

COMPARISON AMONG CPP-ACP, FLUORIDE AND A COMBINATION
OF CPP-ACP/ FLUORIDE AND THEIR ABILITY TO REMINERALIZE
WHITE SPOT LESIONS *IN VITRO*

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ABSTRACT

GUSTAVO OLIVEIRA: Comparison among ACP, Fluoride and a combination of
ACP/ Fluoride and their ability to remineralize white spot lesions *in vitro*
(Under the direction of Timothy Wright)

This study evaluated the ability of a casein-phosphopeptide amorphous-calcium-phosphate paste (CPP-ACP, MI Paste™), a 1.1% NaF toothpaste containing 5000 ppm of fluoride (ControlRX™) and a CPP-ACP paste with 900 ppm of fluoride (MI Paste Plus™) to remineralize white spot lesions *in vitro*. Thirty five crowns of sound human molars were sectioned into four specimens. Artificial white spot lesions were formed in all specimens. Specimens were then randomly assigned to one of the following remineralizing treatments (n=35) with a pH-cycling model over 30 days: Group 1 – Control; Group 2 – MIPaste; Group 3 - F5000; Group 4 – MI Paste Plus. Therapeutic agents were applied following the manufacturers' directions. MicroCT and Light-induced fluorescence were used as methods of assessment. The outcome measures were Lesion Volume ($p=0.185$), Lesion Density ($p=0.048$), Mean Fluorescence Loss ($p<0.001$), and Area of the Lesion ($p<0.001$). Overall, the 1.1% NaF containing toothpaste demonstrated better remineralization ability than CPP-ACP products overtime.

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INTRODUCTION

It is widely accepted that the interrelationship between oral and general health can influence quality of life. According to the The World Oral Health Report of 2003, oral diseases impede activities in school and work causing many productive hours to be lost each year all over the world [Petersen, 2003]. The possible associations between oral health – especially dental caries - and systemic diseases as cardiovascular diseases, diabetes mellitus, and even certain types of cancers, is described in the literature [Hujoel, 2009]. Despite worldwide improvements in the oral health, dental caries is still a major oral health problem in most industrialized countries, affecting 60-90% of schoolchildren and the vast majority of adults [Petersen, 2003].

Dental caries is a pathological condition that results from an unbalance in the physiological process of remineralization/ demineralization of the dental structure [Featherstone, 2006a]. The understanding of dental caries as a multifactorial oral pathology implies a complex treatment plan in which remineralization plays an important role, preventing the progression of the disease and reverting initial signs of demineralization [Backer Dirks, 1966; Hicks and Flaitz, 2007; Silverstone, 1977]. Due to the presence of calcium and phosphate ions in supersaturated state, the whole human saliva has the potential to remineralize tooth structure [Featherstone et al., 1993]. However, if pH

challenges overcome the physiological remineralization process, a therapeutic approach is made necessary.

Fluoride is recognized as a remineralizing agent, interacting with oral fluids on the interface of enamel and subsurface regions of teeth, and combining with calcium and phosphate ions to form fluorapatite [ten Cate and Featherstone, 1991]. However, this element can cause fluorosis, and also be toxic if administered in high enough dosages [Dean et al., 1938; Rose, 2000].

The possible cariostatic potential of dairy products is subject of many reports in the literature [Jensen and Wefel, 1990; Rosen et al., 1984; Shaw et al., 1959]. In 1991, the complex casein phosphopeptide amorphous calcium phosphate (CPP-ACP), derived from a major protein found in milk called casein, is patented in U.S. [Reynolds, 1991]. The complex is presented as an alternative remineralizing agent, remarkably capable of stabilize calcium phosphate, maintaining a state of supersaturation of these ions in the oral environment. As a consequence, the tooth structure would benefit from the high levels of calcium phosphate in the biofilm, and remineralization would occur [Reynolds, 1997; Reynolds et al., 1995]. Although product containing CPP-ACP are already available in the market, the lack of consensus among scientist regarding the remineralizing potential of this complex is evident [Azarpazhooh and Limeback, 2008; Pulido et al., 2008].

The aim of this study was to assess the ability of MI Paste (CPP-ACP containing toothpaste), ControlRX NaF 1.1% (5000 ppm containing toothpaste),

and MI Paste Plus (CPP-ACP/ fluoride 900 ppm containing toothpaste) to remineralize white spot lesions *in vitro*.

The null hypothesis of this study is that there is no difference in the remineralization of the white spot lesions among the MI Paste, ControlRX and MI Paste Plus groups.

