IN SITU RESEARCH PROTOCOL

Study Number: 17-I-112

Title: Evaluation of the Fluoride Dose Response of a Modified In situ Caries Model

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1. RATIONALE AND STUDY OBJECTIVE

Fluoride toothpaste is the most widely used form of fluoride delivery worldwide. Fluoride dentifrices have shown in numerous clinical trials to be effective anticaries agents and have been the subject of several systematic quantitative evaluations (Marinho et al., 2003; Twetman et al., 2003), which provide the highest standard of evidence for the effectiveness of fluoride dentifrice. Marinho et al. (2003) based their conclusions on a meta-analysis of 70 trials of the effectiveness of fluoride dentifrice for the prevention of dental caries in children compared to placebo. They found evidence that the use of fluoride dentifrices has a caries-inhibiting effect (average reduction in Decay, Missing, and Filled Surfaces (DMFS) of 24%) on the permanent dentition. In addition they concluded that the effectiveness of fluoride dentifrice may be relatively greater in individuals with a higher caries experience, with increased fluoride concentration, increased frequency of use, and with supervised brushing. There was no evidence that the effect was dependent on background exposure to fluoridated water. Twetman et al. (2003) reached similar overall conclusions from their systematic review.

The current levels of fluoride dentifrice products that are marketed worldwide generally fall in the range between 1000 – 1500 ppm F, although dentifrices with lower fluoride concentrations are marketed in some countries. All fluoride toothpaste sold in the US is in the 1000-1100 ppm F range. Walsh et al. (2010) reported based on a network meta-analysis that the relative anticaries effects of fluoride toothpastes increased with higher fluoride concentration. Based on 74 trials involving the caries scores (DMFS) in the mixed or permanent dentition, the anticaries effect of fluoride toothpaste was 23% for 1000/1055/1100/1250 ppm F and 36% 2400/2500/2800 ppm F; however, toothpastes with 440/500/550 ppm F and below did not show a statistically significant effect compared to placebo.

Due to the high cost involved in conducting clinical caries trials a number of surrogate measures of fluoride efficacy have been introduced. These include in situ caries models, rat caries models, in vitro demineralization and remineralization studies, and fluoride uptake studies. These model systems have been extensively reviewed at a Conference held in Rochester, NY in 1994 (Adv Dent Res 9:169-340, 1995). While intra-oral models have been in use for the past forty years, a "Consensus Conference of Intraoral Models" (ADA, Chicago, Sept. 1990) clearly identified the need for validation of intra-oral caries models for their potential use as methods of evaluating the efficacy of fluoride dentifrices and other fluoride-containing dental products. Based on this conference and the Rochester Models conference, there is general agreement among researchers in the field that appropriately validated in situ models represent an acceptable approach for testing the anticaries potential of fluoride products.

Previous work by our group has shown that a modification of the Koulourides intra-oral model (Koulourides et al., 1974) has sufficient sensitivity and reproducibility to respond in dose-response manner to meet the requirements for model validation (Proskin et al., 1992). The model, which uses partially demineralized enamel as the starting hard tissue substrate, permits the evaluation of the ability of the test dentifrice to enhance net remineralization. Our current model has been validated based on its response to different dentifrice fluoride concentrations - 0, 250, 500 and 1100 ppm fluoride (Zero et al., 1994;

Zero, 1995; Zero et al., 2005) as well as in several more commercially funded in situ studies testing fluoride dentifrice products that also included fluoride dose response controls (unpublished data). An additional capability of the model involves testing the acid resistance of remineralized enamel specimens. This test provides the capability to compare the acid resistance of the mineral deposited during the in situ remineralization phase after treatment with different test products.

Over the past 10 years we have been experiencing increasing difficulty in recruiting subjects with mandibular partial dentures that meet our inclusion criteria. One of the reasons for the failure is the lack of sufficient room in the posterior buccal flange area of the subjects' partial dentures to accommodate two 4 × 4 mm enamel specimens. In an effort to improve subject recruitment we have redesigned the model so that the enamel specimens (4 mm round) are placed in the posterior denture tooth area of their denture and thus eliminate the requirement for having sufficiently large buccal flange area. We have already identified several potential subjects that only qualify for specimen placement in denture teeth and not in the buccal flange area. We have already successfully conducted an in vitro feasibility exercise to demonstrate the practicality of this technique. Furthermore, we have been placing specimens in the denture teeth location for several other in situ model studies including studies using our new biofilm model and this has not presented to be a problem for the subjects. The alteration of the denture teeth does not affect the occlusal part of the artificial tooth and thus does not alter a functional part of the denture.

An additional aspect of the modified model will be to use bovine enamel and not human enamel as has been our usual practice. We are facing increasing difficulty in obtaining a supply of human teeth for our research studies due to a decreasing supply and anticipated regulatory changes requiring informed consent. Most in situ model systems use either bovine or human teeth as the hard tissue substrate. From the perspective of clinical relevance, human teeth would be the most appropriate to use (Zero, 1995). Bovine enamel are known to have structural and chemical differences and to be more porous than human enamel (Lippert et al., 2013) and it is more vulnerable to acid dissolution than human enamel in an artificial caries model systems (Featherstone & Mellberg, 1981; Lippert et al., 2013; Lippert & Lynch, 2014). However, the resulting lesions are practically indistinguishable from each other (Lippert & Lynch, 2014), and these differences result in quantitative differences and not qualitative differences in their response in in situ model studies (Mellberg, 1992; Lippert & Hara, 2012). Bovine enamel can therefore be considered an acceptable alternative to human enamel.

