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In vivo inhibition of demineralization around orthodontic brackets

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IN VIVO INHIBITION OF DEMINERALIZATION AROUND ORTHODONTIC BRACKETS

by

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THESIS

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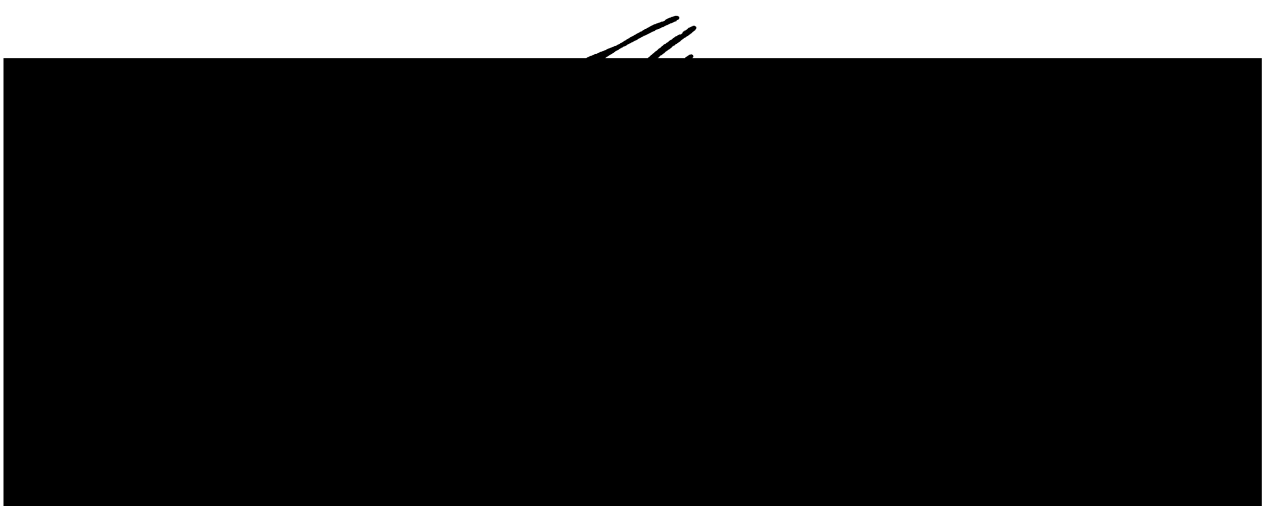

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ABSTRACT

Demineralization caused by dental decay around orthodontic appliances continues to be a problem for patients. Given the long intervals between appointments and poor patient cooperation with fluoride mouthrinse, a compliance-free means of preventing tooth decay would be optimal. To date, there have been no studies published which examine quantitatively *in vivo* the difference in decalcification for fluoride-releasing glass ionomer versus (non-fluoride) composite resin. However, *in vitro* testing using extracted teeth with subsequent exposure to a simulated oral environment indicated that fluoride-releasing glass ionomer significantly reduced enamel mineral loss compared to composite resin during a caries challenge (Banks 1997, Vorhies 1998, Molitor 1998). The hypothesis tested in this study was that fluoride released by glass ionomer cement inhibits the formation of carious lesions around orthodontic brackets *in vivo*.

Orthodontic brackets were bonded on two first premolars previously scheduled for extraction in 21 randomized, consecutively selected patients ages 11-18 years old. Subjects whose teeth were bonded with fluoride-releasing glass ionomer comprised the experimental group, and those bonded with composite resin containing no fluoride were used as controls. The teeth were removed after four weeks, sectioned, and evaluated quantitatively by cross-sectional microhardness testing. Fluoride levels in patient saliva were also measured by the Taves diffusion method for days 0 (baseline), 1, 2, 3, 7, 14, 21 and 28 (tooth extraction) to determine if the fluoride in the glass ionomer influenced overall fluoride levels in the mouth.

There were eleven patients in the experimental group, 10 in the control group. After 1 month in the mouth, there was significantly more demineralization (dental decay)

around the brackets of the control patients than the test patients ($p < 0.01$, Wilcoxan signed-rank test). For whole-mouth fluoride levels, there was no significant overall difference between the groups ($p > 0.05$), nor any noticeable trend within groups ($p > 0.05$).

In summary, the use of fluoride-releasing glass ionomer cement for bonding orthodontic brackets to teeth had a significant effect on inhibition of caries around the brackets. This cariostatic fluoride effect was localized to the individual teeth in the area around the brackets and was statistically significant after 4 weeks.

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I. INTRODUCTION

Despite the advances in orthodontic techniques in recent years, the development of decay around the brackets during the course of orthodontic treatment continues to be a problem. Although the reported incidence varied from study to study, the consensus was that orthodontic appliances significantly increase an individual's susceptibility to this type of decay, commonly referred to as "white spot lesions" (Øgaard B 1986, Mizrahi E 1983, Gorelick L 1982). These "white spot lesions" are due to demineralization of the enamel by organic acids produced by cariogenic bacteria (Featherstone 2000). The variability in the percentage increase in decay cited could likely be explained by the variability in patient population due to differences in local water fluoridation (Isaac 1967), diet (Kirkham 1994), oral hygiene instruction and reinforcement (Artun 1986), and appliance design (Gwinnett 1979, Zachrisson 1978). The prevention of these lesions during treatment is an important concern for the orthodontist, since they are unaesthetic, unhealthy, and potentially irreversible (Øgaard 1989).

Fluoride regimens have been shown to reduce or prevent white spot lesions in orthodontic patients. O'Reilly and Featherstone 1987 showed that a combination of fluoride toothpaste and a 0.05% NaF mouth rinse daily completely inhibited demineralization due to dental caries in subjects where compliance was strictly monitored. However, these traditional forms of administering fluoride such as mouth rinse are limited in their success by patient compliance. Research has shown that only about 13% compliance could be expected from patients asked to decrease their caries risk with a daily fluoride mouth rinse (Geiger et al 1992). In-office topical fluoride treatments have also been suggested to minimize the need for compliance (Zachrisson 1975,Shannon

1981). However, demineralization lesions of significant depth (75 μm) can develop in four weeks, which is shorter than the typical orthodontic appointment interval of 6-10 weeks (Øgaard 1989, Glatz and Featherstone 1985)

To avoid these problems of compliance and treatment intervals, manufacturers of the bonding material that adheres the brackets to the teeth incorporated fluoride into their product to see if this would help prevent or reduce the amount of decay that would develop around the braces. Of the two principal classes of bonding material available, composite resin and glass ionomer, only the latter has been shown to be successful in releasing the incorporated fluoride into the mouth, at least *in vitro* (Banks 1997, Vorhies 1998).

Unfortunately, there have been no studies published which examine quantitatively *in vivo* the difference in demineralization in the presence of fluoride-releasing glass ionomer versus (non-fluoride) composite resin. The most recent studies published on glass ionomers have focused on bond strength and defining the precise mechanism for fluoride re-uptake and release, neither of which address the question of inhibition of demineralization around orthodontic brackets which was the focus of this current study. Of the few studies, which have looked at this question, some were *in vitro* (Vorhies 1998, Donly 1995), whereas others were conducted *in vivo*, but did not provide a quantitative analysis (Gaworski 1999). In 1999, Molitor showed quantitatively with the cross-sectional microhardness technique that there was an inhibition of demineralization around brackets when using glass ionomer, however this was done *in vitro*.

The microhardness testing procedure evaluates volume % mineral loss, which is calculated based on physical properties that can be measured; decalcification or demineralization. Decalcification describes the characteristic of the substrate (enamel) and this loss of the calcium on the tooth surface can be either artificially induced (i.e. exposure to acid solution in the laboratory) or naturally occurring as an early step in the disease process leading to dental decay. Demineralization, on the other hand, implies bacterial involvement and is therefore generally considered equivalent to a "carious lesion", since it develops while a tooth is still in the patient's mouth. The purpose of using microhardness testing in this study is to assess the levels of demineralization on a tooth and employ these values to quantify a patient's carious lesion (the disease entity). This current research provides the first prospective *in vivo* quantitative analysis of the effects of fluoride-releasing glass ionomer on teeth bonded with orthodontic brackets.

II. LITERATURE REVIEW

A. DEMINERALIZATION

Nearly 50% of orthodontic patients exhibit clinically visible white spot lesions during the course of orthodontic treatment of approximately 2 years duration (Basdra et al 1996), with smooth surface lesions increasing up to 50% in prevalence during treatment (Øgaard 1989, Mizrahi 1983, Gorelick et al 1982). Orthodontic treatment has been associated with increased enamel demineralization because of increased plaque accumulation around the brackets (Gwinnett and Ceen 1979) and a more cariogenic bacterial environment (Mattingly et al 1983, Corbett et al 1981). The most common

place for this demineralization to occur in orthodontic patients is the gingival and middle thirds of the facial surfaces (Mizrahi 1983), thus shifting the tendency of demineralization from interproximal to facial/lingual and from posterior to anterior after bracket placement (Zachrisson and Zachrisson 1971, Zachrisson 1975). Apparently, banded and bonded teeth are affected to an equal extent (Gorelick 1982).

It has been documented that plaque accumulation can lead to a white spot lesion. Acidogenic bacteria such as the mutans streptococci group and the *lactobacillus* species in the dental plaque produce acids as they metabolize fermentable carbohydrates (Loesche 1986, Loesche 1972, Newbrun 1989). These acids, primarily lactic and acetic, diffuse through the plaque into the tooth sub-surface, releasing hydrogen ions in the process. Hydrogen ions are able to dissolve the minerals on the tooth surface, moving on to dissolve deeper layers of the enamel sub-surface before the outer layer is completely destroyed. The crystals being dissolved in this deeper enamel lose calcium and phosphate ions, leaving much smaller crystals and hence produce optical changes in the enamel (Featherstone 1985) manifested as a "white spot" lesion. The refractive index of this demineralized area is different than that of the surrounding translucent enamel, making it appear white. This type of subsurface lesion has lost up to 50% of its original mineral, which is then often covered by a surface zone formed by remineralization. This small area of sub-surface demineralization with its "apparently intact" surface layer is called a "white spot lesion" which could potentially progress to become a cavitation in the tooth surface (Featherstone et al 1999). Once this cavitation occurs, it is considered very difficult to reverse, so the tooth would likely require a filling (Øgaard 1989).

On the histologic level, white spot lesions are very complex. A mature white spot lesion has been characterized as having four distinct layers or zones (Gustafson 1957). The deepest layer is the "translucent zone" or "light zone", which acts as the invasive part of the lesion. Immediately superficial to this layer is the "dark zone" which is thought to be the result of remineralization below the surface (Joyston-Bechal 1980). The largest of the four zones is the "body" of the lesion, which makes up the bulk of a white spot lesion. The "surface zone" is the layer considered the most uniquely characteristic of white spot lesions. Since the outer enamel shell in this type of lesion remains relatively intact, the surface of the tooth maintains its original contour (Silverstone 1968). This layer is usually only sufficiently demineralized to allow the penetration of bacterially-produced acids into the deeper layers.

B. REMINERALIZATION

Protective factors, such as salivary calcium, phosphate and proteins, salivary flow, fluoride in saliva, and antibacterial components or agents can balance, prevent, or reverse dental caries. Of these, fluoride is an important agent for combating dental decay. Although the incorporation of fluoride into the apatite structure during tooth development is helpful in preventing future lesions, it is the topical effect that plays a greater role after the tooth has erupted into the mouth (Featherstone 1988). Not only does fluoride inhibit bacterial enzymes and therefore demineralization, it inhibits acid action at the crystal surfaces, and very importantly, enhances remineralization (Featherstone 2000).

As the saliva flows over the plaque on the tooth, salivary components neutralize the acid, raising the pH, which can stop and/or reverse the demineralization process. Remineralization occurs because the saliva is supersaturated with calcium and phosphate, which can drive mineral back into the tooth (ten Cate 1991, Moreno 1977). The partially demineralized crystal surfaces within the lesion act as "nucleators" and new surfaces grow on these crystals. Thus, these processes constitute remineralization, which is defined as the replacement of mineral in the partially demineralized regions of the carious lesion of enamel or dentin (ten Cate 1983).

Fluoride enhances remineralization by adsorbing to the crystal surface and attracting calcium ions, which are followed by phosphate ions, thereby leading to new mineral formation. This newly formed surface coating on the individual crystals does not contain carbonate and has a composition that lies between hydroxyapatite and fluorapatite. Fluorapatite contains approximately 30,000 ppm fluoride and therefore has a very low solubility in acid. The newly remineralized crystal behaves much like this low-solubility fluorapatite, thereby making it much harder for bacterially derived acids to dissolve this remineralized enamel compared to the highly soluble carbonated hydroxyapatite of the original crystal surface (Featherstone 2000).

