ORIGINAL RESEARCH

Remineralization of eroded enamel by a NaF rinse containing a novel calcium phosphate agent in an *in situ* model: a pilot study

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Purpose: An *in situ* study evaluated the remineralization potential of 225 ppm fluoride (F) rinses with and without a calcium phosphate agent (TCP-Si-Ur) on eroded enamel.

Methods: 20 human patients participated in this IRB approved study. Enamel blocks extracted from 20 human molars were assigned to each of the three study phases (G1, G2, G3). Each block was eroded using 1% citric acid (pH = 2.5), with a slice cut from each block to establish baseline lesion parameters (ie, integrated mineral loss ΔZ, and lesion depth LD) using transverse microradiography (TMR). Participants and assigned blocks were randomly divided into three 28-day phases. The blocks were mounted into modified orthodontic brackets and bonded to the buccal surface of one of the subject's mandibular molars. The appliance remained in the subject's mouth for 28 days. Prior to each study phase, participants observed a one-week-washout period using a fluoride-free dentifrice. In each phase, participants brushed with the fluoride-free dentifrice for 1 min, followed by one of the following coded treatments: G1: 225 ppm F + 40 ppm TCP-Si-Ur rinse (1 min); G2: 225 ppm F rinse (1 min); G3: no rinse (saliva-only). After each phase, appliances were removed and specimens were analyzed using TMR.

Results: TMR data (ie, ΔZ and LD) revealed all three groups significantly remineralized eroded enamel (paired *t*-tests, $P < 0.001$). Net mineralization (% change in ΔZ, LD) were as follows (mean (std.dev.): G1: 44.1 (22.6), 30.5 (27.0); G2: 30.0 (7.4), 29.4 (10.5); G3: 23.8 (16.4), 25.7 (15.5). Furthermore, G1 was found to cause significantly more remineralization than G2 ($P = 0.039$) and G3, ($P = 0.002$).

Conclusion: Mouthrinse containing 225 ppm F plus TCP-Si-Ur provided significantly greater remineralization relative to 225 ppm F only or saliva alone.

Keywords: TCP-Si-Ur, fluoride, antierosion, tricalcium phosphate, double-blind

Introduction

Dental erosion, or the chemical wear of tooth enamel, is a well-recognized problem in dentistry which may be caused by frequent consumption of common acidic foods (eg, any citrus food such as apples and oranges) and beverages (sports drinks, orange juice, soft drinks, coffee, etc), or the attack of stomach acids during acid reflux and/or vomiting. Patients can barely detect early enamel erosion due to its smooth and shiny appearance. Their attention is drawn to the disorder when it becomes advanced and symptomatic due to dentin exposure and its associated sensitivity. It is therefore appropriate to develop antierosion therapies that either arrest the progression or promote the remineralization of any developing lesion.

Research assessing fluoride's impact against dental erosion has led, in part, to the following recommendations: either high fluoride levels are required to adequately

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increase enamel resistance to erosion, or fluoride should be administered directly before or immediately following an acid challenge.[1,2] Administration prior to acid challenge is problematic and unreasonable, since it is obvious that persons who are prone to vomiting or acid reflux may not bother to brush their teeth prior to the acidic event. On the other hand, brushing after the event produces significant complications where softened enamel is readily worn through toothbrush/ toothpaste abrasion. For the former recommendation, many people already refuse to use over-the-counter (OTC) levels of fluoride (ie, 1100 ppm fluoride) for personal reasons or purported ill-effects of fluoride;[3] similarly, the recommendation of a daily 5,000 ppm or 12,000 ppm fluoride dentifrice may not be welcoming to all patients. Therefore, opportunities exist in exploring innovative therapies to combat dental erosion.

