis using a non-fluoride prophylaxis and floss. They were issued with a washout toothpaste (Negative Control 0 ppm fluoride, as below) and study toothbrush (Aquafresh® Clean Control Everyday Clean™ toothbrush, GSK Consumer Healthcare, supplied new for each washout and treatment period).

At the first treatment visit, eligible participants were randomised to a treatment sequence according to a schedule provided by the Biostatistics Department of GSK Consumer Healthcare. Given the number of treatment arms, an incomplete block design was employed to reduce the length of participation for each subject and so that all possible pairs of treatments were observed, ideally an equal number of times. Details of study treatments are provided in Table 1.

Toothpastes were supplied in plain white tubes with a label containing protocol number, product code, directions of use/storage and emergency contact number. The examiner, laboratory analysts, study participants, study statistician, data management staff and other employees of the sponsor who could influence study outcomes were blinded to treatment allocation and order of treatment.

Participants used 1.5 g (±0.1 g) of their assigned toothpaste twice a day, applied to a wet toothbrush, and brushed only their natural teeth for one timed minute, ensuring the enamel specimens were not brushed. They then rinsed with 15 mL tap water for approximately 10 s. The first brushing for each participant for each treatment period was under study site supervision with their brushing technique re-assessed at each treatment visit. Participants wore their mandibular partial denture for 24 h a day but could remove it for water-rinsing following eating and for cleaning, taking care to avoid brushing the specimens. Participants could not eat or drink anything for at least 30 min following brushing and were instructed not to eat canned sardines during the study as they could contain high fluoride levels. Participants followed the brushing schedule for 28 days per treatment period and returned to the study centre at days 14 and 28 where the two A-lesion specimens and two B-lesion specimens were removed, respectively.

There was a washout-period of approximately 1 week between each treatment period during which participants followed their usual dental hygiene practices for at least 4 days. They then returned to the study centre for a dental prophylaxis. For the 2–3 days prior to the treatment start visits,

participants discontinued all usual oral hygiene practices (with the exception of inter-dental cleaners) and used only the study washout fluoride-free toothpaste and toothbrush. For the study duration, participants were instructed not to use any other denture cleaning products or denture adhesive on the lower partial denture or a denture adhesive, with the exception of supplied, zinc free, Polygrip, on the upper partial or full denture.

Outcome measures and their assessment

The primary endpoint for analysis was percentage surface microhardness recovery (%SMHR) [31] of the A-lesions with the primary objective being to evaluate if %SMHR was related to sodium phytate dose. SMH was measured by assessing indentation lengths using a Wilson 2100 Hardness Tester (Norwood, MA, USA) equipped with a Knoop diamond, before (B) and after (D) *in vitro* demineralisation of the enamel specimens and after intraoral toothpaste treatment (R). Values were averaged across the two enamel specimens per participant to give a single observation. %SMHR was calculated as = [(D-R)/(D-B)]×100 [32].

Additional endpoints for the A-lesions were measurement of the amount of fluoride incorporated into the enamel (EFU) and the TMR-derived parameters of ΔZ (integrated mineral loss in vol%min×μm), L (lesion depth in μm) and SZ_{max} (surface zone mineral density in vol%min). For the B-lesions, secondary endpoints evaluated included EFU, ΔF (lesion fluorescence loss, determined using quantitative light-induced fluorescence [QLF]) and the TMR-derived variables of ΔM (change from baseline integrated mineral loss), ΔL (change from baseline lesion depth) and ΔSZ_{max} (change from baseline surface zone mineral density).

TMR measurements were made by sectioning the enamel specimens into 100 μm plano-parallel thin slices using a Silverstone-Taylor Hard Tissue Microtome (Scientific Fabrications, Lafayette, CO, USA). Specimens were polished, mounted on plates and x-rayed at 20 kV and 30 mA at a distance of 42 cm for 65 min. Micrographs were examined by Zeiss EOM microscope using TMR software v.3.0.0.11 (Inspektor Research Systems BV, Amsterdam, The Netherlands). Mineral content was calculated from the grey intensity levels of the section images [33, 34]. Lesions were required to have a baseline mineral loss (ΔZ) of 2500 ±600 vol%min × μm and a maximum mineral density at the surface zone (SZ_{max}) of at least 40 vol%min. After treatment, a further section was taken from each lesion specimen for radiography assessment. To avoid potentially compromising the primary endpoint, %SMHR of A-lesions, baseline TMR measures were not performed; TMR data for the A-lesions represents the lesion condition at the end of treatment while TMR data for the B-lesions represents change from baseline.