The purpose of this study is to evaluate the fluoride dose response of different dentifrice fluoride concentrations - 0, 250, 500 and 1100 ppm fluoride of our existing in situ model involving the use of human enamel specimens placed in the buccal flange area of the subjects partial denture with the modified model involving placement of bovine enamel specimens in a denture tooth location.

2. STUDY DESIGN

This will be a double blind, single center, 4-way crossover design study. Two to three days before the start of each treatment period the subjects will have their teeth cleaned to remove all accessible plaque and calculus and will be provided with a non-fluoride

dentifrice to use until their next visit. At the beginning of each testing period, two gauze-covered 4 × 4 mm partially demineralized human enamel specimens will be placed in the buccal flange area of the subject's mandibular partial denture. In addition, two gauze-covered 4 mm round partially demineralized bovine enamel specimens will be placed in the buccal surface of two posterior denture teeth of the same side of the partial denture. Once specimens are placed, subjects will wear their partial dentures twenty-four hours a day and use their assigned toothpaste twice daily, as instructed, until their next visit. Specimens will be removed after two weeks and the subjects will undergo a four to five day washout period followed by another cleaning and two to three day lead in period. This process will be repeated until all subjects have used all four test products. Changes in the mineral content of the enamel specimens will be assessed using the SMH and TMR. Enamel fluoride uptake (EFU) will be determined using the microdrill enamel biopsy technique. In addition the net acid resistance (NAR) and the comparative acid resistance (CAR) of the remineralized enamel specimens will be determined.

2.1 Study Schedule:

	Visit 1	**Visit 2, 5, 8, 11**	**Visit 3, 6, 9, 12**	**Visit 4, 7, 10, 13**
	Screening	Prophy	Begin Tx	End Tx
Informed Consent	**X**			
Medical history review	**X**	**X**	**X**	**X**
Oral Soft Tissue exam	**X**	**X**	**X**	**X**
Oral Hard Tissue exam	**X**	**X (v2 only)**		**X (v13 only)**
Salivary Flow	**X**			
Inclusion/Exclusion criteria	**X**	**X**		
Continuance Criteria		**X**	**X**	**X**
Randomization			**X**	
Adverse Event monitoring		**X**	**X**	**X**
Dental Prophylaxis		**X**		
Placement of specimens			**X**	
Removal of specimens				**X**
Issue washout tooth paste		**X**		
Return washout tooth paste			**X**	
Issue study products and diary			**X**	
Return study products and diary				**X**
Study close-out				**X (v 13 only)**

3. STUDY POPULATION

3.1 Source and Number of Subjects

Potential subjects will be selected from the OHRI's IRB approved database of persons previously accepted into the partial denture panel (IRB #1110007150). Potential subjects will be screened to determine eligibility to participate in this study. 34 adult subjects, between the ages of 18 and 85 years, will be accepted and randomized in the study so at least 24 subjects can complete the study.

3.2 Subject-Selection Criteria

3.2.1 Inclusion Criteria

In order to participate subjects must:

1. provide voluntary, written informed consent;
2. be between 18 and 85 years old;
3. understand and be willing, able and likely to comply with all study procedures and restrictions;
4. be wearing a removable mandibular partial denture with sufficient room in one posterior buccal flange area to accommodate two enamel specimens (required dimensions 12 × 7 mm) and room on the same side to accommodate two 4 mm round specimens in the buccal surface of two posterior denture teeth;
5. be willing and capable of wearing their removable partial denture 24 hours a day for four (4), two-week treatment periods;
6. be willing to allow study personnel to drill specimen sites (as described #4) in their mandibular partial denture;
7. be in good medical and dental health with no active caries or periodontal disease; NOTE; subjects presenting at screening with caries may continue in the study if their carious lesions are restored prior to beginning treatment 1.
8. have a salivary flow rate in the range of normal values (unstimulated whole saliva flow rate ≥ 0.2 mL/min; gum base stimulated whole saliva flow rate ≥ 0.8 mL/min).

3.2.2 Exclusion Criteria

No subject may:

1. currently be pregnant, intending to become pregnant during the study period or breast feeding;
2. currently have any medical condition that could be expected to interfere with the subject's safety during the study period;
3. currently be taking antibiotics or have taken antibiotics in the two weeks prior to the beginning treatment 1;
4. have participated in another clinical study or receipt of an investigational drug within 30 days of beginning treatment 1; or
5. be taking fluoride supplements, required to use a fluoride mouthrinse or have received a professional fluoride treatment in the two weeks preceding specimen placement;
6. be taking or have ever taken bisphosphonate drugs (e.g., Fosamax, Actonel and Boniva) for the treatment of osteoporosis;

3.2.3 Removal of Subjects from the Study

Subjects may withdraw from the study at any time for any reason. The Investigator may also remove subjects from the study at any time. The Investigator will document the reason for withdrawal for any discontinued subjects. Subjects withdrawn for medical reasons will be referred to a physician/dentist by the study personnel and will have their condition monitored to resolution or until deemed clinically non-significant. All subjects who discontinue participation before the completion of the study will be encouraged to return for an exit oral soft tissue examination.