Although saliva and cheese can remineralize eroded enamel,[4–6] there remains a significant need in elevating and extending the level of antierosion protection. As an example, GlaxoSmithKline markets a global toothpaste, Sensodyne® Pronamel (GlaxoSmithKline), that contains fluoride and polymer surfactants that may have promising antierosion benefits.[7] Separately, some researchers have probed the potential benefits of low pH metallic fluorides, hydrofluoric acid, or metallic solutions.[8–10] Ultimately, multimineral treatments including fluoride, calcium, and phosphate may provide the most promising effects,[11] due in part to mineral-matching characteristics.[11] These formulations, however, are not typically compatible with one another in a single-compartment water-based product, as unwanted calcium fluoride may form and therefore reduce fluoride bioavailability and therapeutic efficacy.[12] As such, the ability to combine fluoride and a fluoride-compatible form of calcium in a single-compartment

water-based container, such as a toothpaste or mouthrinse, presents a tremendous opportunity.

Recently we have reported on the *in vitro* antierosion benefits of fluoride plus an innovative fluoride-compatible functionalized tricalcium phosphate (fTCP) material, consisting of beta-tricalcium phosphate (β-TCP), silica (Si), and urea (Ur), TCP-Si-Ur.[13–15] Contributing to the appeal of β-TCP is the fact that it is partially soluble and readily contributes to the mineralization of bone and teeth.[16,17] Silica breaks down in acidic environments and as a result, may provide linking opportunities with hard and soft tissue defects:[18] when combined with β-TCP, it may help provide protection against enamel softening during demineralization. Urea is especially important as it can penetrate enamel without attacking the interprismatic organic material,[19] provide an interface to facilitate interactions among β-TCP, fluoride, and enamel, and may thwart undesirable interactions between fluoride and β-TCP when contained in an aqueous vehicle (eg, mouthrinses). The *in vitro* studies have demonstrated that TCP-Si-Ur enhances the benefits of fluoride by conferring statistically greater remineralization benefits relative to positive and negative controls.[13–15] However, clinical evaluation has not yet been reported. Therefore in this paper, we present the results of a pilot clinical study evaluating remineralization benefits of erosive lesions treated with the above antierosion agent. Of primary interest was whether the fluoride plus TCP-Si-Ur rinse may mimic *in vitro* results and provide greater antierosion benefits relative to fluoride and saliva alone.

Table 1 Paired *t*-test analyses of mean (std. dev.) mineral loss (ΔZ) and lesion depth (LD) for treatment regimes G1, G2, and G3 before and after treatment (N = 18)

Treatment Group	ΔZ_1, Control (vol % · μm)	ΔZ_2, Test (vol % · μm)	P Value[†]	Significant?
G1	318 (105)	167 (60)	7.1×10^{-6}	YES
G2	339 (85)	236 (55)	1.2×10^{-8}	YES
G3	315 (88)	238 (83)	1.3×10^{-5}	YES
	LD_1, Control (μm)	LD_2, Test (μm)		
G1	11.9 (3.4)	7.9 (2.3)	9.6×10^{-5}	YES
G2	12.2 (2.6)	8.6 (1.2)	3.2×10^{-5}	YES
G3	11.9 (2.6)	8.6 (1.8)	2.3×10^{-5}	YES

Note: [†]Paired *t*-test.
Abbreviations: ΔZ, integrated mineral loss; LD, lesion depth.

Table 2 Comparisons of mean (std. dev.) percent (%) change in mineral loss (ΔZ) and lesion depth (LD) for treatment regimes G1, G2, and G3

Comparison[†]	% Change in ΔZ	% Change in ΔZ	P value	Significant?
G1 vs G2	G1: 44.1 (22.6)	G2: 30.1 (7.4)	0.039	YES
G1 vs G3	G1: 44.1 (22.6)	G3: 23.8 (16.4)	0.002	YES
G2 vs G3	G2: 30.1 (7.4)	G3: 23.8 (16.4)	0.501	NO
	% Change in LD	**% Change in LD**		
G1 vs G2	G1: 30.5 (27.0)	G2: 29.4 (10.5)	0.977	NO
G1 vs G3	G1: 30.5 (27.0)	G3: 25.7 (15.5)	0.664	NO
G2 vs G3	G2: 29.4 (10.5)	G3: 25.7 (15.5)	0.787	NO

Note: [†]ANOVA and Tukey's test.
Abbreviations: %, percent; ΔZ, integrated mineral loss; LD, lesion depth.