C. GLASS IONOMER CEMENTS

Fluoride can be incorporated into glass ionomer bonding cements as a strategy for decreasing areas of demineralization around orthodontic brackets without having to rely on patient compliance (Swartz 1984, Sonis 1989). The subsequent release of the fluoride

by glass ionomer cements varies slightly between manufacturers, but typically is greatest on the first day, decreases sharply on the second day, then diminishes gradually (Perrin et al 1994) to virtually undetectable levels by the 90th day *in vitro* (Basdra et al 1996). This fluoride release has also been shown to be effective in inhibiting the growth of cariogenic bacteria, such as *mutans streptococci* (Loyola et al 1994) and in promoting remineralization at orthodontic band margins (Donly et al 1995). As a result, the plaque that accumulates around glass ionomer cement has significantly reduced bacterial concentrations and significantly greater incorporation of fluoride than plaque found around brackets bonded with composite resin, an effect that can last up to six months (Wright 1996, Benelli 1993, Hallgren 1993).

Even though the levels of fluoride release from glass ionomers drop sharply after the first 3 days (Vieira et al 1999), it has been demonstrated *in vitro* that levels of fluoride as low as 0.03 ppm are sufficient to enhance remineralization (Featherstone et al 1986, 1990, 1999). It has also been suggested that the effect of the initial fluoride release changes the properties of the enamel immediately around the brackets, thus reducing possible decay in the most susceptible area (Basdra et al 1996). According to Basdra (1996), these enamel changes that take place secondary to the initial fluoride release can inhibit demineralization adjacent to orthodontic brackets for a period of four weeks. Additionally, glass ionomers have the ability not only to provide an initial fluoride release, but also to uptake fluoride from the environment and re-release it, thereby "recharging" their antibacterial effect (Seppea et al 1992,1995, Takahashi et al 1993, Damen et al 1996, Diaz et al 1995, Creanor et al 1994, Pascotto 1999, De Witte 2000).

The exact fluoride release behavior of a bracket-bonding agent depends on the cement formulation. The total amount of fluoride released from a glass ionomer after being exposed to a fluoride source increases with consecutive fluoridations. According to De Witte et al (2000), this is particularly true for resin-modified glass ionomers, such as the Fuji-LC capsules used in the present study. Fuji LC exhibits a relatively slow release after fluoridation as compared to conventional glass ionomer cement (not resin-modified).

D. PURPOSE / SIGNIFICANCE

The overall objective of this study was to provide clinical evidence that the use of fluoride-releasing glass ionomers for bonding could significantly reduce the amount of demineralization around orthodontic brackets. If successful, this study would show that fluoride-releasing glass ionomer was the material of choice for caries control when bonding orthodontic brackets.

Orthodontists need to find a means of preventing white spot lesions during treatment in a manner that does not rely heavily on patient compliance (fluoride mouth rinse) or on increased office visits (fluoride gel). Recently, there have been several *in vitro* studies that demonstrated the cariostatic potential of glass ionomers (Molitor 1999, Vieira et al 1999, Vorhies 1996, Basdra et al 1996). The current study was the first controlled, randomized clinical trial done on glass ionomer used for cementing orthodontic brackets. If this *in vivo* research corroborated the existing *in vitro* data, it

would set a precedent for a long-term clinical study and set the stage for a new approach to clinical management of caries in orthodontics.

E. HYPOTHESIS

The hypothesis to be tested was that fluoride released by glass ionomer cement would significantly inhibit the formation of carious lesions around orthodontic brackets *in vivo*. The control teeth bonded with non-fluoride composite resin would therefore show significantly more demineralization (decay) around the bracket.

H_0: Control (composite; no fluoride) = Test (glass ionomer; fluoride)

H_1: Control (composite; no fluoride) ≠ Test (glass ionomer; fluoride)

F. SPECIFIC AIMS

1). To conduct a clinical trial in which brackets are bonded to teeth previously scheduled for extraction using either a fluoride-releasing glass ionomer (test group) or a composite resin (control group).

2). To determine if there is inhibition of decay around the brackets for the test (fluoride-releasing glass ionomer) group versus the control group using the cross-sectional microhardness testing technique for a quantitative measure of demineralization.

3). To quantitatively measure and analyze the fluoride levels at days 0,1, 2, 3, 7, 14, 21, 28 in patient's whole mixed saliva using the Taves microdiffusion method and evaluate whether there was a detectable pattern or increase in the overall fluoride levels in the mouth for test group patients (fluoride-releasing glass ionomer) when compared to controls.

III. MATERIALS AND METHODS

A. GENERAL STUDY DESIGN

The study consisted of a parallel groups double-blind randomized controlled clinical trial, UCSF CHR approval number H9136-16814-01. The outline of the study is pictured in **Figure 1**.

Fig 1: RESEARCH FLOW CHART

Orthodontic Clinic Visit: Final Consult Appointment *
Sign orthodontic waiver and contract
Schedule Oral Surgery Consult Visit
Sign research study informed consent **

↓

Hygiene Clinic Visit: Cleaning
Saliva sample
Braces put on 2 teeth for the study

↓

(Collect saliva at home the first 3 days after the cleaning)

↓

Orthodontic Clinic Visit: **Saliva sample**
(1 week after cleaning)

↓

Oral Surgery Clinic Visit: Oral surgery consult
(2 weeks after cleaning) ***Saliva sample***

↓

Orthodontic Clinic Visit: ***Saliva sample***
(3 weeks after cleaning)

↓

Oral Surgery Clinic Visit: Extractions
(4 weeks after cleaning) ***Saliva sample***

↓

Saliva assay
Microhardness Measurements

* normal clinic procedures
** ***study procedures***

1. Tooth Selection

The teeth used in this study were scheduled for removal for other reasons such as orthodontic treatment. Only first premolars were included in this study, since these accumulate significantly more plaque than any other teeth in the mouth (Geiger et al 1992). Only canines show comparable amounts of plaque, and these are seldom extracted for orthodontic purposes, so they were not included in the study.

2. Study Group Assignments

New patients who met the selection criteria (described in detail below) and provided a written informed consent (**Appendix 1**) were randomized to one of two groups; (A) a usual or conventional treatment "control" group, or (B) an "intervention" (test) group. Prior to randomization, an independent dental examiner not involved in the study conducted the first round of clinical and epidemiological exams to assess caries status and determine an orthodontic treatment plan. This patient work-up included an intraoral exam, review of new intraoral radiographs, medical history and a definitive dental history. Those subjects qualifying for and interested in participating in the study were asked to have their parents co-sign an informed consent form. Participants were then randomized into control and intervention treatment groups, which balanced age, gender, risk status and factors not measured (Fleiss 1986). To avoid selection bias, randomization of patients into test and control groups was based on a list compiled by a statistician not directly involved in the study using a computerized statistics program. The group allocation was revealed by an assistant not directly involved in the study only

after the patient had been seated for the initial bonding appointment. All patients received a full mouth cleaning to remove plaque in preparation for bonding.

3. Conventional (Control) Treatment

In preparation for orthodontic treatment, all patients had their restorative needs met and received a dental prophylaxis. No fluoride gel treatment was administered at the time of this cleaning, since it affects the ability of the bracket to bond to the tooth (Meng et al 1998). The cleaning was provided at no charge to the patients. As per usual, the orthodontic treatment plan was made independent of the study by the patient's doctor and attending faculty. Those interested patients who qualified were then randomly assigned to either the intervention or control group and had the two first premolars bonded. with a conventional non-fluoride-releasing composite resin (Transbond XT, 3M Unitek, REF 712-035), following manufacturer's guidelines. After this, no specific study-related recruitment for any procedures occurred with the exception of the salivary assays. Saliva sample collection is described in detail below under lab protocols. Salivary sample collections 1, 2, and 3 were conducted by the patient at home and stored in their freezer, the remaining salivary samples were done on a weekly basis. The investigator scheduled these weekly saliva assays and every attempt was made to coordinate sample taking with their regular orthodontic treatment provided by their doctor to avoid the necessity of patients making extra trips to the clinic (**Figure 1**). The participants were not charged for the salivary assays.

4. Intervention (Test) Treatment

The procedures followed for those patients randomly assigned to the intervention group mirrored the procedures of the control group. Participants had all restorations finished and received a prophy at no charge from the UCSF division of oral hygiene. Those patients assigned to the intervention group had their two premolars bonded with a fluoride-releasing resin-modified glass ionomer (Fuji Ortho LC, GC America Inc., Chicago, Ill.), which is a newer generation hybrid currently available for use in orthodontic bracket bonding. Salivary collections were conducted as described above for the control group, and there was no charge to the patients for these assays.

5. Measures of Compliance

The patients were instructed to brush twice daily with the provided dentifrice containing 1100 ppm NaF. The patient's and/or their parents were asked to fill in a log of their daily tooth-brushing schedule, and free tubes of toothpaste were distributed to the patients and weighed before and after the study to crosscheck compliance. To further aid in compliance with the research protocol, the subjects were also given detailed verbal and written instructions, and check off sheets for their at-home saliva sampling (**Appendix 2**).

6. Clinical Protocol

At the start of the experiment (day 0), each patient had two first premolars bonded with either fluoride-releasing resin-modified glass ionomer (Fuji Ortho LC, GC America

Inc., Chicago, Ill.) or non-fluoride-releasing composite resin (Transbond XT, 3M Unitek, REF 712-035). For calibration and increased accuracy, the amount of both types of cement was standardized to measure 1 mm of material extruded from the applicator tip. This helped minimize the problem of excess cement exuding from below the bracket during bonding, thereby exerting a potentially protective effect on the adjacent enamel simply by covering it. To insure consistency between patients by eliminating inter-operator variability, all the teeth in this experiment were bonded by the investigator and the pre-measured automatically mixed capsule form of the Fuji LC was used (Transbond XT didn't require mixing). Patients were not told which bonding agent was used on the teeth involved in the study. Also, the extracted teeth were scrambled and renumbered by a technician not directly involved in the study to insure blinding of the investigator prior to microhardness testing. Extractions were scheduled in the UCSF oral surgery department to take place four weeks after initial bracket placement. The same oral surgeon supervised all extraction procedures for the study.

7. Study Structure

The primary outcome measure was the 1-month caries increment for the control and intervention groups. The secondary outcome measure was the profile of the fluoride levels in the saliva for the control (non-fluoride) and intervention (fluoride) groups over the course of the 4 weeks of the experiment.

This study did not involve experimental pharmaceutical compounds, only products already available on the market for use by orthodontists for bonding appliances

to teeth. The flow chart in **Figure 1** and the spreadsheet in **Figure 2** provide an overview of the clinical aspect of the research design.

Fig. 2: STUDY SCHEDULE

RESEARCH DAY	?	0	7	14	21	28
DATE						
TIME						
APPOINTMENT	Consult	Cleaning	Samples	Samples	Samples	Extraction
Label Pt Tubes						
Weigh Toothpaste						
Informed Consent						
Pt Flow Chart						
Pt Instruction Form						
Saliva Sample						
2 Brackets Placed						
3 Pt Saliva Tubes/P-film						
Pt Saliva Log						
Pt Toothpaste						
Pt Toothbrushing Log						
Teeth Received						
Payment Mailed						
Saliva Assay						
Microhardness Test						

GREEN: Lab procedures
PURPLE: Patient Duties
BLUE: Clinic procedures

B. SUBJECT SELECTION

1. Patient Population

Subjects were selected from the UCSF Orthodontics Clinic. During the recruitment period, all patients aged 11-18 years who were scheduled for extraction of two or more first premolars were invited to participate and sign a consent form. There were no gender criteria, since the UCSF orthodontic patient population typically demonstrates approximately equal numbers of each gender. There were no ethnicity criteria.

2. Total Number / Number per Group

In order to determine sample size, the *in vitro* study performed under the mentorship of Dr John Featherstone by Matt Molitor (1999) comparing resin-modified glass ionomer (Fuji Ortho LC, GC America Inc., Chicago, Ill.) versus composite resin (Transbond XT, 3M Unitek, REF 712-035) was examined by a statistician. To predict the effect size, the difference in enamel decalcification levels between glass ionomer after 30 day exposure to demineralization / remineralzation pH cycling and the control (composite) was considered. Additionally, the clinical results of O'Reilly and Featherstone (1987) were evaluated for the demineralization levels (ΔZ) around composite-bonded brackets for teeth exposed to fluoride gel (APF)+ fluoride rinse for 30 days versus a control (no fluoride rinse or gel) to determine expected ΔZ value ranges *in vivo*.

Based on these calculations, it was estimated that two patients per group would suffice for a pilot study, with 10 patients total in each of the two groups participating in the main study over the course of one year, with a total of 20 patients overall. This would provide an 80% power to detect differences between the test and control groups.