4. TEST TREATMENTS

The following products applied with a provided, marketed toothbrush will be used in this study:

1. 0 ppm F (placebo, negative control)
2. 250 ppm F as NaF (dose-response control)
3. 500 ppm F as NaF (dose-response control)
4. 1100 ppm F as NaF (positive control)

The test products will be provided by GlaxoSmithKline as part of the ISS funding mechanism using their Sensodyne sodium fluoride/silica toothpaste base formulation. The test products and fluoride-free washout toothpaste will be supplied in plain white tubes. Each tube will have a study label affixed. Each study label will contain, but not be limited to, protocol number, product code letter (for treatment products only), directions for storage, emergency contact telephone number and “For Clinical Trial Use Only”.

Care should be taken with the supplied study products and their labels so that they are maintained in good condition. It is important that all labels remain intact and legible for the duration of the trial. Subjects should be instructed to not remove or deface any part of the study label.

Subjects will also receive a fluoride-free washout toothpaste and standard toothbrush for home use during the 2 to 3 day lead in period.

4.1 Product Use Instructions

Subjects will first clean their natural teeth with the partial removed with a toothbrush and water only. They may also brush their partial denture outside of their mouth with a brush and water to reach the areas not accessible when the partial denture is in place, while being very careful not to touch the specimen sites. For the test dentifrice they will place their partial denture in their mouth and apply a full ribbon of test dentifrice onto the toothbrush. Subjects will be instructed to brush the biting surfaces of their back teeth in all four quadrants of their mouth for a total of one timed minute taking care to not brush the specimen sites. They will then expectorate the toothpaste slurry and rinse with 15 mL of water for 10 seconds and expectorate.

4.2 Treatment Compliance

Product use compliance will be assessed by determining the average weight of each type of test product prior to dispensing (five tubes per product will be weighed and average weight determined). At the end of each treatment period, subjects will be required to return all product containers (including empty ones) to the study site. The tubes will be individually weighed upon return and that number subtracted from the average weight will be recorded on the treatment dispensing log.

The amount of test product used for each study treatment will be compared to daily product usage recorded on the subject's diary. Significant discrepancies will be discussed with the study subject, as needed. Subjects who miss 15% or more treatments within the two week treatment period will be considered non-compliant. The reason for non-compliance will be noted in the subjects study records.

4.3 Randomization Procedures

A unique study number will identify all subjects screened for study participation. Screening numbers will be assigned in ascending numerical order according to appearance at the study site. Subjects who meet all inclusion and exclusion criteria will be randomized into the study. At the first treatment visit, randomization numbers will also be assigned in ascending numerical order according to appearance at the study site.

A randomization schedule will indicate the treatment order sequence. Each subject will complete all four treatment regimens, one treatment regimen in each of the four treatment periods. The randomization schedule will be provided by the statistician, IU Department of Biostatistics.

4.4 Blinding Procedures and Code Breaks

The blind will only be broken in an emergency where it is essential to know which treatment a subject received in order to give the appropriate medical care.

The subject, the study dentist and the laboratory technicians responsible for performing the surface microhardness, TMR and fluoride analyses will be blinded. Only specimens and their tracking sheets without product identifiers will be sent to laboratory personnel.

5. STUDY METHODOLOGY

5.1 Conduct of Study

This study will be performed according to GLP and GCP. SOP's for all procedures are on file with the Quality Assurance Manager of the Oral Health Research Institute.

5.2 Clinical Procedures

Visit 1: Screening – Subjects who have been recruited for the study via a telephone interview will sign in at the study site for this and all subsequent visits. At this time, they will complete an informed consent statement, authorization for the release of health information for research form and demographic form. Upon review of these documents, an update of their medical history/medications and the inclusion/exclusion criteria, each

subject will be given an oral soft/hard tissue exam (OSHT) prior to their acceptance into the study. Their mandibular partial denture will be examined to determine if specimens can be held in one buccal flange area and the posterior teeth of the same side, as previously described. They will then provide a stimulated and unstimulated saliva sample to determine salivary flow rate. If no contraindications to their participation are discovered and the subject meets the study requirements, the subject will be accepted into the study.

Visit 2: Dental Cleaning Treatment Period 1 – Two to three days prior to the beginning of Treatment Period 1, subjects will return for a professional dental prophylaxis. The subject's mandibular partial denture will be prepared to hold the enamel specimens. A minimum area of 12 mm x 7 mm will be drilled out from the buccal flange area of the subject's partial denture and specimen sites in the buccal surface of two denture teeth on the same side of the partial denture will also be prepared. A temporary filling (Coe-Soft) will be placed in the cut out areas of the partial denture. Subjects will be given fluoride-free toothpaste to use at home for the next two to three days until they return for their next visit. They will be encouraged to remove their partial denture at night until their next visit.

Visit 3: Begin Treatment Period 1 – Each subject will receive an OST examination, answer continuation questions and have their medical history/medication information updated. The four enamel partially demineralized specimens will be placed in the buccal flange/posterior teeth areas on one side of the subject's mandibular partial denture. All subjects will be instructed to wear their partial denture containing the enamel specimens 24 hours a day except during the cleaning of their natural teeth (twice per day) and for short periods to rinse their mouth out with tap water after meals and snacks. Subjects will be instructed on the brushing method and perform their first brushing under supervision at the Institute.

Visit 4: End Treatment Period 1 – Two weeks following Visit 3, the subjects will return to the Institute and receive an oral soft tissue examination, answer continuance criteria questions and update their medical history/medication information. The enamel specimens will be removed from their partial denture and the hollows will be filled with a temporary dental filling until the next treatment visit. They will be told to use their regular toothpaste until they return and encouraged to remove their lower partial denture at night.