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Material and methods

Specimen preparation and initial erosive lesion formation

Freshly extracted human molar teeth were collected, sterilized with ethylene oxide gas and examined before storing in 0.1% thymol solution prior to use. 20 teeth without caries, cracks, or enamel malformations were selected and cleaned with pumice to remove the remnants of pellicle and debris/stains from the buccal surface. The buccal surface of each tooth was ground and polished to produce a flat surface. The teeth were painted with two coats of acid-resistant nail varnish except for a window of exposed enamel, measuring approximately 9 mm × 2 mm, on the flat buccal surfaces of the tooth. An early erosive enamel lesion was created on each exposed window through immersion in a static 1% citric acid solution (pH = 2.5) lasting 30 minutes. Following exposure, the nail varnish on all teeth was carefully and totally removed with acetone (GPR, Aldrich, Milwaukee, USA). Using a water-cooled diamond wire saw (Buehler, Düsseldorf, Germany), each erosive lesion was cut into three lesion-bearing blocks (approximately 3 mm × 2 mm) with sound enamel surface at each end of the block. The sound enamel component was used as a reference surface for transverse microradiography (TMR) analysis. A total of three erosive lesion-bearing blocks were obtained from each tooth and used for each leg of the study.

Processing of pre-test control slices and baseline measurements

One tooth slice (control) of approximately 150 μm thick was cut from each experimental block for measurement of the baseline parameters of the lesion and for selection of the suitable lesions for the study. The slices were processed and microradiographed using TMR as described in previous publications.[20] The microradiographs were analyzed with TMR analysis software version 3.0.0.11 (Inspektor Research Systems, Amsterdam, Netherlands) to quantify the parameters of integrated mineral loss (ΔZ, vol % • μm) and lesion depth (LD, μm).[4,20]

Intra-oral appliances

Each of the lesion-bearing tooth blocks was mounted within an intra-oral appliance, a customized orthodontic bracket (Figure 1). The appliance consisted of an orthodontic molar pad with retentive mesh backing, which had a stainless steel band welded to it so that the band closely enclosed each test enamel block. The enamel specimen was retained within the

Figure 1 Photograph of the *in situ* appliance.

bracket using fluoride-free Intermediate Restorative Material. In order to minimize the abrasive effect of tooth brushing on the erosive lesion, the blocks were mounted slightly recessed below the edges of the band. Each tooth successfully completing the fabrication process produced three *in situ* appliances. The appliances were then sterilized with gamma irradiation.[21]