3. Inclusion/Exclusion Criteria

Inclusion Criteria

These criteria were not based on race or gender. Participants were new patients at UCSF orthodontics clinic and:

1. Between the ages of 11 and 18 years old
2. Able to give informed consent themselves and from a parent/guardian in English or Spanish and be unlikely to move away from the area during the study period
3. Willing to participate regardless of group assignment
4. Willing to comply with all study procedures and protocol
5. Residing in San Francisco or other nearby locales with community water fluoridation (to eliminate water fluoridation as a potential confounding variable)
6. Scheduled for extraction of two or more first premolars which must be non-carious and not restored on the buccal surface

Exclusion Criteria

Persons with:

1. Significant past or current medical history especially conditions that may affect oral health or oral flora (i.e., diabetes, HIV, heart conditions that require antibiotic prophylaxis)

2. Medication use that may affect the oral flora or salivary flow (e.g., antibiotic use in the past three months, drugs associated with dry mouth / xerostomia)

3. Drug or alcohol addiction, or other conditions that may decrease the likelihood of adhering to study protocol

4. In-office fluoride treatment within the last 3 months.

C. SUBJECT RECRUITMENT

1. Patient Source

The study was conducted at the Orthodontics Clinic at the University of California at San Francisco. The clinic sees a large number of patients in the age range 11-18 years, who are at a higher risk of caries during their orthodontic treatment due to the high cariogenicity of the typical adolescent diet. Thus, participants were recruited from among all new orthodontic clinic patients 11-18 years old for whom the treatment plan included the extraction of two or more first premolars. This clinic is a 24 -chair

facility with core faculty and staff committed to coordinated academic, research, and patient care services. The clinic currently is attending approximately 1,500 patients, most of whom are seen on a monthly basis and reflect the ethnic diversity of San Francisco.

2. Initial Contact Method

There was a general announcement to the care providers and posted signs in the clinic and elevators (**Appendix 3**) were also used to aid in subject recruitment. All new patients visiting the UCSF Orthodontics Clinic identified as potential candidates during their initial new patient examinations and fulfilling inclusion/exclusion criteria were provided information about the study and invited to participate at the time of their final consultation appointment. If these individuals agreed to participate in the study, both patient and parent signed the consent form, thus completing the enrollment process.

3. Incentives for Participants

Patients meeting the inclusion/exclusion criteria were offered substantial reductions in the fees for standard dental care as an incentive to participate in the study. The cleaning prior to bracket bonding was provided free of charge by the UCSF Oral Hygiene Division. Additionally, the total cost of the orthodontic treatment provided by the UCSF orthodontic residents was reduced by 5.0% for study subjects. At the end of the study, when the two teeth scheduled for extraction and used in the study were removed, a $75 reimbursement was provided directly to the patient using funds from the

UCSF Oral Surgery Department and the UCSF Department of Growth and Development. The total savings for those patients participating in the study was estimated to be approximately $300 for patients without orthodontic insurance coverage and approximately $200 for patients with typical (50%) orthodontic insurance coverage. The fluoride dentrifice (Crest) used during the 4 weeks each patient participated in the study was kindly provided by the Proctor and Gamble Company to Dr. John Featherstone, and given to the participants at no cost.

4. Consent

Informed consent was obtained from those individuals meeting inclusion/exclusion criteria and indicating an interest to participate (**Appendix 1**).

5. Confidentiality of Records

Research records were handled as confidentially as possible. All patient treatment computer records were accessible only by those persons requiring access for treatment purposes and the investigator. All written records were coded and kept in locked file cabinets. No individual entities were used in any printed material resulting from this study.

D. LABORATORY PROCEDURES

1. Study Procedures

The existing clinic computerized patient record system recorded all subject's demographic variables such as age, gender, insurance status, potential explanatory variables (i.e. # visits, time between visits), address, phone numbers and contacts. Additional information such as ethnicity, medical history, dental history and treatment variables were recorded by the care provider in the patient chart at the initial visit. To avoid bias towards an extraction-based treatment plan, an independent dental examiner not involved in the study conducted this first round of clinical and epidemiological exams using radiographs, models, and clinical presentation.

2. Laboratory Protocol

Teeth were assessed quantitatively for demineralization around the bracket by the cross sectional microhardness testing technique (Featherstone et al 1983, 1986, 1990, White and Featherstone 1987)

Saliva samples were collected at days 0 (baseline), 1, 2, 3, 7, 14, 21, and 28 (extraction day). The principal investigator collected saliva samples for all sampling intervals except the day 1,2,3 samples when the subjects collected saliva samples without supervision. The whole saliva samples were assessed for fluoride content. To insure blinding of the investigator during the analyses, the saliva samples were re-labeled by a technician not involved in the study upon arrival at the lab.

3. Demineralization Assessment Procedure

Sterilization

Upon extraction, teeth were placed in 0.1% thymol solution and sterilized with a gamma irradiation (Cs ^{137}) at a dose above 173 krad overnight (White 1994, Vorhies 1998, Molitor 1999). Following sterilization, the collection media was replaced with fresh DDW and thymol.

The use of gamma irradiation to sterilize teeth has been shown to have no detectable effect on dentin structure, as measured by FTIR, UV / VIS / NIR and permeability (White 1994). Because enamel has less organic material than dentin, it has been assumed that the permanent effect on enamel would be less pronounced and therefore negligible for the purposes of this study.

Sectioning

The roots were removed 2 mm apical to the cemento-enamel junction and the crowns hemi-sectioned vertically into mesial and distal halves with a 15 HC (large) wafering blade on an Isomet low-speed saw (Buehler, Lake Bluff, Ill.). The samples were sectioned directly through the slot of the bracket, leaving a gingival portion and an incisal portion. The sectioned tooth with the bracket still attached was then embedded in blocks of Ladd epoxy resin.

Embedding

A PVC ring was lubricated on the inner surface using silicone grease and a Q-tip. Then a piece of 3M double-sided adhesive tape (St Paul, MN) was placed on a flat metal

tray. The polished edge of the ring was placed tightly on the tape, creating a tight seal. Using forceps, the tooth samples were placed in a circular fashion inside the ring; bracket side facing in and the cut side down on the tape. The teeth were pressed firmly against the tape. The ring was labeled with a group letter and a sample number.

Ladd epoxy resin was then mixed to imbed the samples. The resin was prepared by mixing 20 g LX 112 (Ladd Research Industries; Catalog # 21310) 14 g NMA (Ladd Research Industries; Catalog # 21350), 0.5 ml DMP-30 (Ladd Research Industries; Catalog # 21370) in a centrifuge tube. The contents of the tube were mixed and centrifuged at 1,000 RPM for 2 minute in a Centra CLC centrifuge to remove bubbles from the solution. The resin was then poured into the ring slowly, leaving slightly more than 1 cm from the top of the ring. Each sample was then inspected for bubbles, and when found, the bubbles were removed with a dental explorer. The tray was then placed in an oven at 50 °C overnight to cure the epoxy resin. When the resin was set, the tray was removed from the oven and allowed to cool. The block was then removed from the PVC ring and labeled.

Polishing

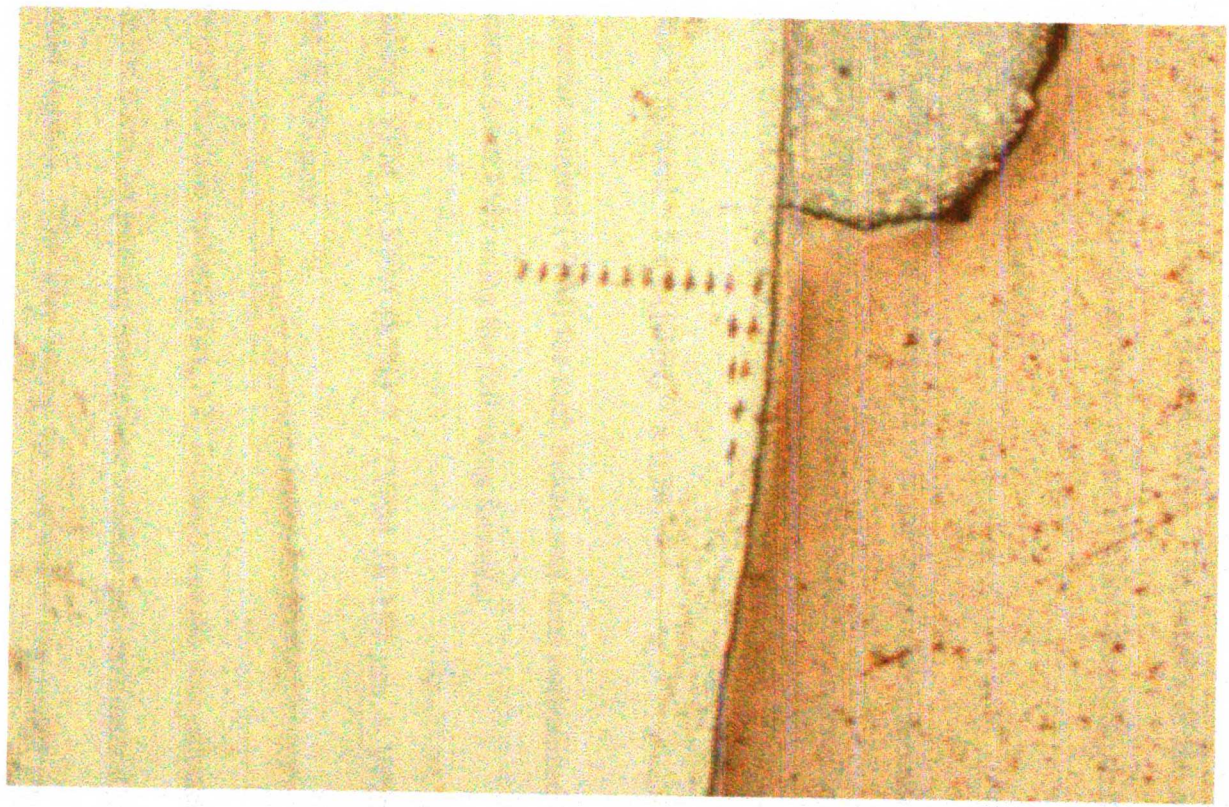
Each sample block was polished on the Ecomet Polisher (Beuhler, Lake Bluff, Il.) on 600 grit silicon carbide paper with the exposed tooth side down for 2 minutes. The block was occasionally rotated slowly on the polisher to ensure that the surface was polished in a uniform manner. Deionized water was sprayed on the polisher to prevent the surface from drying. The block was washed with soap and a toothbrush in cold water, sonicated for 1 minute, and washed again. The block was then polished using a cloth

paper on the polisher. The surface was kept saturated by spraying 6 micron diamond suspension spray (Beuhler, Lake Bluff, Il). The block was polished with the 6 μm paper for 4 minutes, 3 μm paper for 4 minutes, then washed and sonicated as described above. Lastly, it was polished on a new cloth paper with 1 μm diamond suspension for 2 minutes, washed and sonicated. The blocks were examined under the microscope for scratches that could interfere with the microhardness testing (Featherstone 1993).

Indenting

After polishing, the exposed flat hemisectioned surface was indented to test for microhardness cross-sectionally using a Leitz Microhardness tester (Leitz Wetzler Germany #8647) and microscopic examination (Featherstone et al 1983, 1986, 1990, White and Featherstone 1987). The first indent was located 15 μm deep towards the dentin from the enamel surface (lingual) and approximately 100 μm below (gingival to) the cement margin using a 15 p weight for patients #1-8 and increasing to a 25 p weight for patients # 9-21. Subsequent indentations continued into the underlying enamel, increasing in depth from the outer surface by 5 μm each time up to a depth of 50 μm in a V-shaped pattern. A photograph of the indent pattern is included below in **Figure 3**. After this, the indents were made at 25 μm deep intervals into underlying sound enamel in a straight horizontal line up to a total depth of 300 μm using a 50 p weight for patients #1-8 and decreasing to a 25 p weight for patients #9-21 (Meyerowitz et al 1991). The hardness formula was adjusted to allow for the weight differences. The change to the 25 g weight was made because it was expected to give more accurate indentations.

Fig. 3: V-shaped Microhardness Indent Pattern



The set of data representing demineralization for each carious lesion was curve-fitted by means of a Simpson approximation (White and Featherstone 1987), and the area under the lesion tracing was calculated (in units of volume % mineral x μm) and subtracted from the normal enamel value to give the parameter ΔZ, being the relative mineral loss for each lesion (**Table 4** in Results). The lengths of the indents measured were first converted to the Knoop hardness number (KHN) according to the formula:

$$KHN\ (Kg/mm^2) = 13230\ K/L^2$$

Where K is the applied force in grams and L is the measured indentation length in μm (Featherstone 1983). The Knoops hardness number is then converted to volume percent mineral using the following formula (Featherstone 1983):

$$\text{Volume \% mineral} = 5.1 \times (KHN)^{0.5} + 0.24$$

The volume percent mineral (VPM) for each indent was then normalized based on sound underlying enamel set at 85% using the following formula:

Normalized VPM = VPM (85%) 7 / (VPM @ 150 μm + VPM @ 175 μm + ...VPM @ 300 μm)

The overall relative mineral loss, ΔZ, for each sample was calculated by creating a hardness profile curve by plotting normalized volume percent mineral against distance from the enamel surface. The area under the curve that represents ΔZ (μm x vol % mineral) was calculated using Simpson's integration rule (White 1997) and is shown in **Figure 5** and **Figure 6** of the results section. Also, the individual ΔZ values for each lesion in each group was combined to give a mean ΔZ and standard deviation for each of

the fluoride-releasing glass ionomer and non-fluoride-releasing composite resin groups. Analysis of indentation lengths / demineralization was calculated with the aid of Image pro plus 4.0 software which is used for capturing and measuring the image through a microscope (Olympus BX50, Melville, NY) at 500X magnification. Analysis of raw data were performed using Microsoft Excel software programmed to handle the data obtained in the study.