Visits 5-13 – The procedures outlined above will be repeated until each subject has used each of the four test products. At Visit 13, subjects will receive an oral hard tissue examination, have their mandibular partial denture cleaned through sonication, if applicable, have their partial denture repaired (if not going on to another in situ study) and their participation in the study will end.

5.3 Lifestyle Requirements

1. For 2-3 days following each cleaning visit subjects must discontinue all regular oral hygiene practices (products and procedures) and use only the study fluoride-free toothpaste and toothbrush provided, twice daily, with the exception of interdental cleaners, e.g. dental floss, if this is their normal practice.

2. For the 14 days of each treatment period subjects may use only the study product assigned to them and toothbrush provided twice daily, after breakfast and just before going to bed. Subjects may floss if this is their normal practice.
3. Subjects must wear their lower partial denture 24 hours a day, except when cleaning it, during each 14-day treatment period. Subjects will be encouraged to remove their mandibular partial denture at night during the washout and lead in periods.
4. Subjects may use a non-zinc fixative like Poligrip® on their upper denture but no adhesive is permitted in the lower partial denture.
5. Subjects must refrain from eating canned sardines during the course of the study and may not eat hard candy when the specimens are in place.

5.4 Informed Consent Process

Written informed consent will be obtained at the screening visit from each subject after they have had the opportunity to read the document, ask questions and had adequate time to make a decision about their participation. A study representative trained and delegated by the Principal Investigator will review the purpose, procedures risk and benefits of the study prior to the subject's signing of the document. The study representative will sign and date (and give the time) the consent form to confirm the consent process was completed prior to initiation of any study procedures. The subject will be given a copy of the signed document.

5.5 Oral/Hard Soft Tissue Examination

The study dentist will complete an oral soft and hard tissue (OSHT) examination at screening, oral hard tissue (OHT) at Visit 2 and 13 and an oral soft tissue (OST) examination at each study visit. The exams will be conducted via a visual examination of the oral cavity and perioral area utilizing a standard dental light, dental mirror, gauze, and periodontal probe and tongue blade, as needed. The soft tissue structures examined will involve the labial mucosa including lips, buccal mucosa, mucogingival folds, gingival mucosa, hard and soft palate, tonsillar and pharyngeal areas, tongue, sublingual area/floor of mouth, submandibular area, major salivary glands, head and neck and TMJ. Observations will be listed as "Normal" and "Abnormal" and abnormalities will be described.

The hard tissue structures examined will include assessing for enamel irregularities, tooth fracture, pathologic tooth wear, cavitated lesions, residual roots, faulty restorations and implants. Observations will be listed as "Absent" or "Present" and conditions noted as present will be described

5.6 Salivary Flow Assessment

Salivary flow will be assessed during the screening visit. For the unstimulated collection, subjects will sit quietly for five minutes before beginning the test. During the five-minute test time, they will be told to allow their saliva to pool, emptying into a collection cup whenever they feel the need to swallow.

For the stimulated collections, subjects will chew unflavored gum base for one minute and then swallow any pooled saliva. They will then chew the gum base for two minutes, during which time they will empty any pooled saliva into a collection cup.

The samples will be weighed and the salivary flow rates determined. The unstimulated saliva flow rate must be ≥ 0.2 mL/min and the stimulated saliva flow rate must be ≥ 0.8 mL/min for study qualification.

5.7 Diary for Home Use

At the start of each treatment period, subjects will be provided with a diary to record the date, and the time of the morning (a.m.) and evening (p.m.) of each brushing and any deviation from the brushing regimen. In addition, subjects will also record any new or changes in pre-existing medical conditions, medications or treatments or any change in signs or symptoms that may occur.

Subjects will be required to bring the completed diary to the end of treatment visit. Study staff will review the Diary Card with the subject to confirm treatment compliance and clarify listed medical conditions, medications and treatments.

5.8 Intra-oral Appliance

The *in situ* model involves the placement of gauze-covered enamel specimens in the subject's mandibular partial denture (see Figure 1). The subject's denture will be prepared for the study by creating a hollow in the buccal flange area on one side of the partial denture and in the teeth of the same side large enough to accommodate the enamel specimens. Four gauze-covered enamel specimens (two 4 × 4 mm square and two 4 mm round) will be mounted on the partial denture as described previously. The enamel specimens will be mounted in such a manner that the gauze-covered enamel surface of the specimen is flush with the surface of the buccal flange/teeth of the subject's partial denture. The two square enamel specimens will be luted in place in the buccal flange area of the partial denture using a light-cured dental composite (Triad VLC material, Dentsply Int., York, PA). Great care will be taken to avoid contaminating the enamel surface of the specimens with the luting material. The round specimens placed in the buccal surface of the denture teeth will be mounted in place with DentuSil™ - Silicone Soft Reline Material (The Harry J.Bosworth® Company, Skokie, IL) or an equivalent material. The Dentusil™ material will be placed in the drilled sites and the enamel specimens carefully inserted so that they be mounted flush with the buccal surface of the denture teeth when fully seated. Again great care will be taken to avoid contaminating the enamel surface of the specimens with the luting material. Upon completion of the study the subject's partial denture will be repaired. The location where the enamel specimens will be placed on the subject's partial denture are not functional parts of the denture, and the experimental procedures will not cause any permanent damage to the denture.

Prior to placement in the subjects' partial dentures, all enamel specimens will be sterilized by exposure to ethylene oxide.