LITERATURE REVIEW

Caries: a multifactorial disease

Dental caries is a chronic infectious disease that results in the destruction of tooth structure. It is caused by a complex interaction of oral microorganisms in biofilm, diet, and many host factors ranging from societal and environmental factors to genetic and biochemical/immunologic host responses [Zero, 1999]. A decrease in the pH at the plaque-tooth interface will result in dissolution of tooth structure and, consequently, tooth demineralization [Kidd and Fejerskov, 2004]. When the acidogenic bacteria present in the dental biofilm are exposed to and metabolizes dietary sucrose, the pH rapidly decreases from a resting pH of 7.0 to a pH of less than 5.0 within the biofilm fluid and along the interface between the biofilm and enamel surface [ten Cate and Featherstone, 1991]. Then, the critical pH (about pH 5.5) at which enamel starts to dissolve is reached [Patel et al., 1987]. If repeated episodes of prolonged-duration demineralization occur, the equilibrium between demineralization and remineralization is disrupted [Featherstone, 1999]. The consequence of these events is the subtle exchange from a physiological process to a pathological condition, and the development of an incipient lesion is initiated.

“White spot lesion”

The so called “white spot lesion” is the first optically visible evidence of enamel caries and is due to an optical phenomenon which is caused by mineral loss in the subsurface enamel [Gorelick et al., 1982]. Histologically, a well-mineralized surface layer covers a demineralized subsurface zone [Alexander and Ripa, 2000]. This configuration of the demineralized enamel is well characterized by the chalky white opacity that can be perceived when the initial subsurface lesion is dried [Holmen et al., 1985]. However, some white spot lesions may be clinically observed even when wet, depending on the extent and severity encountered in each of these lesions [Holmen et al., 1985]

It has been proven by both *in-vitro* and in human clinical studies that white spot lesions can be remineralized [Altenburger et al., 2009; Hicks and Flaitz, 2007]. Remineralization may re-establish the hardness of the sound enamel to a certain extent and also produce a greater resistance to further dissolution [Silverstone, 1983]. To achieve a positive balance between demineralization and remineralization – inducing the reversal of the lesion - a number of preventive therapies are available including patient education, fluoride administration, use of antimicrobials such as chlorhexidine, diet control and the use of artificial sweeteners such as xylitol and, more recently, the application of caseinphosphopeptide-amorphous calcium phosphate (CPP-ACP) to promote remineralization.

Fluoride

Over 70 years since Dean and colleagues identified fluoride as being responsible for differences in caries prevalence between communities, fluoride remains the “gold standard” in prevention and treatment of the caries disease [Dean et al., 1938]. Its safety and its efficacy against dental caries has been extensively studied and discussed over the years [Griffin et al., 2007]. In trace amounts, fluoride increases the resistance of tooth structure to demineralization and is the only modality that is applied on a population basis for caries prevention [Backer Dirks, 1966]. For these reasons, the Centers for Disease Control and Prevention identified water fluoridation as one of 10 great public health achievements of the 20th century [MMWR, 2008].

Fluoride exerts its anticaries effect by different mechanisms. It not only enhances the precipitation of fluorapatite into tooth structure, but also remineralizes incipient, non-cavitated carious lesions, following the same process [ten Cate and Featherstone, 1991]. Another front of action involves interaction with microflora, especially inhibiting the enzymatic production of glucosyltransferase by bacteria [Shani et al., 2000]. Most investigators consider the primary mechanism of caries prevention from fluoride therapies as being related to its ability to promote remineralization [ten Cate and Featherstone, 1991]. Obviously, the foundation of the first two anticaries mechanisms stated above is the fundamental ability of the disassociated fluoride ion to combine with calcium and phosphate ions, and replace the lost hydroxyapatite with a new and more resistant tissue that is enriched in fluoride (is not usually fluorapatite which

is present in teeth in only very small amounts). Fluoride enhances remineralization of partially dissolved enamel or dentin crystals by combining with calcium and phosphate that comes primarily from saliva. Remineralization is the natural repair process for the non-cavitated carious lesion. Fluoride speeds up remineralization and promotes the deposition of mineral on the partially demineralized crystallite remnants inside the carious lesion. The crystal solubility is markedly reduced due to the loss of carbonate during the demineralization and the incorporation of fluoride during remineralization [Featherstone, 2006b].