4. Saliva Sampling Procedure

Whole stimulated saliva was collected and standardized by volume for assessment of fluoride levels. Each subject was asked to chew on a 2 x 2 inch square of Parafilm,™ and 2 ml of saliva was spit into a pre-labeled sterile 50 ml centrifuge tube. After collection, the saliva was stored at 4°C no longer than 1 week for later fluoride analysis.

The Taves (1968) microdiffusion method was used to evaluate the saliva samples for fluoride content. Saliva samples were vortexed and 1 ml of the supernatant was transferred into the microdiffusion dish of a Taves diffusion apparatus. The volume of the samples was adjusted to 3 ml with double deionized water, and 0.1 ml of 1.65 mol/L NaOH added to the central trap. One ml of 6 mol/L HCl, saturated with hexamethyldisiloxane was added to the sample before the dish was sealed. The samples were rotated for 18 h on a rotary shaker at 80 rpm to allow the fluoride to diffuse as HF and be collected in the NaOH trap. At the end of the diffusion period, the NaOH traps were removed. The samples contained in the traps were dried at 65° C for two hours, buffered with 1 ml of 0.34 mol / L acetic acid (final pH 5.0).

The fluoride content (μg) was calculated from a standard curve constructed from standards prepared from a 100 ppm fluoride stock solution (Orion Research Inc) in concentrations of 0, 0.01,0.02, 0.03, 0.04, 0.05, 0.075, 0.1, 0.25, 0.5, 1.0, 5.0, 10.0 ppm. These standards were microdiffused at the same time as the samples and the fluoride concentrations were measured by a fluoride ion-specific electrode (Model 960900, Orion Research, Inc., Boston, MA). The mV reading for each standard and sample was recorded after the electrode has been submerged for 2 minutes. All readings were followed by a thorough rinsing and drying of the electrode. A fluoride assay plot was created for each standard using a calibration curve of mV vs. log [F] (**Figure 4**) for the standards was created using the IGOR Pro 3.14 software (Wavemetrics Inc., Lake Oswego, OR) on a Macintosh computer. From this calibration curve, the following formula was utilized for calculating the experimental fluoride concentrations:

$$X = Y\text{-}b / m$$

X = log [F ppm], Y = recorded value of the experimental solution (in mV), b = the y-intercept, and m = the slope of the curve.

The fluoride concentration was then calculated for each of the solutions utilizing the following formula: [F ppm] = antilog [X]

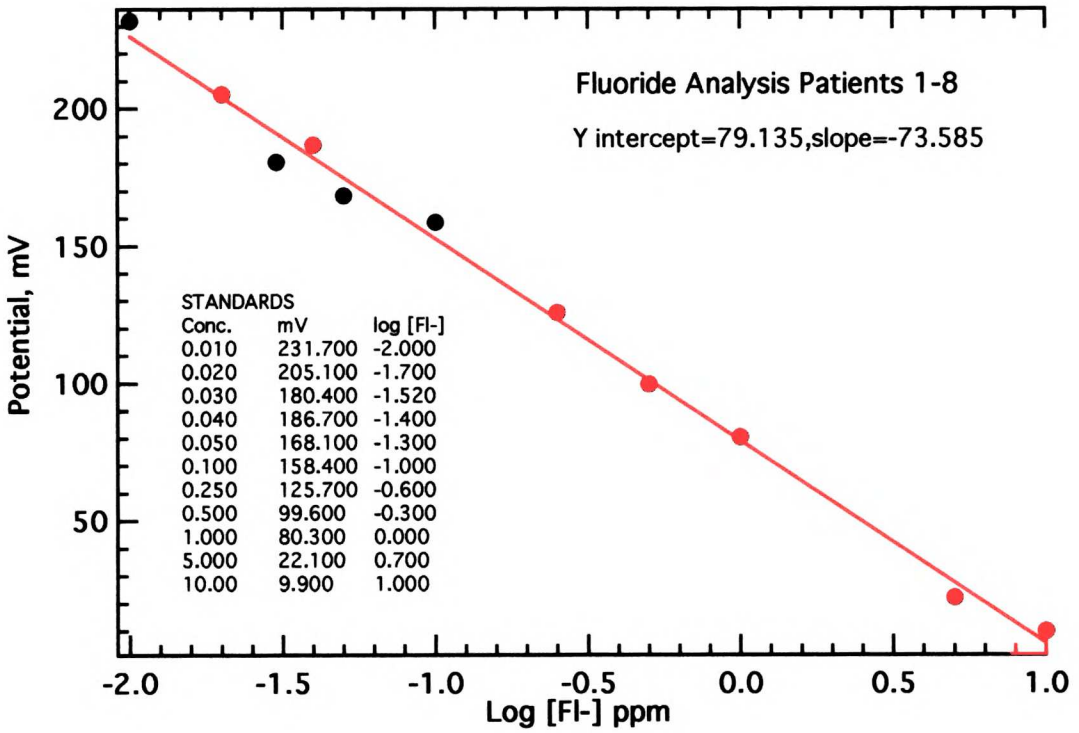


Figure 4: Example of standard curve for fluoride

E. METHODS OF DATA ANALYSIS

1. Intention –to-Treat (ITT) Sample

The primary analyses used the intention-to-treat (ITT) approach (Fisher et al 1990, Gillings and Koch 1991) i.e. using patients in the groups to which they were randomized to limit intentional and unintentional biases as well as to establish a basis for statistical analyses.

2. Protocol Compatible (PC) Sample

Supplemental analyses used the protocol compatible (PC) approach, which excludes patients who have not adhered to the protocol sufficiently.

3. Data Analyses

One extreme outlier was noted in the test group when the microhardness values were compiled. After removing this outlier, the data were normally distributed and a two-sided T-test was used to compare the enamel relative mineral loss (ΔZ) values for the fluoride-releasing glass ionomer versus the composite. The data were also analyzed with the outlier, for which the non-parametric Wilcoxan Rank Sum test was considered the most appropriate form of assessment. For evaluating the fluoride levels in patient saliva, a 2 sample t-test was employed and comparisons were made on a per-day as well as an overall group comparison. Statistical calculations were performed with the aid of Statview 4.5 (Abacus Concepts, Inc.) software program, Microsoft Excel and statxact3.

IV. RESULTS

A. NUMBER / CHARACTERISTICS OF SUBJECTS

Of the 25 patients who met the inclusion / exclusion criteria after the initial CHR approval on October 26, 1999, twenty-four of these subjects were involved in this study. There was one subject who refused to participate in the study on the grounds that there would potentially be a delay of approximately one week for the start of the designated

orthodontic treatment. After that incident, the oral surgeon and the primary hygienist involved in the study increased their availability for research study subjects and there was no longer any delay in the start of conventional orthodontic treatment for the patients.

Of the 24 patients involved in the study, data is complete for 21 patients: 11 in the test group and 10 in the control group. The reasons for 3 of the patients not completing the study were all a result of circumstances beyond their control. One patient was involved in a serious car accident and could not attend the last appointment due to the resultant facial injuries. Another patient arrived at the final appointment to find that the orthodontic faculty had made a last-minute change in the plan of which teeth to extract, and the teeth that had been selected for the study would no longer be extracted. This type of occurrence was avoided with subsequent patients by obtaining a copy of an extraction form with the faculty signature committing to the extraction pattern prior to bonding brackets on the study teeth. A third patient completed the study, however, it appears that an assistant to the oral surgeon accidentally gave the teeth to the wrong investigator and they were not recoverable. All 3 of these patients still benefited from the incentives offered by the study since their failure to complete the study was not the result of lack of compliance.

For the 21 patients who completed the study, and from whom the data were analyzed, 8 (38%) were male, 13 (62%) were female, which is comparable to the gender distribution of patients seeking orthodontic treatment in the United States (slightly more females than males). The ethnicity breakdown was: 9 Hispanic patients (43%), 5 African Americans (24%), 4 Caucasian (19%) and 3 Asian (14%). This represents a higher proportion of Hispanic patients than originally predicted, which is likely reflective

of the steady increase in the Hispanic (predominantly Mexican-American) population in California. The age range of the patients was 11-18 years old, with a mean of 13.19 years old and a standard deviation of 1.91 years (**Table 1**).

TABLE 1: Patient Demographics

Patient	Age at start	Gender	Ethnicity
1	14	M	AA
2	12	F	AA
3	14	F	H
4	11	F	H
5	15	M	A
6	13	M	C
7	13	M	H
8	12	M	C
9	16	F	H
10	15	F	H
11	12	F	H
12	13	M	AA
13	12	F	H
14	16	M	H
15	14	M	AA
16	12	F	AA
17	9	F	H
18	17	F	C
19	11	F	A
20	13	F	C
21	13	F	A
Average SD	13.2 1.9	M= 8 (38%) F= 13 (62%)	H= 43% AA= 24% C= 19% A= 14%

Note:

1. All ages rounded to the nearest whole number
2. One patient (#17) fell below the 11-18 year old target age range
3. M= Male, F= Female
4. H= Hispanic, AA= African American, C= Caucasian, A= Asian

Patient compliance with the research protocol was monitored through the use of the log the patient was required to fill out (**Appendix 2**) and by weighing the toothpaste provided to the patient for use during the study. The majority of the patients (14/21= 67%) remembered to return their saliva logs that recorded the day and time of the at-home saliva collection (samples from days 1-3), respectively. There was a lower rate of return for the tooth-brushing logs that the patients were supposed to return at the end of the study period when they arrived for their extraction appointment (9/21=43%). Apparently, not enough emphasis was placed on the fact that the toothpaste was not a gift after the cleaning like the toothbrush was, so many patients kept the tube and continued to use it after the teeth were extracted. However, an analysis was run for the 7 tubes of toothpaste that were returned (7/21=33%), and all tubes showed that a decrease in the volume of toothpaste had occurred during the 4 week period. On average, the starting weight of the toothpaste tubes was 191 grams with a standard deviation of 0.45, while the tubes were returned with a large range between heavy use of toothpaste (only 71 g weight) to light use (164 g) with an average return usage weight of 112 g, standard deviation 34.7 grams. Seventy-one grams in 28 days would coincide with brushing twice a day, allowing 1 gram per brush load. It was an assumption of the study that variations in toothpaste use and brushing technique were equalized between groups as a result of the randomization process.

Sample Size and Power Analysis

Since some attrition of subjects is always expected in a clinical project, 24 patients were enrolled with the goal of having 20 patients complete the study. This target number of 20 was based on the amalgamation of data from the similarly structured *in vivo* work by O' Reilly et al (1987) using composite resin cement with and without fluoride mouth rinse and an *in vitro* version of the current project (Molitor 1999). **Table 2** below shows these statistical predictions which recommend 10 patients for each of the 2 groups, based on achieving a significance of $p< 0.05$ and a power of 80%.

TABLE 2: Statistical Predictions

Sample size selection based on microhardness testing data from O'Reilly et al (1987) and Molitor (1999) assuming a 2-sided t-test:

Parameters	Predicted Values
Test significance Level (α)	0.05
Test Group Mean ΔZ (volume % mineral)	500
Control Group Mean ΔZ (volume % mineral)	1000
Difference in Means	500
Common Standard Deviation	377
Effect size (Diff. Mean/SD)	1.33
Number per Group	10
Power (%)	80

B. DEMINERALIZATION AROUND BRACKETS

Twenty-one patients successfully completed the 4-week duration of the clinical research, which is one more than the projected minimum. The individual mineral loss (ΔZ) values for each subject are presented in **Table 4**. Although the sample size was small, the difference in demineralization directly below the brackets for the test group (fluoride-releasing glass ionomer) and the control group (non-fluoride composite resin) was so sizeable (**Table 5**) that a power of 99% was achieved for this study.

To verify the reproducibility of the measurement method for the indent lengths, 10 separate measurements were made for both a long (carious) indent and a short (sound enamel deeper than 150 μm) within the same tooth by the same person who did all the measurements for the study. For the shorter, non-carious indent, the average measurement length was 35.6 μm with a standard deviation of 0.25, while for the longer indent measuring 55.8 μm (mean); the standard deviation went up proportionally to 0.53 (**Appendix 4**). Also, the data were verified for quality assurance prior to revealing the group assignations by repeating any measurements that seemed unusual.