Mouthrinse preparation

The fluoride mouthrinses were prepared under Good Manufacturing Practices by Nanotech as follows. A cylindrical
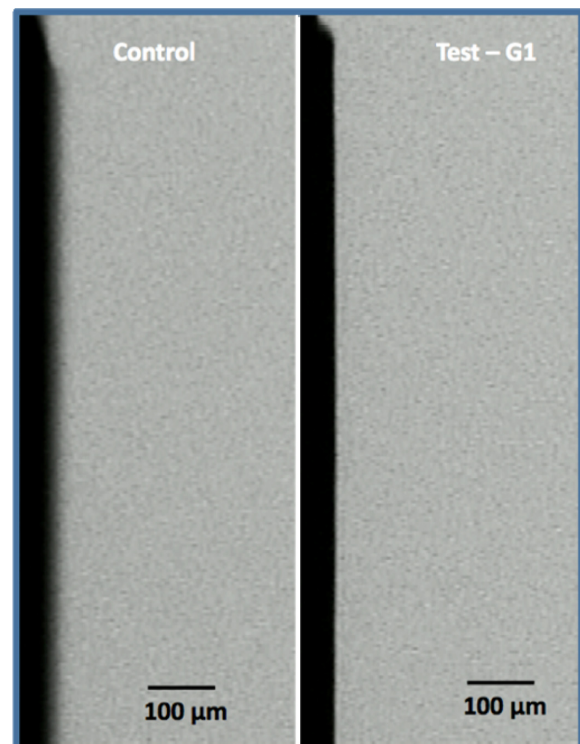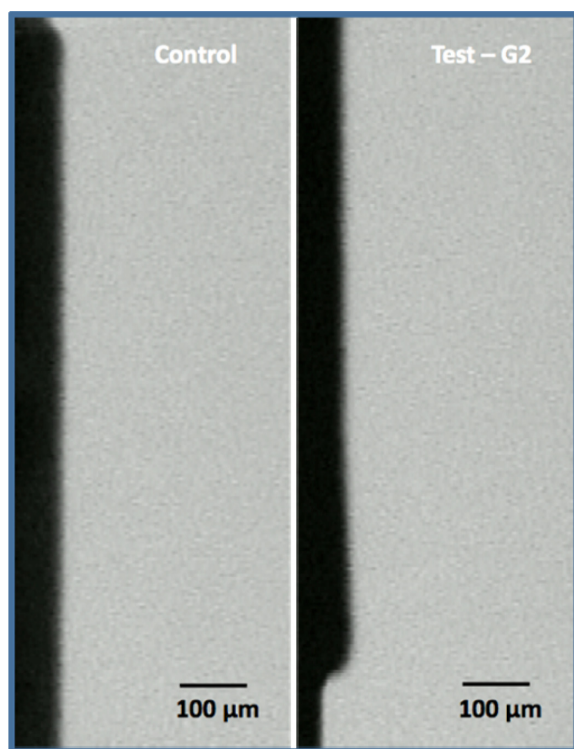

Figure 2 Example microradiographs of enamel initially eroded (left – Control) and after treatment with treatment regime G1 for four weeks (right – Test).

Figure 3 Example microradiographs of enamel initially eroded (left – Control) and after treatment with treatment regime G2 for four weeks (right – Test).

10 gallon high-density polyethylene (HDPE) tank with a spigot (Saint-Gobain Performance Plastics, Muncie, IN) was wiped down with 70% reagent alcohol (Fisher Scientific, Pittsburgh, PA) then filled with 6 gallons of steam distilled water. 225 ppm F from sodium fluoride (Fisher Scientific, USP/EP/BP grade) was added and stirred for 5 minutes using a Stir-Pak laboratory stirrer (Cole-Parmer, Vernon Hills, IL) to ensure complete dissolution. Next, 0.1% w/w sodium methyl parabens (EP grade; Fluka, St. Louis, MO, USA) was slowly added and mixed for five minutes. Visual inspection was used to ensure complete dissolution and that no powder remained on the sides of the container or mixer blade. This same process was used to add 0.05% w/w sodium propyl parabens (Spectrum Chemical, nongraded, New Brunswick, NJ, USA). The solution was then dispensed into individual and coded rectangular 1 L HDPE Nalgene containers. The 10 gallon tank was wiped down with alcohol again and a second solution containing 225 ppm F, 0.1% w/w sodium methyl parabens, 0.05% w/w sodium propyl parabens, and 0.004% w/w TCP-Si-Ur (added last) was made using the above procedure. The solution was continually stirred while being dispensed into coded 1 L Nalgene containers to prevent TCP-Si-Ur from settling out. Each mouthrinse system has a pH of 8. The identities of the coded mouthrinses were kept blinded to the personnel at University of Texas Health

Science Center at San Antonio (UTHSCSA) until completion of the study.

Subjects selection

The study was approved by the Institutional Review Board of the UTHSCSA. Twenty healthy adults, aged 18–50 years, from different ethnic origins and socioeconomic status, participated in this study. The subjects were identified with code numbers. After providing informed written consent, subjects underwent a complete intraoral examination and completed a medical history questionnaire. The inclusion criteria were: having at least 22 teeth with a past history of dental caries but no clinically active caries, periodontal disease, or other oral pathology, and having a mandibular first molar with sound, unrestored buccal surface. Other inclusion criteria were normal salivary function with unstimulated and stimulated salivary flow rates greater than 0.2 mL/min and 0.7 mL/min, respectively, measured according to the Sreebny and Valdini procedure,[22] and not taking any antibiotics or medications which could affect saliva flow rate. The sample size calculation was based on a hypothesized reduction of 60% in mean mineral loss (ΔZ) in each experimental group relative to control, using a one-sided test with 5% significance level and 80% power.