FIGURE 1

Lower Partial Denture Appliance with specimens placed in the buccal flange and buccal surface of posterior denture teeth

5.9 Adverse Events Assessment

Subjects will be questioned at each visit regarding any general health or oral complaints and symptoms they have experienced since baseline. Any findings will be documented on the AE CRF. In the event of subjects reporting AEs outside the scheduled clinical visit, they are assessed at the earliest opportunity by the Investigator.

All AEs, regardless of severity or relationship to the test product, will be recorded. Serious AEs include any events resulting in death, decreased life expectancy, life-threatening situations, persistent or permanent disability/incapacity, hospitalization, or congenital anomaly/birth defect. Within 24 hours, the Investigator will submit a written report documenting the circumstances of the serious AE.

6.0 LABORATORY METHODS

6.1 Specimen Preparation

Extracted human teeth will be used as the hard tissue test substrate for the preparation of the 4 x 4 mm specimens in the existing situ caries model. The teeth will be collected and transported to OHRI in a saturated thymol solution. Upon receipt, the teeth will be sorted and cleaned. The teeth will then be stored in saturated thymol solution during sample preparation procedures.

Teeth will be selected based on the following criteria:

- Free of caries and major restorations;
- No discoloration and no markings, such as cracks, when viewed under a microscope at 20× magnification;
- Sufficient tooth surface to provide a large size specimen to meet study requirements.

Bovine teeth will be used as the hard tissue test substrate for the preparation of the 4 mm round specimens in the modified in situ caries model. Upon receipt at OHRI, the teeth will be sorted, cleaned and stored in 0.1% thymol solution during sample preparation procedures.

Teeth will be selected based on the following criteria:

- Have no discoloration and no markings, such as cracks, when viewed under a microscope at 20× magnification.
- Sufficient tooth surface to provide a large size specimen to meet study requirements.

Approximately two specimens will be obtained from the buccal surface of each human or bovine tooth. For the 4 × 4 mm specimens, longitudinal sections approximately 3 mm in thickness will be made parallel to the selected tooth surfaces. The tooth sections will then be cut into 4 mm × 4 mm specimens using a Buehler Isomet low-speed saw. Each block will be ground and polished to create flat surfaces [Zero et al., 1990]. – For the 4 mm round specimens, a core of enamel 4 mm in diameter will be prepared from each bovine tooth by cutting perpendicularly to the buccal surface with a hollow-core diamond drill bit.

The square and round specimens will be ground and polished to create planar parallel dentin and enamel surfaces. All grinding/polishing will be done on a polishing surface moistened with deionized water (dw). The dentin side will be ground flat using 500 grit silicon carbide paper, followed by grinding and polishing of the enamel side. A small orientation cut will be placed on each block (see Figure 2 and 3). The enamel surface of each specimen will be ground using 1200 grit silicon carbide paper followed by 2400 and then 4000 grit silicon carbide paper. After the grinding procedures are completed, the enamel specimens will be sonicated in deionized water (dw) for two minutes and then placed under running dw for three minutes. The polishing step will involve the use of a 1 micrometer (μm) diamond suspension on a polishing cloth. The enamel specimens will then be rinsed under a steady stream of dw, sonicated for two minutes in 2% microliquid soap and then rinsed again with dw for three minutes. Resulting specimens will have a thickness range of 1.8 to 2.2 mm. The specimen will have a minimum polished surface of 3 mm × 3 mm in the center of the enamel surface.

Figure 2

4 x 4 mm Human Enamel Specimen

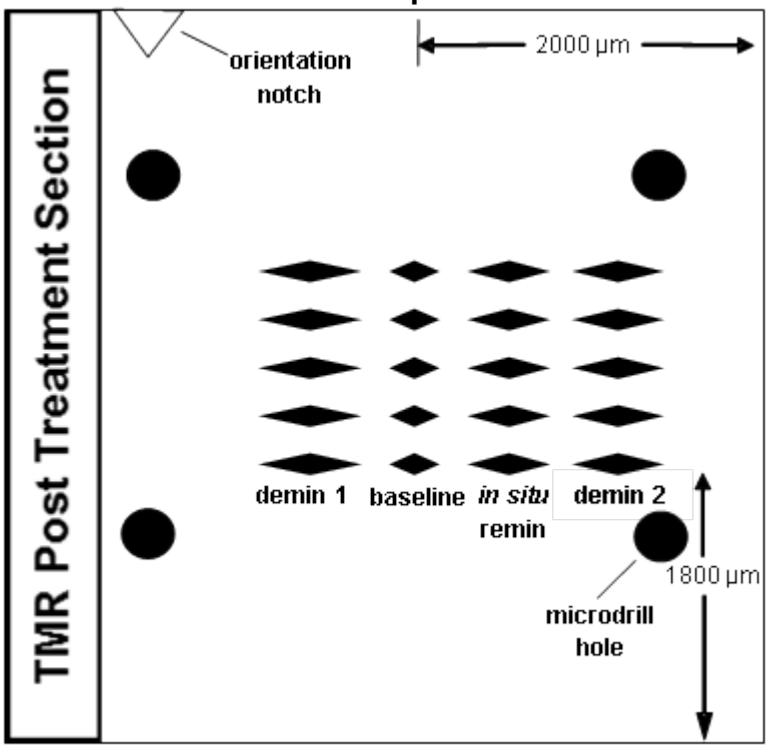
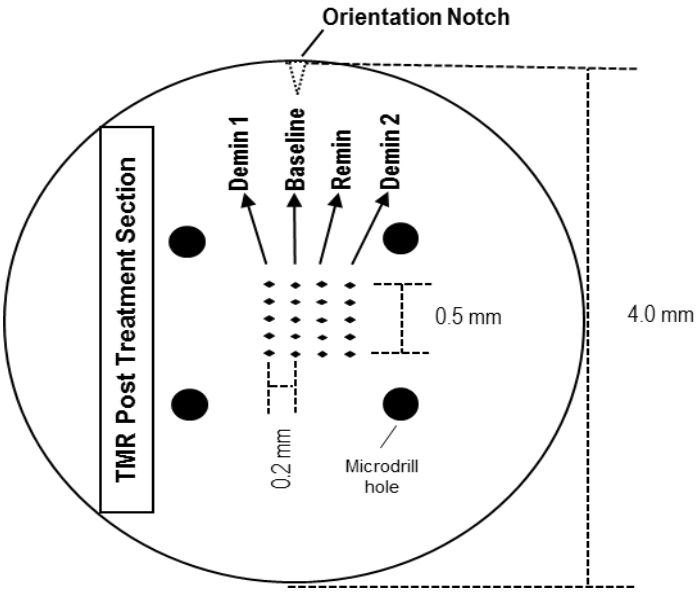