Additionally, internal calibration of the microhardness machine was tested with a series of 10 indents made with a 100g weight on a polished stainless steel block (serial # 355401181) designed especially for the purpose. The results of the series of 10 indents were then compared to the known values for indent length published for stainless steel and the Knoop hardness number for the steel block being used, which is stamped on the block. The results showed a good approximation to the expected values, with an average indent length of 46.1 +/- 0.6 μm compared to the standard of 48 μm and an average

Knoop Hardness number of 670 +/- 17.4 which was very close to the value of 671 stamped on the block (**Table 3**). Stainless steel was selected since its values are well known and the indent length of 48 μm is similar to that expected for normal enamel (45-50 μm with a 50 g weight), or for carious enamel in the present study using a 25 g weight.

TABLE 3: Knoop Hardness
Stainless Steel indented with 100 g weight

Indent #	Indent Length (microns)	Knoop Hardness #
1	46.1	670
2	45.1	703
3	45.8	679
4	46.3	664
5	45.6	685
6	46.7	653
7	46.6	655
8	46.5	664
9	45.8	679
10	47.1	644
Mean	46.2	670
SD	0.597	17.4
Standard	48.0	671

Note:
1. The indent length for stainless steel (48 microns using a 100g weight) is similar to sound enamel (45-50 microns using a 50g weight).
2. The Knoop hardness number standard for stainless steel (671) is stamped on the block
3. The standard length for stainless steel indented by a 100 g weight can be found in the Knoop Hardness Guide (ASTM standard for dental materials).

This comparison to the ASTM standards was in addition to the internal calibration per tooth provided by the indent pattern and the ΔZ formula which uses the indents in sound enamel at depths from 150 μm inwards in each tooth to normalize the hardness measurements made in the outer layers of that tooth. The mean of the sound enamel values $\geq$ 150 μm is adjusted to 85% for each tooth and all outer measurements are adjusted proportionally. In this way, each tooth serves as its own negative control where the potentially carious surface is compared to the non-carious inner enamel, since in 4 weeks there is only enough time for surface lesions to develop.

Of the 11 patients in the test group, one was an extreme outlier, with a level of decalcification higher than any other patient, even of those in the control group (**Table 5**). The young girl's mother was interviewed as to the patient's dietary habits during the time of the clinical trial, which coincided with Thanksgiving and ended just before Christmas. Her mother mentioned that the girl was spending a lot of time away from home during that period preparing for and performing "The Nutcracker" ballet, where the patient would have had access to vending machines without parental supervision.

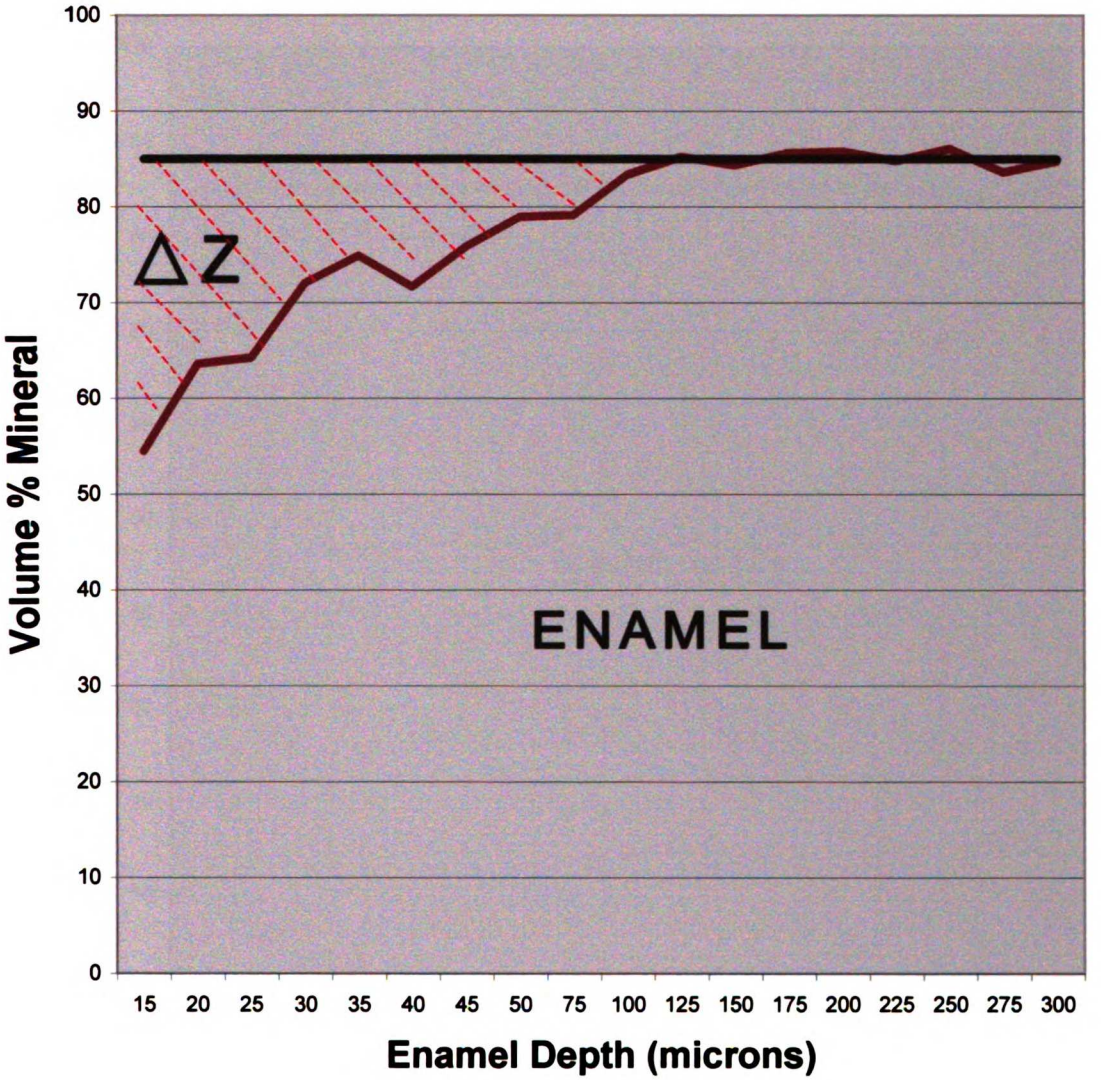
TABLE 4: Relative Mineral Loss, △Z (Volume % x microns) Values per Subject

SUBJECT #	CONTROL △Z	SUBJECT #	TEST △Z	*SUBJECT #	*TEST △Z
2	360	1	267	1	267
3	897	5	-57.5	5	-57.5
4	274	7	512	7	512
6	1228	9	-135	9	-135
8	1194	11	575	11	575
10	712	13	490	13	490
12	766	14	-227	14	-227
15	795	18	-292	18	-292
17	987	19	219	19	219
16	842	20	244	20	244
				21	1853
Mean (SD)	805 (310)		160 (319)		314 (593)

Note:

1. * Test values include the outlier (patient # 21)

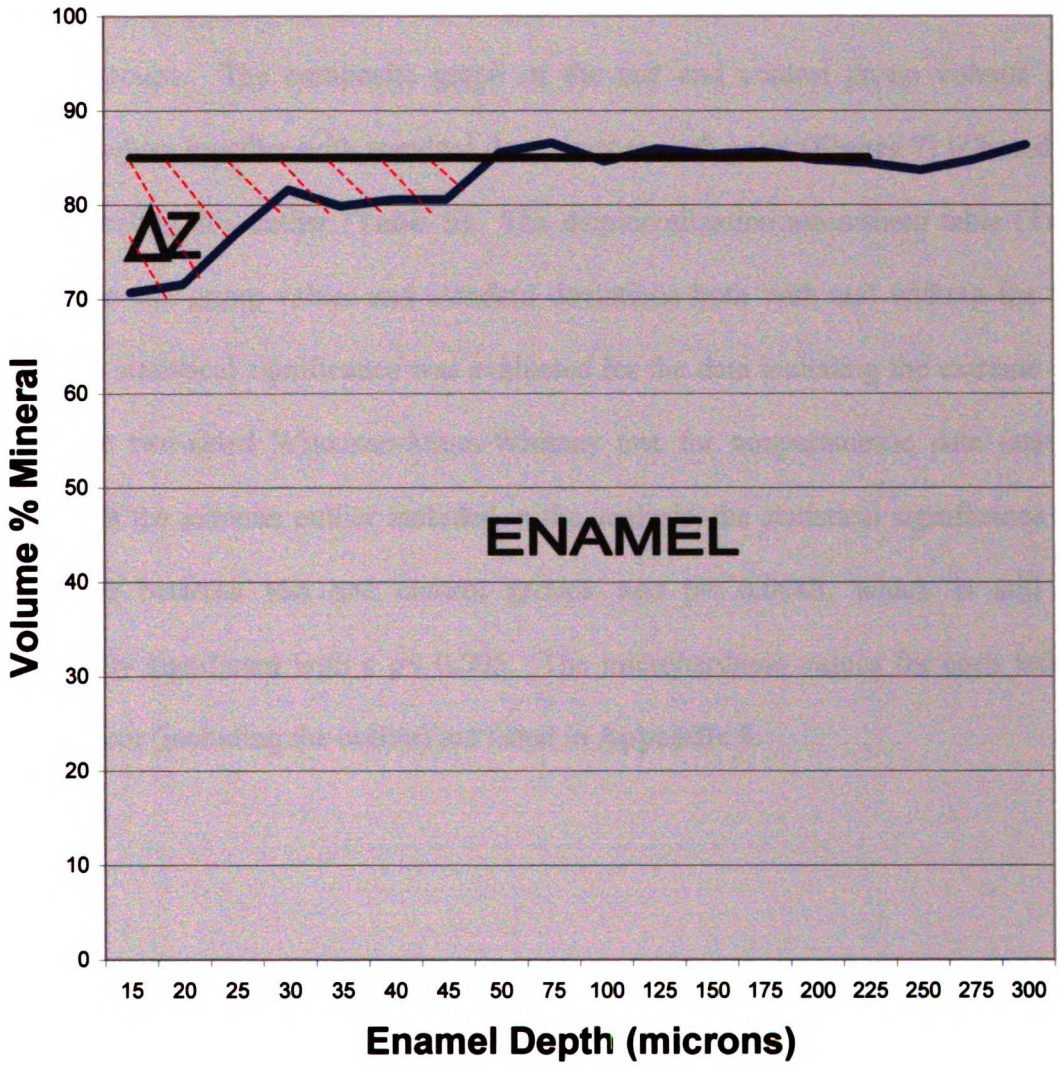
Fig. 5: Control Group:
Delta Z (overall relative mineral loss)



NOTE:

1. 150-300 um = underlying sound enamel
2. 85% is the "normalized" value for sound enamel

Fig. 6: Test Group:
Delta Z (overall relative mineral loss)



NOTE:

1. 150-300 um = underlying sound enamel
2. 85% is the "normalized" value for sound enamel

The data were analyzed with and without this sample. When the data were analyzed excluding this one extreme outlier using a 2 sample t-test with equal variances (statxact3) there was a high statistical significance of $p= 0.0002$ between the test and control groups. The composite graph of the test and control group volume percent mineral values together with standard deviations at each point (**Figure 7**) is based on the data excluding this outlier (**Table 5**). The demineralization assessment table (**Table 5**) shows the test group values and standard deviations both with and without the outlier. Also, the statistical significance was evaluated for the data including the extreme outlier, using the two-sided Wilcoxan-Mann-Whitney test for nonparametric data (statxact3). Even with the extreme outlier included in the analysis, the statistical significance for the difference between test and control groups was $p= 0.0048$, which is still highly statistically significant with a $p< 0.005$. The microhardness values for each indent for each patient (including the outlier) are listed in **Appendix 5**.