Study procedure

This study was comprised of three distinct phases during which the subjects were exposed to the following treatments in a randomized crossover design: **G1**: Test mouthrinse containing 225 ppm fluoride (NaF(aq)) plus 40 ppm TCP-Si-Ur; G2: Control mouthrinse containing 225 ppm fluoride (NaF(aq)); G3: No mouthrinse (exposure to saliva alone). Each phase lasted for 28 days, and was preceded by a 7-day washout period to balance for residual effects of previous product. The three *in situ* appliances made out of the three tooth blocks originating from the same tooth were assigned to one subject. Following this, the first of the three assigned appliances was bonded onto the buccal surface of the chosen lower molar tooth, in accordance with current principles of orthodontic practice. Subjects received oral and written instructions to brush their teeth two times daily with a fluoride-free dentifrice (Tom's of Maine® Silly Strawberry fluoride-free toothpaste, Tom's of Maine, USA) for 1 minute on each occasion followed immediately by rinsing with their assigned mouthrinse. For those subjects using G1 and G2 mouthrinses, the subjects were provided with one bottle of their respective mouthrinse – enough for 28 days, to be used two times daily. Subjects were instructed to swish 10 mL of the rinse for 1 minute in the mouth and spit. The rinse was used after brushing in the morning and then lastly before

Figure 4 Example microradiographs of enamel initially eroded (left – Control) and after treatment with treatment regime G3 for four weeks (right – Test).

going to bed. Subjects were instructed to neither rinse their mouth nor take any drink for at least 30 minutes after rinsing. Also included in the instructions was to record the number and time of toothbrushing each day in the diary provided; refrain from the use of any other oral hygiene products for the duration of the trial; maintain their normal dietary habits; and return the remaining toothpaste and mouthrinse after each study phase. The weight of toothpaste and mouthrinse were measured before and after the study phase. This was done to monitor compliance, ensure that the subjects discontinue the use of the previous product, and ensure uniformity in the use of the oral hygiene product, which may otherwise unduly influence the de-/remineralization cycle during the study periods. After each 28-day period, the appliance was detached, and after the washout period the next appliance was cemented in place on the same tooth as the first appliance. This procedure was repeated until the three phases were completed by each subject. At the detachment of the appliance, any bonding agent left on the tooth surface was carefully and completely removed with composite-removing burs.

Post-study TMR processing

After detachment, the blocks were removed from their respective appliances, and an enamel slice (about 150 µm thick) was cut from each block and processed for microradiography

as described above for the control. Although the control slices had been microradiographed and analyzed for selection of the appropriate lesions, they were microradiographed again along with the post-test slices and both analyzed together for quantification of ΔZ and LD. This enabled both groups to be microradiographed and analyzed under the same conditions. This process yielded the following information:

1. The pre-test ('Control') TMR parameters (ΔZ_1 and LD_1) of the lesions.
2. The post-test ('Test') TMR parameters (ΔZ_2 and LD_2) of the lesions.
3. The pre-test ('Control') and post-test ('Test') TMR images of the lesions.

With respect to TMR data, the mean (N = 18, two subjects dropped out due to noncompliance) values of the pre-test and post-test lesion parameters (ΔZ and LD) for each test product (G1, G2, and G3) were compared to determine the eroded enamel response to each treatment modality. However, to enable comparisons among the three experimental phases, percentage changes in lesion parameters relative to the control parameters were determined for each treatment group. We note that percentage change is commonly used for ranking and comparison in order to provide that, for instance, although the three blocks came from the same tooth, the lesion parameters for the blocks may differ at baseline. The percentage change in mineral loss (ΔZ) (ie, net remineralization) and lesion depth (LD) (ie, net modification of lesion size) were calculated in equations (1) and (2), respectively:[4]

$$\% \text{ change in } \Delta Z = \frac{\Delta Z_1 \text{ (control)} - \Delta Z_2 \text{ (test)}}{\Delta Z_1 \text{ (control)}} \times 100 \quad (1)$$

$$\% \text{ change in } LD = \frac{LD_1 \text{ (control)} - LD_2 \text{ (test)}}{LD_1 \text{ (control)}} \times 100 \quad (2)$$