Figure 3

4 mm Round Bovine Enamel Specimen



6.2 Lesion Creation

The square and round enamel specimens will be partially demineralized using a modification of the method described by White [1987]. The 4 x 4 mm human enamel specimens will be immersed for 24 and the 4 mm round bovine specimens will be immersed for 18 hours at 37º C under static conditions in 40 ml of an acid buffer (0.05 mol/L lactate), 50% saturated with respect to hydroxyapatite and with 0.2% (wt/vol) carbopol 907 (BF Goodrich Co., USA) added (pH adjusted to 5.0 using KOH), and then rinsed thoroughly with deionized water. The demineralized enamel specimens will then be stored in a moist environment to prevent dehydration.

6.3 Lesion Quality

Lesions will be inclined towards an overhead light until a reflection is obtained. Acceptable lesions with an intact surface-zone will have a continuous, uniform shiny surface, with no matt areas. Lesions with matt area(s) will be rejected. The following criteria will be used to select specimens for inclusion in the study:

- The lesioned areas of each specimen should be of equal and uniform opacity; and
- The lesioned areas of each specimen should possess a surface shine when exposed to light, thereby indicating an intact surface.

6.4 Efficacy Measurements and Evaluations

6.4.1 Surface Microhardness

The SMH test will be used to assess changes in the mineral status of partially demineralized enamel specimens. SMH will be measured using a Wilson 2100 Hardness Tester. Each enamel specimen will be secured on a 1-inch square acrylic block with sticky wax and then placed on the microhardness tester. Five baseline indentations spaced 100 µm apart will be placed with a Knoop diamond under a 50 gram load in the center of a flattened, polished sound enamel specimen. SMH will be determined by measuring the length of the indentations using Wilson 2100 - Clemex CMT Software (version 6.0.011). For enamel specimens to be acceptable for use in the study, the mean of the five baseline indentation length must be 43 ± 3 µm with a standard deviation of ≤ 3µm.

After in vitro demineralization, the enamel specimens will be again SMH tested by placing five indentations 100 µm to the left of the baseline indentations (see Figures 2 and 3). To qualify for the study, the mean (n = 5) indentation lengths of the partially demineralized specimens must be 120 ± 20 µm with a standard deviation of ≤ 10µm. After 14 days of intra-oral exposure the enamel specimens will be again SMH-tested by placing five indentations 100 µm to the right of the baseline indentations (see Figure 2 and 3). The extent of remineralization will be calculated based on the method of [Gelhard et al., 1979].

$$\%SMH\ recovery = (D1-R)/(D1-B) \times 100$$

B = indentation length (µm) of sound enamel specimen at baseline
D1 = indentation length (µm) after in vitro demineralization

R = indentation length (μm) after intra-oral exposure.

6.4.2 Enamel Fluoride Uptake

The microdrill enamel biopsy technique as described by [Sakkab et al., 1984] will be used to analyze the fluoride content of the partially demineralized enamel specimens. Each enamel specimen will be mounted perpendicular to the long axis of a drill bit attached to a specially designed microdrill, and drilled to a depth of ~100 μm through the entire lesion (four cores per specimen; see Figures 2 and 3).

The drilling and sample collection will be performed in a static-controlled atmosphere to prevent loss of enamel powder due to charging effects. The enamel powder sample, pooled from the four drilling samples, will be transferred to an analyzer cup cap. 20 microliters (μl) of 0.5 Molar (M) Perchloric Acid ($HClO_4$) will be added to the enamel powder and the cap gently swirled to dissolve the powder. To the analyzer cap containing the 20 μl of $HClO_4$/enamel powder, 40 μl of citrate/ ethylene-diamine-tetraacetic acid (EDTA) buffer and 40 μl of de-ionized water will be added and immediately analyzed for fluoride content using a fluoride specific electrode and pH/Ion meter. The diameter of the drill hole will be determined using a calibrated microscope interfaced with an image analysis system. The amount of fluoride-uptake by enamel will be calculated based on the amount of fluoride divided by the area of the enamel cores and expressed as μg F/cm^2.