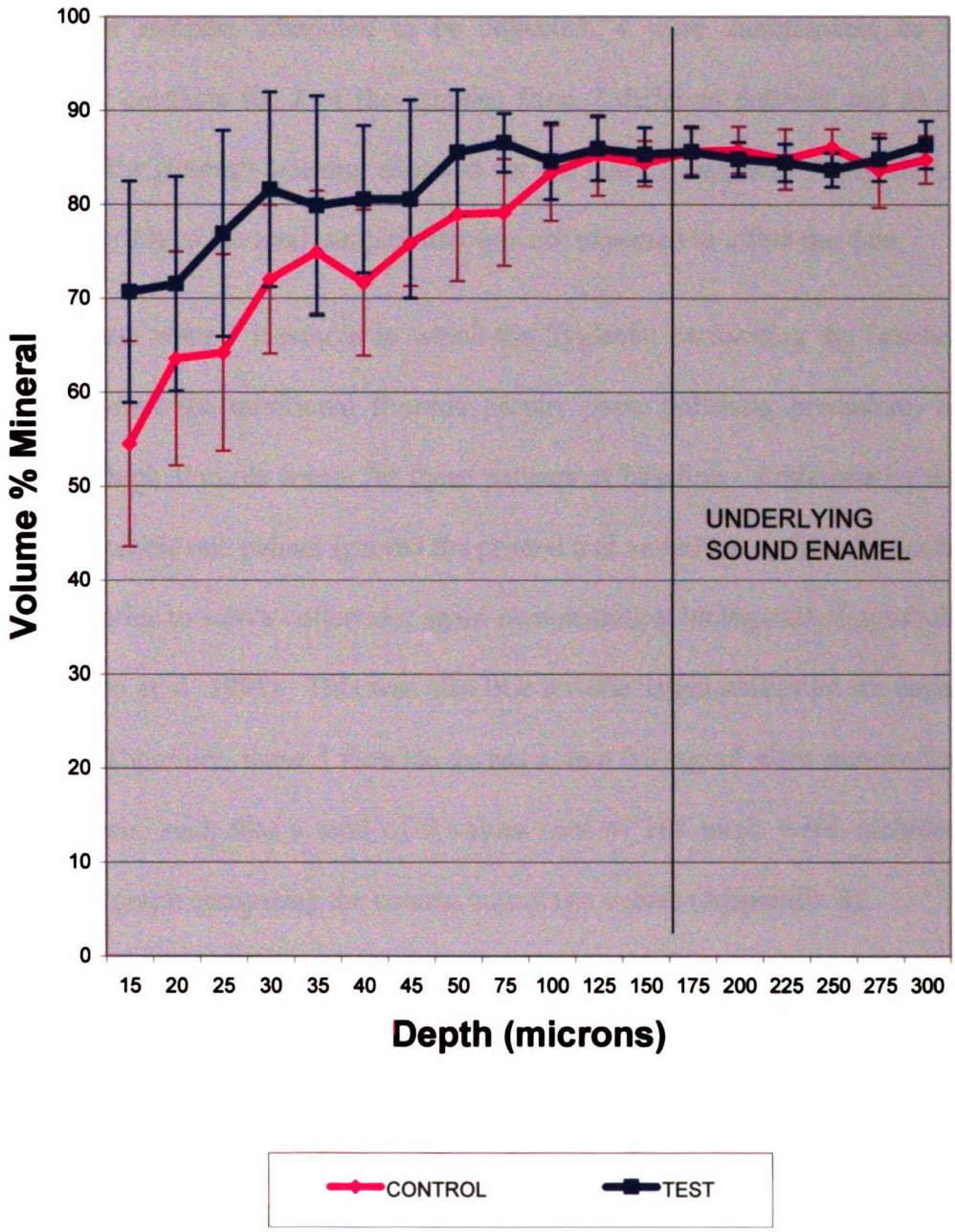
TABLE 5: Group Demineralization Assessment at each depth from the surface

Depth from surface (μm)	CONTROL (n = 10)		TEST (n = 10)		TEST * (N = 11)	
	Volume % Mineral Mean	Volume % Mineral Std Dev	Volume % Mineral Mean	Volume % Mineral Std Dev	Volume % Mineral TEST Mean*	Volume % Mineral TEST Std Dev*
15	54.5	10.9	70.7	11.8	69.4	12.0
20	63.6	11.4	71.5	11.4	71.0	11.0
25	64.2	10.5	76.9	11.0	74.5	13.2
30	72.0	7.92	81.6	10.4	79.3	12.4
35	74.9	6.53	79.9	11.7	77.0	14.7
40	71.7	7.78	80.5	7.85	79.0	9.02
45	75.8	4.53	80.6	10.6	80.0	10.2
50	78.9	7.13	85.5	6.69	84.8	6.83
75	79.1	5.68	86.6	3.12	85.1	5.60
100	83.3	5.08	84.6	4.08	83.7	4.85
125	85.2	4.30	85.9	3.41	84.9	4.64
150	84.4	2.41	85.3	2.85	85.3	2.71
175	85.6	2.48	85.6	2.69	85.8	2.61
200	85.8	2.50	84.8	1.84	84.8	1.75
225	84.8	3.22	84.5	1.99	84.3	1.97
250	86.1	1.94	83.7	1.80	83.8	1.80
275	83.6	3.95	84.8	2.30	84.8	2.18
300	84.8	2.54	86.4	2.54	86.2	2.45
	ΔZ Mean 805	**ΔZ Std. Dev.** 310	**ΔZ Mean** 160	**ΔZ Std.Dev.** 319	**ΔZ Mean** 314	**ΔZ Std. Dev.** 593

NOTE: 1. Depths from 150-300 microns are assumed to represent healthy enamel

2. * test values include the outlier (patient # 21)

Fig. 7: Volume % Mineral: Control vs. Test



C. SALIVA FLUORIDE LEVELS

There were 8 saliva samples collected from each of the 21 patients. Of the 168 total saliva samples scheduled to be collected, 4 were unobtainable as a result of scheduling conflicts for 2 of the samples from 2 different patients and an unexpected change in the patient's housing situation for the other 2 samples. Altogether, this was a loss of only 2% of the total samples and was not expected to affect the data.

There were 3 instances in which the hygienist performing the baseline cleaning forgot to omit the traditional fluoride prophy paste polishing procedure, resulting in unusually high fluoride levels for these patients at baseline. Additionally, there were 3 occasions where one patient ignored the printed and verbal instructions to not brush or eat one hour prior to saliva collection, again demonstrating biologically improbable fluoride levels (Zero et al, 1992). This was also true for one saliva collection for each of 2 other patients. Therefore, these 5 fluoride values above 0.3 μg/ml were removed prior to the data analysis, such that a total of 9 values (out of 168 total) were excluded from the composite graph comparing the control versus test values (**Appendix 6**).

Table 6 summarizes the data by day as the mean (SD) for each group. There was no significant difference between the test and control intraoral fluoride levels ($p= 0.06$) when the data at each time point were analyzed using a two-sample t-test assuming equal variances as seen in **Figure 8**.

Fig. 8: Intraoral Fluoride Levels: Control vs. Test

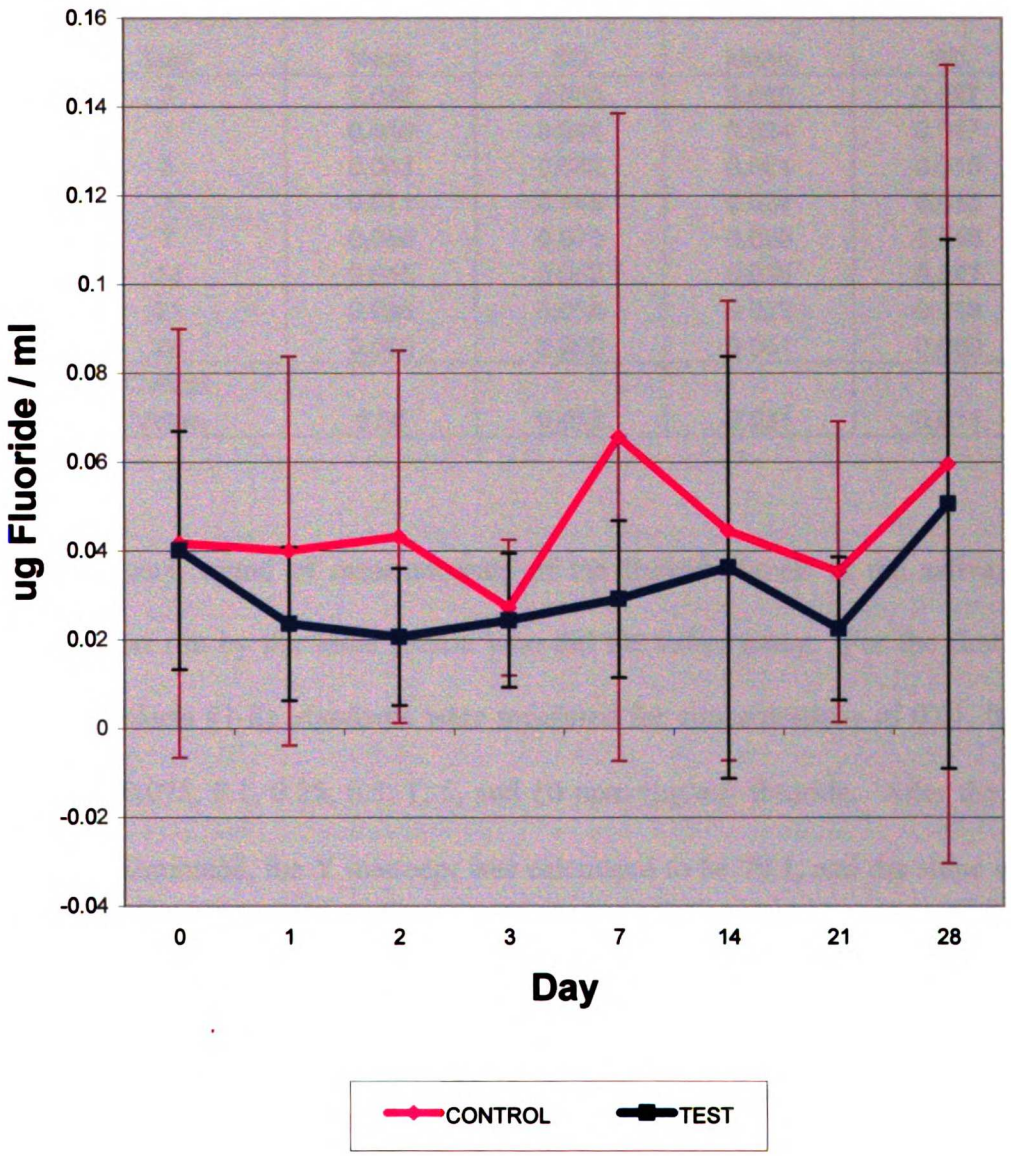


TABLE 6: Group Mean Intraoral Fluoride Levels

	CONTROL (ppm F)		TEST (ppm F)	
Day	Mean	SD	Mean	SD
0	0.042	0.048	0.040	0.027
1	0.040	0.044	0.024	0.017
2	0.043	0.042	0.021	0.015
3	0.071	0.140	0.024	0.015
7	0.066	0.073	0.029	0.018
14	0.045	0.052	0.036	0.047
21	0.035	0.034	0.023	0.016
28	0.060	0.090	0.051	0.060
Overall Value	0.05	0.013	0.031	0.011

For each round of measurements of the fluoride levels in the saliva, a set of standards was run by the same person who did the saliva assay. For the first round of patients (subjects #1-8), standards were measured for concentrations of 0.01, 0.02, 0.03, 0.04, 0.05, 0.075, 0.1, 0.25, 0.5, 1, 5, and 10 ppm (μg/ml) fluoride. After the outlier at 0.075 was eliminated, the Y intercept was calculated to be 79.1, and the slope was –73.6 (**Figure 4** in Materials and Methods). The measurements for most patients usually fell in the upper part of this curve between 0.01 and 1.0 and for this experiment; both the control and test group averages were within this range. The second series of measurements done for patients #9-21 used similar standards: 0.01, 0.02, 0.04, 0.06, 0.1, 0.25, 0.5, 1, 3, 5, 10 and 25 ppm (μg/ml) fluoride. After the 2 outliers at 0.25 and 3 were deleted, a Y intercept of 62.1 and a slope of –51.2 were obtained. Although there was a slight variability for the slope and Y intercept for the first and second round of measurements,

this was reflective of and comparable to other tests done in the laboratory at these times (the first and second measurements were done 9 months apart). Since each assay uses at least 10 standards and results are calculated from the standard curve concentration, values are internally consistent and directly comparable from one time to the next.

V. DISCUSSION

Previous studies have shown that glass ionomers have the ability to decrease the incidence of demineralization around orthodontic brackets, however no study has quantified the effect *in vivo* (Benelli 1993, Hallgren 1993, Basdra al 1996, Chadwick 1995). The hypothesis of this study was that carious lesions around brackets could be prevented or minimized solely by using a fluoride-releasing glass ionomer cement instead of the traditional composite resin. Based on this background research, it was expected that this fluoride effect would be of such a magnitude that a significant difference in demineralization between test (glass ionomer) and control (composite resin) teeth would be apparent in just 4 weeks in the patient's mouth without the need for large numbers of subjects to complete the study. Based on the quantitative results from the research reported here, and the highly significant differences found the hypothesis of this study can be accepted.

A. SUBJECTS

The high statistical significance of the results supporting the hypothesis indicates that the sample size for the test and control groups was adequate. This was due, in part, to the high rate of completion for subject involved in the study (88%) and also the even higher rate (96%) of enrollment in the study of those patients that did qualify (only one potential subject refused to participate). This recruitment success can be attributed to a combination of the minimal levels of discomfort / inconvenience incurred by the study, the short duration of the patient participation portion of the project, and to the substantial financial incentives provided to both the parents and directly to the patient.

Hispanics, African-Americans, and females were more heavily represented than originally predicted (**Table 1** in Results), however, this should not have had any influence on the results, since the patients were randomly assigned to the test and control groups which would compensate for small differences such as these. Compliance with returning written logs and toothpaste was lower than expected, but the importance of returning these was not stressed by the investigator, and patients were not reminded. The emphasis was instead placed on compliance with appointment days and times, and with this, there was a very high success rate with only 2 patients missing one appointment each (the initial cleaning appointment) such that of the 105 appointment that the 21 patients were expected to attend (5 appointments each), there was a failure rate of just 2%. Overall, then, it can be assumed that most patients adhered fairly well to the research protocol and that any differences in compliance were minimized by the randomization process.