Changes in mineral content (ΔM), lesion depth (ΔL) and maximum mineral density at the surface-zone (ΔSZ_{max}) were calculated as respective post-treatment values minus baseline values. Positive numbers denote remineralisation, increase in lesion depth and increase in SZ_{max}, respectively; negative numbers denote further demineralisation, decrease in lesion depth and decrease in SZ_{max}, respectively.

For QLF, the B-lesion specimens were air-dried for at least 10 min before QLF measurements were performed using the QLF-D Biluminator 2 (Inspektor Research, the Netherlands). A sound enamel reference area had previously been denoted/protected on each specimen through the use of clear nail varnish. Acquired QLF images were analysed using dedicated QLF Analysis software (C4 Research Software Suite, Inspektor Research Systems BV, Amsterdam, The Netherlands). ΔF_{base} values were recorded at a threshold level of 5%, i.e., a minimum of 5% fluorescence loss between sound and uncovered demineralised enamel. After completion of the 28-day treatment period, QLF images were obtained on all B-lesions again (ΔF_{post}). The change that occurred in lesion fluorescence loss ($\Delta\Delta F$) following treatment was calculated as follows: $\Delta\Delta F = \Delta F_{post} - \Delta F_{base}$, where positive numbers denote remineralisation, negative numbers denote further demineralisation.

EFU was determined using the microdrill enamel biopsy technique, as previously described [35]. Four microdrill samples from each enamel specimen were pooled and a value for fluoride content determined. Participant-wise measurements were calculated separately for A- and B-lesions as the mean values from the two respective enamel blocks.

Safety

Assessments of tolerability were made with respect to OST abnormalities and adverse events (AEs) reported by participants following the first dental prophylaxis until 5 days following last administration of study treatment. Each AE was assessed for intensity (mild, moderate, severe) and whether it was related to study treatment, according to clinical judgement. Safety assessments were based on the safety population, defined as all participants who were randomised and received at least one dose of study toothpaste.

Statistical analysis

This was an exploratory study to examine the effects of phytate (at two concentrations, the primary focus of the study) and zinc (the secondary focus) separately and together in a regular fluoride toothpaste on remineralisation and prevention of further demineralisation of enamel. Given the

exploratory nature of this comparison, no formal sample size calculation was performed. However, to ensure validity of this established model (and permit comment on comparison between the fluoride toothpastes), a formal size calculation was performed on the Placebo and F-only controls. This study aimed to randomize approximately 62 participants to ensure approximately 50 completed the entire study. In this incomplete block design, with 50 evaluable participants this study had >90% power at a 5% significance level (using 2-sided testing) to detect differences between the Placebo and F-only groups for the main comparators (%SMHR for A-lesions, TMR [ΔM] for B-lesions).

The intent-to-treat (ITT) population, on which all efficacy analyses were performed, was defined as all participants who were randomised, received study toothpastes at least once and provide at least one post-baseline efficacy assessment. The per protocol (PP) population was defined as all participants in the ITT population who had at least one efficacy assessment considered unaffected by protocol violations.

Validity of the study assessment measures were predefined as acceptable if there was a statistically significant difference ($p < 0.05$) between the F-only and Placebo toothpastes, favouring the former. If model analysis method validity was not achieved, no further analysis of any endpoint was performed using that technique. In all analyses, if a participant was missing an enamel specimen, the mean was computed over the available enamel specimens. All pairwise treatment comparisons were performed using 2-sided testing at the 5% significance level. The assumption of normality of residuals was investigated and met for all endpoints. No adjustment for multiple comparisons was employed as the primary comparison had been defined.

%SMHR was analysed using an analysis of covariance (ANCOVA) model with participant as a random effect and fixed effects for study period and treatment. The covariates were based on participant-level (mean across treatment periods) and period level (period level minus participant level) baseline and pre-treatment acid challenge enamel SMH/indentation lengths. Primary comparisons were: F-only vs Placebo (validity of model, fluoride effect); 0.425% Phy/F vs F-only; 0.85% Phy/F vs F-only; 0.425% Phy/F vs 0.85% Phy/F. Linear and quadratic contrasts were fitted to establish whether there was a dose-response relationship of phytate (0%, 0.425%, and 0.85% all containing 1150 ppm fluoride). Secondary comparisons were: 0.85% Phy/Zn/F vs Zn/F; Zn/F vs F-only; 0.85% Phy/F vs 0.85% Phy/Zn/F.