Statistical analysis

Statistical analysis of the data was conducted using SPSS statistical software (PASW Statistics 17.0; SPSS Inc., Chicago, IL), with a level of significance (α) selected at 0.05. The mean values of the lesion parameters, mineral loss (ΔZ) and lesion depth (LD), were calculated for the pre- and post-test groups of each of the treatment modalities (G1, G2, and G3). The data were examined for normality using the Kolmogorov–Smirnov test with $P = 0.05$. The pre-test and post-test lesion parameters (ΔZ and LD) for each group were compared using paired t-tests at the 95% confidence level (CL). To compare remineralization ben-

efits among treatment groups, one-way analysis of variance (ANOVA) was used to determine if differences existed among the groups at the 95% CL. Observed differences were then analyzed post-hoc using the multiple comparison Tukey's HSD test.

Results

Two subjects were withdrawn due to noncompliance by established compliance monitoring factors such as the amount of mouthrinse used. The data (mean ± std. dev.) demonstrate the initial erosive period produced consistent integrated mineral loss (approximately 320 vol % • μm) and shallow lesion depths (approximately 12 μm) for each set of 18 specimens. Table 1 reveals significant differences between the pre- and post-test ΔZ and LD in each group, indicating significant remineralization in all three treatment groups. Complementing the overall remineralizing effect of the study groups, representative TMR images for specimens subjected to treatments G1, G2, and G3 are shown in Figures 2, 3 and 4 respectively. In Figure 2, the radiographs revealed a clear difference between the initial erosive lesion, which manifests a more opaque surface, and the remineralized specimen (ie, well-resolved surface) after four weeks of G1 treatment. Similarly, Figures 3 and 4 also demonstrate some differences after G2 and G3 treatments, although the contrast is not as clear as that shown in Figure 2; overall, toothbrushing followed by the G1 treatment appears to promote the greatest remineralization.

Table 2 shows the inter-group comparison based on the calculated percent change in the lesion parameters. Among the three treatment groups, toothbrushing followed by one-minute rinsing with G1 (fluoride plus TCP-Si-Ur) significantly imparts a remineralizing benefit relative to toothbrushing plus G2 (fluoride only) or G3 (saliva only). Although the treatments G2 and G3 did not statistically break, there is a directional trend favoring the G2 treatment after toothbrushing. With respect to the change in lesion depth, there were no significant differences among the three groups. The depth corresponding to G1 and G2 were nearly equivalent and both provided some directional trending toward shallower lesions relative to G3.

Discussion

In vitro and *in situ* studies have demonstrated the remineralization properties of saliva on erosive lesions.[4–6] Thus, the overall remineralizing effect observed for each of the three treatment groups in this *in situ* study is consistent with existing knowledge and highlights the importance of saliva in mineralizing erosive lesions. Even further, the model appears appropriate for assessing the remineralization potential of candidate systems, including saliva, fluoride, and calcium phosphate systems.

It is important to mention that exposure of human enamel to 1% citric acid (pH = 2.5) solution produced significant erosive effects, with the formation of large craters as observed visually with a light microscope. The TMR images reveal a shallow erosive lesion, with initial lesion depths of about 12 μm. Thus, the low pH conditions used to form the shallow lesion led to large pockets of tissue loss. This contrasts to lesions produced by orange juice (pH ~ 3.8), for instance, whereby the erosive pockets are not as extensive and the lesion depth extends to almost 60 μm.[4] Hence, the sensitivity to remineralization in this *in situ* study will be strongly influenced by the preparation of the initial erosive lesion in enamel.