By virtue of its benefits, various types of vehicles have been developed and proposed to enhance exposure of the population to fluoride. Within the broad spectrum of alternatives such as fluoride supplements and topical treatments with materials like fluoride varnishes, fluoride toothpastes play an important role in preventing dental caries [Ten Cate, 2004]. Fluoridated toothpaste can effectively deliver a variety of different concentrations of fluoride [Stookey et al., 1993].

Inside this concept of delivery, the debate around fluoride concentration needed for treatment of incipient lesions still persists. However, it appears that the so-called advanced subsurface lesions, i.e. that are 150 microns deep, could benefit from increased remineralization and decreased demineralization when submitted to treatment with higher fluoride-content toothpaste (5000ppm) instead of traditional products (1500ppm) [ten Cate et al., 2008].

On the other hand, the administration of fluoride will expose patients to potentially toxic side-effects. For example, it is known that excessive doses can

cause fluorosis during ages when tooth development is occurring and in large enough quantities can even be toxic [Rose, 2000]. Taken together the safety issues and dosage limitations are considered an important limitation of fluoride therapies thereby opening the window for the development of new remineralizing products with fewer possible side-effects or that are potentially more effective than fluoride.

Dairy products and the development of CPP-ACP

In the late 1950's, dairy products were discovered as a food group that could be effective in preventing dental caries [Shaw et al., 1959]. Studies were conducted using dairy products to verify the correlation with caries control. Epidemiological reports suggested that children [Petti et al., 1997] and adolescents [Petridou et al., 1996] with low incidence of dental caries drank more milk than those with high caries incidence [Aimutis, 2004]. More recently, studies shown that children with high consumption of soft drinks relative to consumption of milk and real fruit juice are at higher risk of developing dental caries [Kolker et al., 2007; Lim et al., 2008]. The theory behind this possible characteristic involved a high calcium and phosphate content and the presence of the phospho-protein casein [Levine, 2001], although other fractions of milk could also play an important role [Grenby et al., 2001].

Caseins account for roughly 80% of the total protein in bovine milk, and exist primarily as calcium phosphate stabilized micellar complexes [Eigel et al., 1984]. This protein, when incorporated into dental biofilm, is associated with an

increase in the content of calcium and phosphate inside this environment [Reynolds, 1987], which are the principal components of hydroxyapatite.

From insoluble acid casein to soluble sodium caseinate, many forms of this protein were evaluated since attention to its apparent anticariogenic activity was initially observed [Bavetta and Mc, 1957; Reynolds and Black, 1987; Reynolds and del Rio, 1984]. The problem encountered in trying to add the protein to food was the required high levels at which the protein would impart its anticariogenic properties. The levels found to be effective would fail in combine organoleptic properties and the virtue of preventing enamel demineralization [Aimutis, 2004; Reynolds and Black, 1989]. Since adding casein in its original form to products for public consumption was clearly not feasible, one probable solution was to break apart the molecule in active peptides.

In order to verify the efficacy of the casein molecule when broken apart, it was found that the peptides released by trypsin during a tryptic digestion of caseinate did not destroy the supposed ability to prevent enamel demineralization *in situ* [Reynolds, 1987]. Furthermore, elevated concentrations of these casein peptides, phosphorous and calcium were found in plaque [Reynolds, 1987]. This findings prompted investigators to focus their work on casein peptides.

In 1987, a study demonstrated that the peptides responsible for caseinates supposed anticariogenic activity were the calcium-phosphate-stabilizing casein phosphopeptides (CPP) [Reynolds, 1987]. The cluster of phosphoseryl residues contained in these peptides can greatly increase the

solubility of calcium phosphate [Reeves and Latour, 1958] by forming colloidal casein-phosphopeptide amorphous-calcium-phosphate complexes [Schmidt, 1982].

In summary, “CPP-ACP would acts as a calcium phosphate reservoir, buffering the activities of free Ca and PO₄ ions in the plaque fluid, helping to maintain a state of supersaturation of these ions, thereby depressing enamel demineralization and enhancing mineralization” [Reynolds, 1991].

Many studies have been completed regarding the anticariogenic potential of CPP-ACP *in vitro*. In a group of specific-pathogens-free rats infected with *Streptococcus sobrinus*, Reynolds and colleagues found that the application of CPP-ACP solutions (100 µL) twice a day significantly reduced smooth-surface caries activity [Reynolds et al., 1995]. They found that the reduction was observed in a dose-response fashion, with 0.1% w/ v CPP-ACP producing 14% reduction, and 1.0% w/ v CPP-CP a 55% reduction [Reynolds et al., 1995]. However, these percentages were obtained when compared with distilled water control. Besides, the CPP-ACP group presented similar remineralizing performance as the 500 ppm F⁻ group.