Participant-wise change in mineral content (ΔM) of TMR values at week 4 and participant-wise EFU at 2 and 4 weeks were secondary efficacy variables. Participant-wise measurements were calculated as the mean values from the two enamel blocks for A- and B-lesions. Treatment comparisons were

performed using a mixed model ANCOVA including participant as a random effect and fixed effects for study period and treatment. It also included the participant-level (mean across treatment periods) baseline and period level (period level minus participant level) baseline ΔZ measurement as covariates. EFU and ΔΔF scores were analysed using analysis of variance (ANOVA) with participant as a random effect and fixed effects for study period and treatment for both A and B-lesions. Linear and quadratic contrasts were fitted to establish whether ther

e was a dose-response relationship of phytate (0%, 0.425%, and 0.85% all containing 1150 ppm fluoride). Secondary comparisons were: 0.85% Phy/Zn/F vs F-only; Zn/F vs F-only; 0.85% Phy/F vs 0.85% Phy/Zn/F.

All TMR parameters (change in mineral content [ΔM], change in the lesion depth [ΔL] and maximum mineral density at the surface-zone) for A-lesions at week 2, change in the lesion depth (ΔL), maximum mineral density at the surface-zone of TMR and change in lesion fluorescence loss (ΔΔF) of the B-lesions at week 4 were exploratory efficacy variables. All TMR parameters at week 2 for A-lesions were analysed the same way as EFU. Change in the lesion depth (ΔL), maximum mineral density at the surface-zone and change in lesion fluorescence loss (ΔΔF) of the B-lesions were analysed in the same way as ΔM of TMR at week 4. Baseline (participant and period level) lesion depth, SZmax and ΔF were used as covariates, respectively.

Results

Of 58 screened participants, 45 were randomised to treatment and 42 completed the study between 8 Feb 2016 and 11 Aug 2016 [Figure 1]. Of the randomised participants, 55.6% were female and mean age was 64.3 (range 36–80) years. The study failed to recruit sufficient participants per the planned recruitment target. This was due to unavailability of enough participants with appliances of adequate size to support four enamel specimens as required. The study was designed so that all possible pairs of treatments would be observed, ideally an equal number of times. However, due to participant recruitment and early withdrawal, where subjects did not complete all periods, the numbers in each treatment comparison were not exactly balanced [Figure 1]. Given that this was an exploratory study, and no formal sample-size calculation had been performed for the primary focus (phytate dose response), the decision was made to continue the study with the available participants. The decrease in power due to the small reduction in the number of planned randomised participants was considered to be modest.

Surface Microhardness Recovery (%SMHR)

Indentation lengths and %SMHR for A-lesions are shown in Table 2 with adjusted mean %SMHR shown in Figure 2A. All groups demonstrated statistically significant ($p<0.0001$) remineralisation except Placebo ($p=0.1093$). Table 3 shows that the %SMHR for the F-only group was statistically significantly higher than the Placebo, demonstrating model validity for this measure. No significant differences were observed between the fluoride toothpaste groups, thus the addition of phytate at 0.425% or 0.85% did not statistically significantly attenuate or improve the effect of fluoride. The linear and quadratic tests did not indicate a dose-response trend of phytate. The addition of 0.3% $ZnCl_2$ to the 0.85% sodium phytate formulation combined with 1150 ppm fluoride produced no significant change in %SMHR, nor did it significantly attenuate the 1150 ppm fluoride effect in the absence of phytate.

TMR values: A and B-lesions

Adjusted mean TMR values are shown in Figure 3 with results detailed in Supplemental Table 1. TMR values for the F-only group were statistically significantly higher than the Placebo ($p<0.05$ for all) demonstrating model validity for this measure. Consistent with %SMHR, no differences were observed between the fluoride toothpaste groups. The linear and quadratic trends indicated no clear dose-response of phytate.

EFU and QLF: A and B-lesions

Results are shown in Figure 2B ($\Delta\Delta$F), 2C (A-lesion EFU) and 2D (B-lesion EFU) along with Supplemental Table 2. The F-only treatment was statistically significantly superior to the Placebo for all measures ($p<0.001$) demonstrating model validity. Consistent with the other measures, no statistically significant differences were observed between the fluoride toothpaste groups, with the linear and quadratic trends indicating no clear dose-response of phytate. Positive $\Delta\Delta$F values demonstrated remineralisation of lesions in all groups.