Treatment G1 conferred the highest level of net remineralization (ie, percent change in ΔZ) among the treatment groups and was found to be statistically greater relative to both G2 and G3. The G1 mouthrinse contained 225 ppm fluoride plus a fluoride-compatible functionalized calcium phosphate system (fTCP) that has been designed to work synergistically with fluoride to improve remineralization of eroded enamel. The remineralization of the G1 system in this study, which is statistically superior to fluoride-only (G2), can then be attributed to the inclusion of TCP-Si-Ur. Therefore, combination of fluoride plus TCP-Si-Ur appears to provide significant antierosion benefits, and is an important outcome of the study which may be useful in the search for novel antierosion therapies. Existing approaches include application of low pH hydrofluoric, titanium or stannous fluoride systems, as well as or iron solutions, to prevent or repair eroded enamel;[8–10] however, the simple combination of calcium, phosphate, and fluoride in a single neutral aqueous may be appealing for several reasons: the mineralizing properties of these minerals are well-established, the economic, aesthetic and/or sensory characteristics may offer advantages over metal-containing formulations, and the neutral pH reduces risk of possible soft-tissue irritation and/or tooth demineralization.[11,23] Additionally, the calcium-phosphate-fluoride combination may be advantageous for those experiencing xerostomia or hypersensitivity. Importantly, the present study also confirms the results from our *in vitro* investigations which also demonstrated remineralization benefits.[13–15] It follows that this *in situ* study also serves to link *in vitro* experiments to the clinical setting.

Among the three treatment regimens, none were found to be statistically different from one another in terms of net change in lesion depth (ie, percent change in LD). However, we note directional trends favor the G1 and G2 treatments. Independently, all three groups narrowed the erosive lesion by about 30%. In a comparable four-week *in vitro* study with orange juice (pH ~ 3.8), the net change in lesion depth with respect to natural and artificial saliva remineralized eroded enamel up to 66%.[4] Thus, despite the narrow lesion depths, the large mineral-loss pockets formed by the initial 1% citric acid challenge, along with the natural remineralization potency of saliva, may frustrate the ability to detect significant differences across the groups. In the future, modifying the characteristics of the initial acid challenge may improve distinction among treatment groups.

Although clear directional trends exist in both the net remineralization and net change in lesion for treatment groups G2 and G3, we believe this requires some commentary since these groups did not break statistically. As all subjects were inherently exposed to fluoridated water throughout the study (0.8 ppm F), we recognize it may be possible that the 'saliva-only' group, G3, may not totally be without some effects of fluoride. However, we believe this effect contributes minimally, based on the clear directional trends favoring G2 treatments. As is always a risk, it is possible that all of the subjects in the G3 phase may not have maintained restricted use of other oral hygiene products. In fact, some strong complaints were vocalized to clinical personnel regarding the unpleasant taste of the Tom's of Maine® Silly Strawberry toothpaste, and the study coordinator proposed that some of the subjects may have reverted back to their personal fluoride toothpaste during the G3 phase. So while full subject compliance is difficult to enforce, this may have been a factor especially given the modest number of subject participants; therefore, using a fluoride-free paste that is more pleasing to the taste as well as including more study participants (ie, 30) may further improve treatment differences. And though significant remineralization of the erosive lesion over a four-week period within each group were observed, due to the powerful effects saliva impart on weakened enamel, it may be that a less-aggressive and dietary-relevant erosive lesion (such as that created with orange juice or carbonated soda which are major contributors to dental erosion), may be required to further tease out differences between saliva and fluoride-only groups. As such, future studies employing this *in situ* model will employ these recommendations.

Conclusion

In this pilot study, the rinse containing 225 ppm F + TCP-Si-Ur provided significantly greater remineralization potential relative to 225 ppm F and saliva. These promising results also provide a strong link to prior *in vitro* results, which also demonstrated significant antierosion potential of fluoride plus TCP-Si-Ur. The *in situ* model demonstrated sensitivity to remineralization of erosive lesions by saliva, fluoride, and fluoride plus TCP-Si-Ur. Therefore, the model appears appropriate for evaluation of antierosion therapies.

Disclosure

The authors report no conflicts of interest in this work.

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