Another *in vitro* study demonstrated that after a ten-day period, 1.0% CPP-calcium phosphate (pH 7.0) solution promoted a 63.9 ± 20.1% of remineralization of enamel subsurface lesion when compared to solutions with lower concentrations of CPP- stabilized free calcium and phosphate ions [Reynolds, 1997]. One negative side is that the research design was not made to mimic intra-oral conditions, which may compromise any relevant conclusion.

More recently, a pH-cycling fashioned study was conducted to verify the ability of CPP-ACP containing tooth mousse to remineralize enamel lesions as compared with a fluoride containing toothpaste. It was observed that the CPP-ACP containing tooth mousse was able to remineralize initial enamel lesions, and performed even better when applied as topical coating after the fluoridated toothpaste was used [Kumar et al., 2008].

Two studies completed in 2009 have also investigated the remineralization ability of CPP-ACP. In the first, the authors reported that milk containing CPP-ACP increased mineral content of subsurface enamel lesions up to 164% relative to control milk *in situ* [Walker et al., 2009]. Another group found that significantly more post-orthodontic white spot lesions regressed with a CPP-ACP based remineralizing cream, when compared with a placebo after 12 weeks, *in vivo* [Bailey et al., 2009].

On the other hand, Lennon and colleagues concluded that a highly fluoridated acidic amine fluoride (AmF) gel was able to protect bovine enamel against erosion while casein/ calcium phosphate, 250 ppm fluoride, or a combination of casein/ calcium phosphate and 250 ppm fluoride proportioned almost no protection [Lennon et al., 2006]. Following the same line of findings, an *in-vitro* study evaluated the inhibition of enamel demineralization produced by MI Paste (GC America, Alsip, IL), which is a CPP-ACP containing product, fluoride and a combination of both, in a caries progression pH-cycling model. The comparisons were made with artificial saliva and NaF 5000 ppm. It was concluded that the MI Paste group did not inhibited lesion progression, while NaF

5000 ppm demonstrated to be the most effective in reducing lesion progression [Pulido et al., 2008]. Nonetheless, it appears that a consensus among scientists about the efficacy of CPP-ACP, alone or combined with fluoride, is yet to be established.

MATERIALS AND METHODS

Specimen preparation

Thirty five caries-free extracted human third molars were collected over a period of a month, through the cooperation of the oral and maxillofacial surgeons of the University of North Carolina (UNC) Oral and Maxillofacial Surgery Department, and kept refrigerated and hydrated in thymol 0.01% until specimen preparation. Teeth with fluorosis, enamel or dentin defects, or presenting any type of dental restoration or sealant were not used.

The roots were amputated and the crowns sectioned twice splitting one tooth in 4 quadrants of approximately equal size. Once sectioned, one quarter of the tooth quadrants were randomly assigned to either group 1, 2, 3 or 4 (n=35). All 4 quadrant samples of the same tooth were identified with the number of the correspondent tooth (1 to 35), as well as the location within the tooth structure (Facial-Mesial, Facial-Distal, Lingual-Distal, Lingual-Mesial). This experimental approach allowed each tooth to serve as its own control thereby helping reduce the effects of uncontrollable confounders related to inherent differences in the structure and composition of individual teeth [Gladwell et al., 2006]. After that, the enamel surfaces were protected with polyvinyl siloxane material (Clinician's Choice, New Milford, CT), and all specimens were mounted in phenolic rings (Buehler, Lake Bluff, IL) and embedded in epoxy resin (Buehler, Lake Bluff, IL).

The next step was to remove the aprismatic layer of enamel from all specimens. For this procedure, an Ecomet Grinder-Polisher (Buehler, Lake Bluff, IL) machine was used. The thickness of each specimen was measured with a digital caliper to serve as initial reference. Then, all specimens were ground/ polished by water-cooled abrasive papers up to 1200-grit. During this process, the specimens were constantly measured to verify the amount of tooth structure removed, which was always around 100 μm . At this point, photographs of each specimen were made with a Dissect Microscope (Nikon, Melville, NY).

To ensure that the demineralizing solution would contact only the ground and polished enamel surface, all specimens were covered with a color-free acid-resistant nail varnish (Revlon Nail Enamel) using a brush under microscope. All surfaces were coated except for the flat-ground enamel area. After that, the specimens were subjected to demineralization.