Safety

Supplemental Table 3 details all treatment-emergent AEs (TEAEs). Overall 79 TEAEs were reported by 57 participants, of which 51 TEAEs were oral. However, few TEAEs were considered treatment related, one each in the 0.85% Phy/F group (angular cheilitis), 0.85% Phy/Zn/F group (dry mouth) and F-only (lip dry) groups. Two non-treatment related TEAEs (one of mild urinary tract infection, one [serious and severe] of invasive ductal carcinoma in breast) led to participant withdrawal. All other TEAEs were mild or moderate in intensity and most had resolved by the end of the study.

Discussion

The primary aim of this study was to evaluate the potential impact of phytate on the ability of fluoride to reduce dental caries. The *in situ* model design employed in this clinical study has previously been used as a surrogate predictor of clinical efficacy for caries [24, 31]. The principal advantage of this model is that efficacy information on the early stages of the caries process can be obtained in a relatively short time-scale in well-controlled conditions. A further advantage of the study design is the incorporation of two lesion types. As seen previously, the B-lesions underwent

net demineralisation [28] whereas the A-lesions underwent net remineralisation [24]. Since fluoride exerts its anti-caries effect by both reducing demineralisation and promoting remineralisation, it is advantageous to be able to study both phenomena simultaneously.

In this study, a three-level dose-response for phytate (0%, 0.425% and 0.85% sodium phytate) was employed to determine its impact on fluoride's ability to promote remineralisation and inhibit demineralisation of enamel in the oral cavity, and hence the potential impact on fluoride's anticaries activity. The effect of zinc was investigated using a 0.3% $ZnCl_2$ formulation (with 0.5% sodium citrate added to stabilise zinc ions in solution). Other formulations were included to allow an investigation of any modulation of the effect of phytate by zinc and vice versa. This treatment set also allowed study of the effect of phytate (at 0.85% sodium phytate) and zinc in combination on fluoride's actions in the model.

While the study failed to recruit sufficient participants per the planned recruitment target, a statistically significant difference between the 0 ppm (Placebo) and 1150 ppm fluoride (F-only) toothpastes (negative and positive controls, respectively) was observed for all endpoints, for both lesion types. Therefore, all endpoints were regarded as valid and permit comment on the potential fluoride efficacy of formulations tested. None of the endpoints showed a statistically significant difference between the positive control (F-only) and any of the treatments containing phytate or zinc for either of the lesion types. Further, there was no clear dose-response of phytate (within the range of 0–0.85%) on any of the endpoints investigated, as evidenced by no statistically significant linear or quadratic contrasts. Thus, within the limitations of this exploratory study (no formal sample-size calculation on the primary focus and a potentially modest reduction in the expected power), neither phytate nor zinc ions, alone or together, appear to meaningfully affect fluoride's ability to promote remineralisation or prevent demineralisation of enamel in this model, within the dose-range tested.

Notwithstanding this conclusion, there are trends in the data with respect to phytate. In many measures, amongst the phytate dose-response toothpastes, the phytate-containing formulations performed numerically less well than the F-only formulation (A-lesions: %SHMR, all TMR measures, EFU; B-lesions: TMR ΔM and ΔL, QLF and EFU). This observation suggests that there may be a slight negative impact of phytate on fluoride efficacy in this model and warrants further investigation.

In relation to other studies performed on phytate [23] or zinc [22], these results stand in marked contrast. In a single-treatment erosion orientated *in situ* study (conducted on lesions representative of erosive lesions [plaque-free]), phytate (at the 0.85% sodium phytate concentration used in this

study) significantly inhibited fluoride-promoted remineralisation, and also appeared to inhibit fluoride's ability to protect against demineralisation [23]. In a similar zinc-oriented single-treatment *in situ* study (also using the 0.3% zinc chloride concentration used in this study), zinc inhibited fluoride-promoted remineralisation, but, intriguingly appeared to add to fluoride's ability to protect against demineralisation [22]. The single-treatment erosion-orientated *in situ* model is focused on exploring recovery from the very earliest signs of enamel demineralisation. However, the strength of the effects observed in the erosion-focused *in situ* studies make it remarkable that the overall effect of phytate and zinc (individually or together) was marginal at most in the present study. For zinc, it may well be that the observed additive effect to fluoride in protecting against demineralisation is important in the daily intra-oral remineralisation/demineralisation cycle, balancing out any inhibition of remineralisation. Perhaps more likely is the fact that the single-treatment model uses plaque-free enamel surfaces, whereas the present study measures effects on plaque-covered surfaces. So, while the plaque-free model allows understanding of potential enamel chemistry from treatments to be studied, any such effects may be much less relevant when the surface is covered by a dense plaque biofilm, as was the case for the model used in this study and is usual for nascent caries lesions [36].