6.4.3 Net Acid Resistance test

To test whether the dentifrice imparts acid resistance to the enamel after 14 days of intra-oral exposure, a second in vitro demineralization will be repeated following the same protocol as described above on the two human and two bovine enamel specimens. SMH will be then evaluated by placing 5 indentations 100 μm to the right of the indentations placed in situ remineralization (See Figures 2 and 3). The %NAR will be calculated by the method of Corpron [Corpron et al., 1986]:

$$\% \text{ Net Acid Resistance} = [(D1-D2) / (D1-B)] * 100$$

B= Indentation length (μm) of sound enamel at baseline
D1= Indentation length (μm) after first in vitro demineralization
D2= Indentation length (μm) after second in vitro demineralization

Acid resistance is indicative of any protection that the treatments and intra-oral exposure may afford the enamel specimens. The net loss of enamel due to clinical caries is the result of multiple cycles of demineralization and repair (remineralization). It is well established that repaired enamel is more resistant to subsequent acid challenges.

6.4.4 Comparative Acid Resistance test

This measure takes a different approach to understanding whether the enamel formed during remineralization is more resistant to acid than the original enamel. Using the data from the four centrally located enamel specimens, the equation used will compare explicitly the reduction in SMH brought by the first and second acid challenges:

% Comparative Acid Resistance = [(D2-R) / (D1-B)] * 100

B= Indentation length (μm) of sound enamel at baseline
R= Indentation length (μm) of enamel after in situ remineralization
D1= Indentation length (μm) after first in vitro demineralization
D2= Indentation length (μm) after second in vitro demineralization

6.4.5 Transverse Microradiography

Sections, approximately 100 μm in thickness, will be cut parallel to the orientation cut (as shown in Figures 2 and 3), after completion of the SMH analysis, using a Silverstone-Taylor Hard Tissue Microtome (Scientific Fabrications Laboratories, USA). The sections will be placed in the TMR-D system and X-rayed at 45 kV and 45 mA at a fixed distance for 12 s. An aluminium step wedge will be X-rayed under identical conditions. The digital images will be analyzed using the TMR software v.3.0.0.18. Sound enamel will be assumed to be 87% v/v mineral.

Lesions will be analyzed after in situ demineralization and the following three parameters calculated:

1. Integrated Mineral Loss - ΔZ= [(lesion depth x 87) - area under the curve*]
2. Lesion Depth – L (83% mineral i.e. 95% of the mineral content of sound enamel)
3. Maximum mineral density at the surface-zone – SZmax

Note; *area under the curve which relates volume % mineral at distances from the specimen surface with respect to section thickness.

6.5 Laboratory Data

After the analyses are completed, all enamel specimen surface microhardness, transverse microradiography and enamel fluoride uptake data will be reviewed by the principal investigator before transfer to the statistician. The investigator will document this review and documentation will be filed with the laboratory study files.

6.6 Specimen Retention

Laboratory specimens will be retained by the study site for twelve months following database lock.

7. STATISTICAL ANALYSES AND SAMPLE SIZE JUSTIFICATION

7.1 Statistical Analyses

The mean % SMH recovery will be calculated using the five sets of indentations within each enamel specimen. The mean fluoride uptake will be calculated using the four samples within each enamel specimen. The mean % SMH recovery, mean fluoride uptake, mean acid resistance and mean ΔZ, L, SZ_{max} will be computed for the two enamel specimens within a subject for each specimen location (buccal flange or tooth) by treatment; if a subject is missing an enamel specimen, the available enamel specimen will be used. Analyses of fluoride uptake will be performed after using a natural logarithm transformation because the measurements generally are not normally distributed. The validation of the new model will involve two types of evaluations. 1) Analysis level evaluation: The treatments will be compared using analysis of variance models (ANOVA) suitable for a crossover study. The models will include random effects for subject and fixed effects for study period, product, specimen location, and the location-by-product interaction. The random effects will model the correlation and variances over time within a location and the correlation between locations. The primary comparisons of interest will be to determine if the specimens placed in the tooth find the same comparisons to be statistically significant as the specimens placed in the buccal flange. In addition, although likely to be underpowered, the analyses will also include an evaluation of the equivalence of the two locations. This will involve calculating a 95% confidence interval for the ratio between the tooth and buccal flange locations for the relative difference between treatments (treatment difference divided by the positive control mean). A 5% significance level will be used for all tests. No alpha-level adjustments for multiple comparisons adjustments will be applied. 2) Data level evaluation: Agreement between the data from the tooth and buccal flange locations will be evaluated using Bland-Altman plots and intraclass correlation coefficients (ICCs).

7.2 Sample Size Justification

Based on previous studies we estimate the standard deviation of the differences between treatments in the crossover model to be 10% for % SMH recovery. With a sample size of 24 subjects completing the study, the study will have 80% power to detect a difference between any two treatments of 6% for % SMH recovery, assuming two-sided tests each conducted at a 5% significance level. Due to expected dropouts during the crossover study, the study will enroll 34 subjects.

8. DATA QUALITY ASSURANCE

This study will be conducted in compliance with the US Code of Federal Regulations (CRF) governing informed consent (21 CFR 50), Institutional Review Board (IRB) (21 CFR 56), and with applicable regulations governing Investigator conduct (21 CFR 312).

9. OBLIGATION OF THE INVESTIGATOR

9.1 Advertising

All potential advertising materials used to recruit subjects for this study will be submitted to the relevant IRB.