B. DEMINERALIZATION

Specific Aim #1 of this study was to assess the effectiveness of a fluoride-releasing glass ionomer cement in the prevention of demineralization around orthodontic brackets in human mouths when compared to composite resin. In order to achieve the most accurate results possible, the possibility for error was minimized whenever possible. Selection bias was minimized by using a computer-generated randomized list provided by a statistician for patient group assignments, and both the patient and investigator were blinded as to the group assignment of each individual.

In addition to double-blinding, accuracy was optimized by calibration whenever possible. Both the control and test cements were measured to the same amount, and inter-operator differences were eliminated, since the investigator did all the tooth bonding and the microhardness measurements. Also, the ability of the investigator to show consistency in measuring the microhardness indents on the computer was assessed by repeatedly measuring the length of 2 different indents; one short indent in sound enamel and one longer indent in demineralized enamel. This reproducibility data were encouraging, with an average over 10 indents of 35.6 +/- 0.25 for the short indent and 55.8 +/- 0.53 for the longer indent (**Appendix 4**).

Aside from the calibration aimed at reducing the human error, there was an internal calibration of the microhardness measurements built into the ΔZ formula which compares the outer, potentially carious surface enamel to the sound enamel on the inner part of the same tooth. This allows for normalization of the microhardness data on a per-

tooth basis, such that tooth-to-tooth variability is eliminated, even when a different weight is used for the indents. Even the microhardness machine was put through calibration testing against the known number for Knoop hardness for a stainless steel standard block, and a weight was chosen that would give similar indent lengths for those obtained in the present study. The mean Knoop hardness number calculated from a series of 10 indents on the stainless steel demonstrated that the machine was performing in accordance to external standards and was able to produce consistent, accurate indents (Knoop hardness number for stainless steel of 670 +/- 17.4 compared to the expected 671).

To ensure the most thorough interpretation of the data possible, the microhardness data were analyzed following the recommendations of a statistician both with and without an extreme outlier (subject #21). The existence of this outlier in the test (glass ionomer group) exhibiting a carious lesion significantly larger than that of any other subject, even those in the control group, most likely indicates that a high caries challenge can overcome even the local protective effects of the fluoride from the glass ionomer. This 13 year-old patient had no history of caries in her newly acquired permanent dentition, but had increased her consumption of sweets around the time of the clinical trial due to large amounts of time spent away from home rehearsing for a ballet performance.

A Wilcoxan signed rank test was performed to accurately assess the potential difference between the 2 groups while taking into account and minimizing the distorting effect of this outlier, and the result showed a statistical significance of $p< 0.005$. This high statistical significance was even more impressive when this outlier was excluded and the data were analyzed using a t-test for equal variances ($p= 0.0002$). It should be

noted that 3 patients in the test group showed some signs of minimal demineralization ($\Delta Z \geq 300$) and one patient in the control group did not show an appreciable amount of demineralization ($\Delta Z < 300$) but this individual variation did not affect the overall significance of the difference between the 2 groups.

These results demonstrate that teeth bonded with glass ionomer showed significantly less enamel mineral loss when compared to teeth bonded with composite resin in a group of adolescents. This suggests that, at least in the short term, teeth bonded with glass ionomers are significantly more resistant to demineralization due to dental caries than those bonded with composite, even in a patient population such as adolescents with braces who are known for their high caries risk (Vorhies 1998, Donly 1995, Molitor 1999). It is possible that in the typical situation where patients have braces on all teeth, not just 2 study teeth, with wires and elastics compounding the plaque build-up, that the difference in the effect of the two bonding agents would be even more apparent.

C. SALIVA FLUORIDE LEVELS

Specific Aim #2 of this study was to assess whether or not the fluoride-releasing effect (if any) of the glass ionomer was restricted to the area around the bracket, or whether there was a whole-mouth increase in fluoride levels. All patients were asked not to use any fluoride supplements during the study with the exception of the toothpaste they were provided for the duration of the study.

It has been shown in many studies that glass ionomer exhibits a high initial release of fluoride, which then tapers off. In this study, there was no such rapid rise in

fluoride levels in whole mixed saliva in the mouth after bonding of the brackets with the glass ionomer on two teeth per subject. There were no statistically significant differences in fluoride levels from baseline measurements (pre- bracket-bonding) for either the control or test groups, or between the groups when the whole-mouth saliva was measured. This suggests that the fluoride release from the cement was not sufficiently large to affect the whole mouth, thus the effects seen in the microhardness studies were localized to the tooth with the bracket. For both groups, the mean whole saliva levels of fluoride (0.05 +/- 0.013, 0.031 +/- 0.011 ppm) fell far below the commonly accepted minimum concentration of fluoride thought necessary to inhibit demineralization, although they were (for both groups) at the 0.03 ppm F minimum level required for enhancing remineralization (Featherstone et al 1986, 1990, 1999). Again, this result may have been different had there been brackets on every tooth in the mouth as is typically the situation during orthodontic treatment.

Despite the absence of a more global, whole mouth effect on the fluoride levels in patients bonded with glass ionomer, the results from the microhardness testing clearly indicate that the local ability of fluoride release from the glass ionomer cement is significant when compared to the traditional composite resin cement. The onset of demineralization due to dental caries on the tooth surface below the bracket margin was successfully inhibited in the test group (glass ionomer).

D. CLINICAL IMPLICATIONS / SIGNIFICANCE

Since bands fit on the posterior teeth circumferentially, they are held in place primarily through mechanical retention and glass ionomers have been widely used for this. For bonding brackets onto the teeth, however, retention is achieved solely as a result of the bond strength of the cement used. Concerns about the bond strength of some of the earlier orthodontic glass ionomers have therefore led many practitioners to use composite resin preferentially over glass ionomers when bonding brackets. However, recent research (Shamaa et al 1999) has shown that the newer versions of glass ionomer orthodontic cement such as the Fuji Ortho LC used in this study demonstrate adequate clinical bond strength when compared to composite resin if the tooth is first conditioned with acid etch (as with composite).

Clearly, if one cement were simply comparable to another, then it would not matter which one a clinician chooses to use. The unique ability of the fluoride-releasing glass ionomers to inhibit demineralization around the orthodontic brackets therefore becomes an important point. It would not be ethical to extract teeth for quantitative analysis of decalcification at the end of a typical 2-year treatment, however, this study was able to show that at least in the short term, the fluoride release from Fuji LC glass ionomer cement is effective in protecting the tooth against decay with the clinical significance expected to increase over the typical treatment time of 2 years.

This information was obtained in a carefully controlled *in vivo* study that was assessed by quantitative analysis. The results of this study therefore confirm the predictions from *in vitro* studies demonstrating that these glass ionomer cements can

"recharge" their ability to inhibit demineralization when exposed to fluoride dentifrices, rinse or gel (Molitor 1999). The once abstract concept of the practitioner preventing or minimizing the development of "white spot" lesions during treatment without relying on patient compliance simply by changing the type of cement used is now apparently feasible.

VI. CONCLUSIONS

1. The use of a glass ionomer cement (Fuji Ortho LC) significantly reduced enamel mineral loss due to dental caries around orthodontic brackets in patients' mouths compared to Transbond XT composite resin during a 4-week period.

2. The fluoride released by the glass ionomer cement had a significant local effect, but the amount used for only 2 brackets in the mouth bonded with this cement did not produce an increase in measurable overall levels of fluoride in the whole-mouth saliva.

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APPENDIX 1: Consent Form

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO
CONSENT TO BE A RESEARCH SUBJECT

***In Vivo* Comparison of Demineralization around Orthodontic Brackets with Fluoride Releasing Glass Ionomer versus Non-Fluoride Composite Resin**

A. PURPOSE AND BACKGROUND

John D. B. Featherstone, PhD, MSc and Jasmine M. Gorton, DMD in the Departments of Restorative Dentistry, Dental Public Health and Hygiene, and Growth and Development are looking for a way to reduce tooth decay around braces. This will be done by measuring the decay around braces bonded with a **non-fluoride** bonding cement compared to a **fluoride**-releasing bonding cement. To see how much fluoride is actually present in the mouth, fluoride levels in spit and plaque will be measured.

I am being asked to participate because I am an adolescent and have teeth that need to be taken out as part of my othodontic treatment.

B. PROCEDURES

If I agree to be in this study, the following will happen:

1. Before treatment, I will receive a cleaning.
2. I will have a 50/50 chance of being placed in either of the test groups. Neither my doctor nor I will make this choice. Both groups will have braces put on 2 teeth that have already been scheduled to be removed (otherwise these teeth would have been removed without having braces on them).

If I am assigned to either group:

1. I will provide spit samples at the initial study visit, at 1 week, 2 weeks 3 weeks and 4 weeks later. I will also collect my own spit at home on the first, second and third day after my initial study visit and store these in the freezer until my next visit to the clinic.
2. I will provide the saliva samples by chewing on a 1 x 1 inch square of wax and spitting into a tube until around a teaspoon of spit is collected.
3. I will not eat, drink, brush my teeth or use mouthwash for at least 1 hour before my scheduled spit collection.
4. I will allow braces to be placed on 2 teeth that have been scheduled to be removed prior to the start of the study.
5. I will be given a tube of toothpaste to use and asked to fill in a log of my daily toothbrushing schedule (2 times daily). I will not use another dental product, including mouthwash, during the study (floss O.K.). I will also be given verbal and written instructions, and check off sheets for the at-home saliva sampling.

6. Participation in the study will take approximately 2 hours over a 4-week period. There will be approximately 5 visits: Initial spit collection and 2 braces (45 minutes), 1,2,3,4 week spit collection (1 hour total) and spit collection at home on days 1,2,3 after the first visit (15 minutes total).
7. Every effort will be made to schedule the study visits at the same time as my regular dental visits to the Hygiene (cleaning) Clinic, Orthodontics Clinic and Oral Surgery Clinic although this may not always be possible due to scheduling conflicts.
8. All study procedures will be done at the UCSF Orthodontics Clinic.

C. RISKS AND DISCOMFORTS

The two braces put on my teeth for a month might rub against my cheeks for the first few days I get them on. Food might get caught in the braces when I eat. These are the usual discomforts that can happen with braces.
Also, research shows that using fluoride in dental materials and toothpaste is safe.

My personal information will be handled as confidentially as possible. All my records will be seen only by people involved in my regular orthodontic treatment and the researcher. My name will not show up in print (ie magazines) because of this study.

Treatment and Compensation for Injury
If I am injured as a result of being in the study, treatment will be available. The costs of such treatment may be covered by the University of California depending on a number of factors. The University does not normally provide any other forms of compensation for injury. For further information about this, I may call the committee on human research at (415) 476-1814.

D. BENEFITS

There is no benefit to me for being in this study. The information from this study will hopefully help doctors find the best way for stopping tooth decay around braces.

E. ALTERNATIVES

If I don't participate in this study, my teeth that need to be removed will be taken out without having braces put on them first. I can still be a patient in the UCSF Orthodontics Clinic and get braces on the rest of my teeth (as usual) without having to provide saliva samples. I will continue using the toothpaste of my choice.

F. COSTS

The cleaning done by the UCSF Hygiene Department immediately before starting the orthodontic treatment will be done for free (usually $65-80).

I won't have to pay for the braces that are put on the 2 teeth that are going to be taken out, but I will still have to pay for my standard orthodontic care (the rest of my braces). However, I will receive a 5 % discount on the total UCSF resident's orthodontic treatment price (a value of approximately $170). If I have dental insurance, it will be accepted for all standard orthodontic procedures that the insurance covers (in accordance with standard UCSF dental clinic policies). Since the 5% discount is taken off the total price at the beginning, both my insurance company and myself would end up saving money (so if my insurance coverage pays 50% of total, we each save $85 off the total price).

When it is time to get my teeth taken out to get ready for my regular braces treatment, the UCSF Oral Surgery Department can take them out for the usual price. However, after I leave their clinic, they will send a check for $75 written out to me (the adolescent patient) as a thank you for having finished the study (it usually takes 4 weeks for the check to be sent). If I have dental insurance, it will be accepted for all procedures that the insurance covers (in accordance with standard UCSF dental clinic policies). The $75 check will be sent to me whether or not I am covered by any insurance.

If I qualify for this study, I can start now, even if I have some teeth that need to be filled. However, if I have tooth decay my orthodontic resident will ask me to go to my dentist to get my teeth fixed before I get the rest of my braces treatment done. This has nothing to do with the study, so I can see the dentist at the same time I am doing the study if I want to, and I will have to pay the dentist like I usually do.

The study sponsor will pay for the test supplies, including the tube of toothpaste I am asked to use, the braces and bonding material used on the 2 teeth selected for the study

The study sponsor will pay for all of the spit/plaque collection and testing for all study participants.