This study involved a variety of different techniques to examine effects on enamel. Interestingly, while the TMR-derived ΔM values show that the B-lesions demineralised further during the study, the QLF data suggest they have remineralised for all treatment groups. This result appears surprising; however, it does mirror previous observations [28]. One explanation may be that QLF is more sensitive to the condition of the surface zone than expected. Whilst ΔM values for these lesions showed overall demineralisation compared to baseline, the ΔSZ_{max} values showed that the surface zone remineralised compared to baseline. This hypothesis would benefit from further investigation.

In conclusion, the present *in situ* caries model study found no statically significant difference in %SMHR, TMR parameters, EFU or QLF measures between the fluoride toothpastes with or without phytate. These results suggest that phytate, incorporated at up to 0.85% sodium phytate into a fluoride or fluoride/zinc toothpaste, has little impact on the ability of fluoride to affect remineralisation of surface-softened (A-) lesions or prevent demineralisation of subsurface (B-) lesions. Similarly, the results also show that the presence of zinc (as zinc chloride at 0.3%) does not impact fluoride efficacy in this model. However, these observations should be confirmed in a formally powered in situ study. All study treatments were generally well-tolerated.

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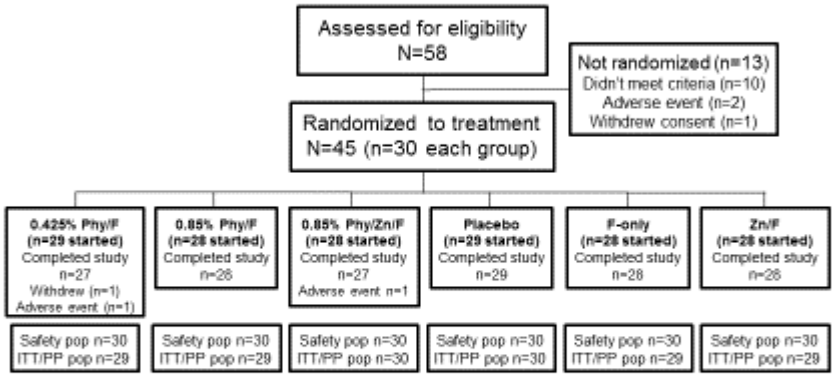

Figure 1: Participant disposition throughout study

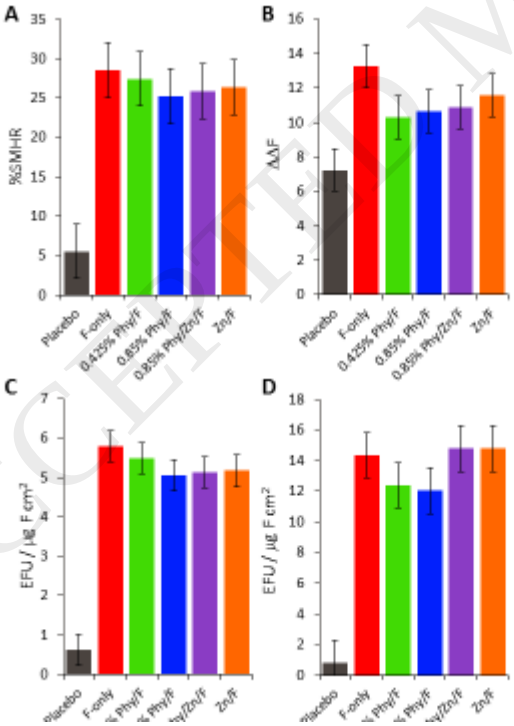

Figure 2: Adjusted mean (standard error) of A) %SMHR for A-lesions, B) change in QLF for B-lesions, C) EFU for A-lesions and D) EFU for B-lesions (ITT population)