9.2 Institutional Review

The IRB approval for this study will be obtained from:

Indiana University, Office of Human Subjects
Lockefield Building, Suite 3338
980 Indiana Avenue
Indianapolis, IN 46202-2915

9.3 Subject Consent

Written informed consent will be obtained from all subjects prior to their enrollment into the study. The consent form will comply with all applicable regulations governing protection of the participants in the study, and include basic elements specified in the US. Code of Federal Regulations, 12 CFR 50.25(a). A signed copy of the consent form will be given to the subject and the original one will be retained by the Investigator. In order to ensure confidentially, the Investigator will store the consent forms separate from the CRFs.

9.4 Data Collection

Data will be collected on source documents/CRFs created by the Investigator.

9.4.1 Case Report Forms

Case report forms will be used for recording all data not entered directly into computers. The Investigator will be responsible for maintaining original consent forms, CRFs and other source documentation.

The CRFs that will be used, unless otherwise indicated are:

- Inclusion/Exclusion Criteria
- Continuance Criteria
- Oral soft tissue
- Oral hard tissue
- Adverse event
- Serious adverse event
- Subject Accountability

9.5 Adherence to Protocol

The final protocol constitutes the conduct of the study. The Investigator is required to adhere to this final protocol. Any reasonable alternatives, variations, or deviations from the protocol must first be approved by the IRB. Any clarification to the protocol will be documented in the study file.

9.6 Records Retention

At the conclusion of the study, all records, relevant medical/dental records, copies of CRFs, and informed consent forms for all subjects treated with the drug or employed as a control in the study are retained by the Investigator for a period of time not less than 10 years.

9.7 Investigator's Final Report

Following the completion of the study the Investigator shall prepare an integrated clinical and final study report. The final report will include a general description of the conduct of the study including protocol deviations, subject withdrawals, a discussion of AEs, safety data, and laboratory analysis. This report will be approved and signed by the Investigator.

10. REFERENCES

Clinical aspects of de/remineralization of teeth: proceedings of Models Conference 1994. Rochester, New York, June 11–14, 1994. Adv Dent Res. 1995;9:169–340.

Corpron RE, Clark JW, Tsai A, More FG, Merrill DF, Kowalski CJ, Tice TR, Rowe CE. Intra-oral effects of a fluoride-releasing device on acid-softened enamel. J Am Dent Assoc 1986;113:383-388.

Featherstone JD, Mellberg JR. Relative rates of progress of artificial carious lesions in bovine, ovine and human enamel. Caries Res 1981;15(1):109–14.

Gelhard TBFM, ten Cate JM, Arends J: Rehardening of artificial enamel lesions in vivo. Caries Res 1979;13:80-83.

Koulourides, T, Phantumvanit, P, Munksgaard, EC, Housch, T. An intraoral model used for studies of fluoride incorporation in enamel. J Oral Pathol. 1974;3:185–196.

Lippert F, Butler A, Lynch RJM. Characteristics of methylcellulose acid gel lesions created in human and bovine enamel. Caries Res 2013;47(1):50–5.

Lippert F, Hara AT. Fluoride dose-response of human and bovine enamel caries lesions under remineralizing conditions. Am J Dent 2012;25(4):205–9.

Lippert F, Lynch RJ. Comparison of Knoop and Vickers surface microhardness and transverse microradiography for the study of early caries lesion formation in human and bovine enamel. Arch Oral Biol. 2014;59(7):704-710.

Marinho VC, Higgins JP, Sheiham A, Logan S (2003c). Fluoride toothpastes for preventing dental caries in children and adolescents. Cochrane Database Syst Rev 1:CD002278.

Mellberg JR: Hard-tissue substrates for evaluation of cariogenic and anti-cariogenic activity in situ. J Dent Res 1992; 71: 913–919.

Proskin HM, Chilton NW, Kingman A. Interim report of the ad hoc committee for the consideration of statistical concerns related to the use of intra-oral models in submissions for product claims approval to the American Dental Association. J Dent Res 71 1992;(Spec Iss):949-952.

Sakkab NY, Cilley WA, Haberman JP. Fluoride in deciduous teeth from an anti-caries clinical study. J Dent Res 1984;63:1201-1205.

Twetman S, Axelsson S, Dahlgren H, Holm AK, Kallestal C, Lagerlöf F, et al. Caries-preventive effect of fluoride toothpaste: a systematic review. Acta Odontol Scand 2003;61:347-355.

Walsh T, Worthington HV, Glenny AM, Appelbe P, Marinho VC, Shi X. Fluoride toothpastes of different concentrations for preventing dental caries in children and adolescents. Cochrane Database Syst Rev 2010;1:CD007868.

White DJ. Use of synthetic polymer gels for artificial carious lesion preparation. Caries Res 1987;21:228-242.

Zero DT: In situ caries models. Adv Dent Res 1995;9:214–230.

Zero D, Chin JR, Kelly S, Eckert G, Venell JA, Engelman EE. Efficacy of natural toothpastes in an in situ caries model. J Dent Res 84(Spec Iss A): Abst. 3259, 2005, (www.dentalresearch.org).

Zero D, Featherstone JDB, Fu J, Hayes AL, Vogel GL. Response of a de/remineralization in situ model to fluoride dentifrice (abstract). Caries Res 1994;28:208.

Zero DT, Rahbek I, Fu J, Proskin HM, Featherstone JDB. Comparison of the iodide permeability test, the surface microhardness test and mineral dissolution of bovine enamel following acid challenge. Caries Res 1990:24:181-188.