G. INCENTIVES

The free services and discounts mentioned above total approximately $300, based on the standard orthodontic fees in the UCSF Orthodontics Clinic, the oral surgery reimbursement of $75 and the current cost of a cleaning. The direct savings will be less if I have insurance that covers my orthodontic treatment.

H. QUESTIONS

This study has been explained to me by Dr. Gorton and my questions were answered. If I have any other questions about the study, I may call Dr. Gorton at (415) 476-6100 x 50875 or Dr. Featherstone at (415) 476-5802.

I. CONSENT

I have been given copies of this consent form and the Experimental Subject's Bill of Rights to keep.

PARTICIPATION IN RESEARCH IS VOLUNTARY. I have the right to decline to participate or to withdraw at any point in this study without jeopardy to my dental care.

If I am a University of California employee I will not participate in this study during my work hours.

If I wish to participate, I should sign below.

(Co) Investigator	Subject's Signature	Parent's Signature	Date

** For patients under the age of 18, the parent/guardian must also sign the consent form

APPENDIX 2: Patient Packet

PATIENT FLOW CHART

Orthodontic Clinic Visit: Final Consult Appointment *
Sign orthodontic waiver and contract
Schedule Oral Surgery Consult Visit
Sign research study informed consent **

↓

Hygiene Clinic Visit: Cleaning
Saliva sample
Braces put on 2 teeth for the study

↓

(Collect your own saliva at home the first 3 days after the cleaning appointment)

↓

Orthodontic Clinic Visit: ***Saliva sample***
(1 week after cleaning)

↓

Oral Surgery Clinic Visit: Oral surgery consult
(2 weeks after cleaning) ***Saliva sample***

↓

Orthodontic Clinic Visit: ***Saliva sample***
(3 weeks after cleaning)

↓

Oral Surgery Clinic Visit: Extractions
(4 weeks after cleaning) ***Saliva sample***

* normal clinic procedures
** ***study procedures***

PATIENT INSTRUCTION FORM

1. I will provide spit samples at the initial study visit, at 1 week, 2 weeks 3 weeks and 4 weeks later. **I will also collect my own spit at home on the first, second and third day after my initial study visit and store these in the freezer until my next visit to the clinic. I will be given instructions and check off sheets for the at-home saliva sampling.**
2. I will provide the saliva samples by chewing on a 1 x 1 inch square of wax and spitting into a tube until around a teaspoon of spit is collected.
3. **I will not eat, drink, brush my teeth or use mouthwash for at least 1 hour before my scheduled spit collection.**
4. I will allow braces to be placed on 2 teeth that have been scheduled to be removed prior to the start of the study.
5. **I will be given a tube of toothpaste to use and asked to fill in a log of my daily toothbrushing schedule (2 times daily). I will not use another dental product, including mouthwash, during the study (floss O.K.). I will return the tube of toothpaste when I finish the study (the day my teeth are extracted).**

NAME:		
SAMPLE #	DATE	SPIT COLLECTION TIME
1		
2		
3		

1. Spit into the tube up to the line
2. Store tube in the freezer until your next appointment
3. Record the day and time you spit

NAME:

	DATE	BRUSHING TIME #1	BRUSHING TIME #2
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
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25			
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27			
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30			

APPENDIX 3: Study Announcement for Recruitment

UCSF

VOLUNTEERS NEEDED FOR
TOOTH DECAY PREVENTION STUDY

ADOLESCENTS MAY QUALIFY TO PARTICIPATE

If they are between 11 and 18 years old
and have been prescribed tooth extraction for orthodontic treatment

Participants will receive a free dental cleaning, 5% discount on their orthodontic fee, and a $75 reimbursement check
(estimated total value **$300**)

Ask your Orthodontic Resident or call:

Dr. Jasmine Gorton
(415) 476-6100 x 50875

University of California, San Francisco
Department of Growth and Development
Department of Restorative Dentistry

APPENDIX 4: REPRODUCIBILITY DATA:

Repeated measures of the same lesion for one indent in a carious zone and one indent in sound enamel (μm)

CARIOUS TOOTH 1A		NORMAL TOOTH 1B	
Indent #	Length (μm)	Indent #	Length (μm)
1	55.0	1	35.5
2	55.8	2	35.5
3	56.1	3	35.5
4	55.0	4	35.5
5	56.1	5	35.5
6	55.0	6	35.5
7	56.1	7	36.3
8	56.1	8	35.5
9	56.1	9	35.5
10	56.3	10	35.5
AVG	55.8	AVG	35.6
SD	0.53	SD	0.25

APPENDIX 5: Microhardness Testing: Control vs. Treatment

Modified from original formula and assumed sound enamel = 150-300μm

Date: 4/13/01

Sample ID: **Patients #1-9** **Patients #10-21**

Force (g): 15p for 15μm 25p for 15-300μm
50p 20-300μm

CONTROL: Normalized Volume % Mineral

Distance (μm)	2 (C)	3 (C)	4 (C)	6 (C)	8 (C)	10 (C)	12 (C)	15 (C)	17 (C)	16 (C)	average	std. dev.
15	46.43	44.73	41.72	42.99	62.42	57.08	63.67	69.83	68.18	47.91	54.49	10.93
20	75.73	76.06	52.78	65.19	64.19	58.16	68.02	72.04	65.09	38.64	63.59	11.39
25	61.57	69.69	44.78	47.28	67.35	69.08	69.85	78.25	68.60	65.70	64.22	10.46
30	74.21	73.37	78.75	66.78	73.05	71.24	79.58	53.07	70.23	79.99	72.03	7.92
35	80.66	77.84	81.63	63.37	78.46	70.69	77.70	78.25	76.24	64.30	74.91	6.53
40	67.17	77.69	78.55	63.33	56.62	76.56	78.97	66.26	74.27	77.26	71.67	7.78
45	70.68	74.57	82.32	75.26	76.81	79.77	79.62	66.73	76.07	76.63	75.84	4.53
50	81.45	88.71	88.13	72.46	78.89	87.52	70.34	76.28	72.73	72.97	78.95	7.13
75	86.30	78.92	86.18	71.64	70.63	84.31	81.51	80.30	74.33	77.28	79.14	5.68
100	88.35	78.92	86.18	81.87	77.26	82.80	81.51	78.25	84.50	93.79	83.34	5.08
125	86.79	78.92	89.87	85.97	89.88	79.92	78.98	87.28	87.05	87.57	85.22	4.30
150	81.55	83.33	87.89	85.45	88.29	82.05	81.49	84.05	84.40	85.12	84.36	2.41
175	87.83	80.63	87.57	88.75	83.32	87.53	86.16	84.05	85.21	85.17	85.62	2.48
200	84.28	86.74	84.54	81.87	88.29	87.49	84.16	83.28	89.62	87.60	85.79	2.50
225	83.37	86.70	84.54	87.59	88.24	84.26	89.31	84.05	79.92	79.99	84.80	3.22
250	84.84	85.23	88.13	87.59	88.29	87.33	86.28	86.45	84.40	82.10	86.06	1.94
275	84.80	85.22	81.01	81.87	75.25	82.03	84.16	89.84	84.48	87.41	83.61	3.95
300	88.33	87.16	81.32	81.87	83.32	84.31	83.45	83.28	86.99	87.61	84.76	2.54
DZ	360.09	896.90	273.51	1227.86	1194.42	711.78	766.37	794.62	986.88	842.00	**805.44**	**309.85**

TEST: Normalized Volume % Mineral

Distance (μm)	1 (T)	5 (T)	7 (T)	9 (T)	11 (T)	13 (T)	14 (T)	18 (T)	19 (T)	20 (T)	average	std. dev.
15	80.96	55.63	73.79	78.07	73.65	45.09	76.11	78.54	79.43	65.68	70.70	11.80
20	69.50	70.30	58.24	93.24	71.84	65.71	90.60	67.53	64.11	64.39	71.55	11.42
25	84.25	75.55	72.63	75.19	78.18	51.94	87.25	86.26	69.41	88.43	76.91	10.98
30	84.80	85.88	82.03	99.91	71.33	67.57	88.03	89.45	67.79	79.17	81.59	10.36
35	99.94	83.83	75.52	97.07	75.51	64.35	84.94	76.67	73.83	66.91	79.86	11.71
40	81.08	78.29	75.53	97.07	77.37	70.53	87.24	86.20	79.58	72.60	80.55	7.85
45	79.82	78.02	60.08	95.12	75.53	72.62	88.09	92.01	75.67	88.61	80.56	10.56
50	84.25	90.91	75.53	95.10	85.59	85.48	90.40	91.94	79.43	76.65	85.53	6.69
75	82.32	90.34	86.41	81.31	85.63	86.22	90.60	88.63	88.76	85.42	86.56	3.12
100	84.30	81.47	84.20	78.55	81.00	92.84	88.05	84.01	87.72	83.98	84.61	4.08
125	82.91	88.90	85.82	84.76	82.48	86.27	80.62	91.97	87.09	88.61	85.94	3.41
150	83.36	82.36	87.54	86.31	89.02	87.02	81.96	84.01	82.42	89.39	85.34	2.85
175	84.76	82.71	85.84	85.28	82.45	83.99	84.89	88.63	91.36	86.18	85.61	2.69
200	82.91	85.68	85.82	81.77	85.59	87.05	82.70	83.96	85.47	86.98	84.79	1.84
225	84.78	86.52	82.10	86.87	85.56	86.24	84.94	81.16	82.42	83.93	84.45	1.99
250	84.76	85.17	83.66	84.76	80.94	83.27	82.01	86.26	84.69	81.12	83.66	1.80
275	87.71	86.16	85.84	83.23	82.45	81.17	87.90	84.01	83.16	86.23	84.79	2.30
300	86.71	86.40	84.20	86.79	88.98	86.27	90.60	86.98	85.47	81.16	86.36	2.54
DZ	267.28	-57.52	512.19	-135.21	574.56	490.36	-226.72	-291.73	219.19	243.74	**159.61**	**318.88**

APPENDIX 6: Fluoride Assay Data Summary: no outliers

CONTROL	Baseline	Day 1	Day 2	Day 3	Day 7	Day 14	Day 21	Day 28	STDEV	Mean/Pt
B(C)	0.151	0.027	0.061		0.042	0.031	0.016	0.008	0.049	0.048
C(C)	0.018	0.026	0.021	0.022	0.022	0.021	0.023	0.025	0.003	0.022
G(C)	0.024	0.030	0.025	0.030	0.028	0.028	0.025		0.003	0.027
H(C)	0.014	0.018	0.018	0.008	0.009	0.010	0.008	0.030	0.007	0.014
J(C)		0.059	0.148	0.061	0.039	0.170	0.057	0.101	0.051	0.091
P(C)	0.031	0.020	0.066	0.031	0.208		0.010	0.022	0.070	0.055
Q(C)	0.000	0.025	0.045	0.025	0.025	0.030	0.113	0.021	0.034	0.036
R(C)	0.067	0.011	0.006	0.014		0.014	0.067	0.020	0.027	0.028
S(C)		0.159	0.024	0.020	0.176	0.081	0.019	0.022	0.069	0.072
V(C)	0.030	0.025	0.015	0.035	0.040	0.014	0.013	0.288	0.094	0.057
Average/Day	0.042	0.040	0.043	0.027	0.066	0.045	0.035	0.060		0.045
SD	0.048	0.044	0.042	0.015	0.073	0.052	0.034	0.090		0.012

TEST	Baseline	Day 1	Day 2	Day 3	Day 7	Day 14	Day 21	Day 28	STDEV	Mean/Pt
A(T)	0.027	0.033	0.011	0.010	0.031	0.055	0.025	0.021	0.014	0.027
D(T)	0.025	0.021	0.020	0.009	0.010	0.009	0.017	0.022	0.006	0.017
I(T)	0.023	0.018	0.012	0.023	0.034	0.170	0.047	0.133	0.060	0.058
L(T)	0.000	0.011	0.014	0.042	0.034	0.022			0.015	0.021
M(T)		0.011	0.014	0.038	0.022	0.019	0.013	0.044	0.013	0.023
N(T)	0.031	0.016	0.013	0.011	0.016	0.025	0.014	0.009	0.007	0.017
O(T)	0.045	0.068	0.027	0.015	0.024	0.000	0.005	0.005	0.023	0.024
T(T)	0.074			0.015	0.068	0.024	0.017		0.029	0.040
U(T)	0.024	0.029	0.062	0.055	0.051	0.050	0.026	0.032	0.015	0.041
W(T)	0.070	0.016	0.019	0.021	0.009	0.013	0.007	0.171	0.056	0.041
X(T)	0.082	0.014	0.013	0.029	0.021	0.012	0.053	0.017	0.025	0.030
Average	0.040	0.024	0.021	0.024	0.029	0.036	0.023	0.051		0.031
SD	0.027	0.017	0.015	0.015	0.018	0.047	0.016	0.060		0.011

Control Group Average	**0.045**	**Treatment Group Avg**	**0.031**
Control Group SD	**0.012**	**Treatment Group SD**	**0.011**