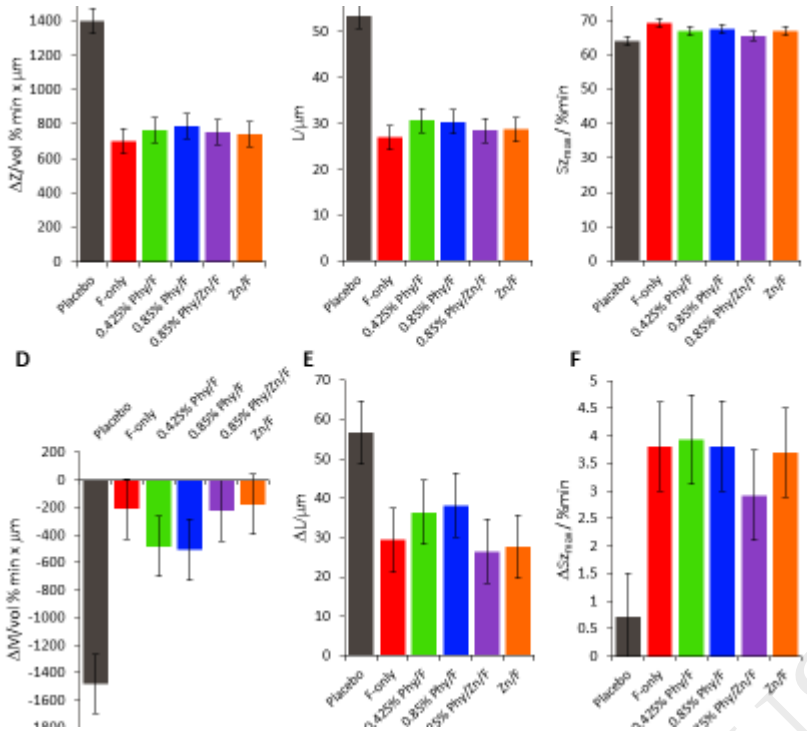

Figure 3: TMR values for study treatments (A–C: A-lesions, D–F: B-lesions; adjusted mean ±standard error): A) Integrated mineral loss (ΔZ), B) Lesion depth and C) SZ_{max}; D) Integrated mineral loss (ΔZ), E) Lesion depth, F) SZ_{max} (ITT population)

Table 1: Description of study treatments*

Name	Phytate (as sodium phytate, w/w)	Zinc (as zinc chloride, w/w)**	Fluoride (ppm F, as NaF)
0.425% Phy/F	0.425% w/w	-	1150
0.85% Phy/F	0.85% w/w	-	1150
0.85% Phy/Zn/F	0.85% w/w	0.3%	1150
Placebo	-	-	-
F-only	-	-	1150
Zn/F	-	0.3%	1150

***All toothpastes were formulated in a silica abrasive base that contained sodium lauryl sulphate as the primary surfactant; **Zinc-containing toothpastes included sodium citrate to stabilise the ionic form of zinc.

Table 2: Indentation lengths and %SMHR means for A-lesions (ITT population)

Indentation lengths (SE)	0.425% Phy/F	0.85% Phy/F	0.85% Phy/Zn/F	Placebo
Baseline	43.73 (0.071)	43.49 (0.081)	43.54 (0.076)	43.57 (0.072
After demin	114.92 (0.599)	114.82 (0.608)	114.30 (0.578)	113.95 (0.594
After treatment	96.36 (0.990)	95.60 (0.924)	97.33 (0.924)	108.57 (1.047
%SMHR Mean (95% CI)	27.42 (20.55, 34.29)	25.20 (18.33, 32.08)	25.89 (19.00, 32.79)	5.54 (-1.26, 12.
P value	<0.0001	<0.0001	<0.0001	0.1093

Table 3: Differences in %SMHR for A-lesions (ITT population)

Treatment comparison		**Adjusted mean diff (95% CI)**	**p-value (% change**)**
Linear contrast*		P = 0.3555	
Quadratic contrast*		P = 0.8545	
F-only vs Placebo		**22.96 (16.02, 29.90)**	**<.0001** (414.3%)
0.425% Phy/F vs	F-only	-1.09 (-8.11, 5.94)	0.7603 (-3.8%)
	0.85% Phy/F	2.21 (-4.81, 9.23)	0.5335 (8.8%)
0.85% Phy/F vs	F-only	-3.30 (-10.34, 3.74)	0.3555 (-11.6%)
	0.85% Phy/Zn/F	-0.69 (-7.65, 6.27)	0.8443 (-2.7%)
0.85% Phy/Zn/F vs	Zn/F	-0.45 (-7.46, 6.55)	0.8985 (-1.7%)

Zn/F	vs	F-only	-2.16 (-9.34, 5.03)	0.5533 (-7.6%)

*0.425% Phy/F, 0.85% Phy/F, F-only; **Second treatment used as reference in calculating (Difference/Reference)*100