Lesion formation

For this procedure, a demineralizing solution was made and consisted of 15% G8 Gel (Fisher Scientific, Pittsburgh, PA), 0.1m lactic acid-sodium lactate (Sigma-Aldrich, St. Louis, MO), and 1ppm NaFl (Fisher Scientific, Pittsburgh, PA). The pH of the solution was adjusted to 4.15 [Feagin et al., 1985]. Gel systems were proven to produce lesions that are histologically comparable to natural lesions [Wefel et al., 1995]. All specimens were divided in 2 different containers and the same solution was used to cover the specimens in both containers up to one inch over the top surface of each specimen. The specimens

were kept inside an incubator at 37°C. On the sixth week, the white spot lesions could already be visualized at the enamel surfaces. For this reason - and also to protect against enamel loss - the containers were removed from the incubator and placed at room temperature, around 10 degrees lower, to slow down the demineralizing process. The specimens were kept in solution for 2 weeks more, totaling 8 weeks of demineralization process and, during the last couple of weeks, no pH correction was done and, at the end, the pH was measured to be 4.3. Pressurized warm water was used to remove all gel from each specimen. After visual analysis, all specimens were placed in a container with deionized water (pH 7.5) and kept at room temperature. New photographs of each specimen were taken again with the dissecting microscope.

Experimental groups

The tests utilized three different products available in the market for professional: a CPP-ACP containing toothpaste (MI Paste™, GC America, Alsip, IL), a 1.1% NaF toothpaste containing 5000 ppm of fluoride (ControlRX™, 3M, St. Paul, MN) and a CPP-ACP paste with 900 ppm of fluoride (MI Paste Plus™, GC America, Alsip, IL). Specimens were randomly allocated in one of the following four experimental groups (n=35): Control (no treatment), F5000 (ControlRX™), MI Paste (MI Paste™), and MI Paste Plus (MI Paste Plus™). The formulations of each product used are described in Table 1. The computer-aided randomization was done in a manner that each specimen from the same tooth was distributed to one of the four different groups. The treatment phase of the

experiment begun after each group was created. For this purpose, each treatment was strictly applied using each manufacturer's directions, every day, for a period of 30 days, as described in Table 2. The treatments were carried out in the mornings and evenings, depending on the treatment proposed, and were applied in the form of slurry, formed by one part of the product and three parts of artificial saliva, directly over the lesions as has been previously published [Pulido et al., 2008]. This ratio is commonly used in toothpaste studies and the mixtures were done on a daily basis. The artificial saliva used for this purpose was always removed before the batch was divided between the four containers of each group. Once the treatment application time was finished, the specimens were rinsed in deionized water for 5 seconds to remove the treatment solution. Immediately after the morning treatment, all specimens were submitted to pH cycling, as described below. Before the evening treatment application, all specimens were placed back in their own artificial saliva container for 10 minutes.

pH-cycling conditions

Standard pH-cycling conditions were used in a daily schedule of 3 cycles, each of 0.5 hours (30 minutes) of demineralization and 2.5 hours (150 minutes) of remineralization [ten Cate and Duijsters, 1982], followed by a "night" period of 6 hours in artificial saliva. Between each remineralization/ demineralization period, the specimens were thoroughly rinsed with deionized water. The artificial saliva consisted of 2.2 g/L gastric mucin, 0.381 g/L NaCl, 0.231 g/L CaCl₂, 0.738

g/L KH_2PO_4 , 1.114 g/L KCl, 0.02% sodium azide, trace of NaOH to pH 7.0 [Wongkhantee et al., 2006]. The pH of the artificial saliva was checked daily and the solution replaced weekly. As stated above, each different group had its own container of artificial saliva to avoid any type of contamination by other treatment solutions. However, to ensure standardization, the artificial saliva used in each of these containers was always made in a single batch and then separated. The remineralization solutions contained 1.5 mM CaCl_2 , 0.9 mM KH_2PO_4 , 130 mM KCl, and 20 mM Hepes pH 7.0, and the demineralization solution 1.5 mM CaCl_2 , 0.9 mM KH_2PO_4 , and 50 mM acetic acid adjusted to pH 5.0. The pH-cycle solutions were refreshed daily. The amounts of each solution were large enough to prevent from becoming saturated with or depleted of mineral ions [ten Cate et al., 1995].

Outcome Measures

Four different variables were analyzed throughout this study. Lesion Volume and Lesion Density, acquired with a MicroCT imaging system (SCANCO μCT 40, Scanco Medical, Bassersdorf, Switzerland), are measured in cubic millimeters (mm^3) and milligrams of hydroxyapatite per cubic centimeters (mg HA/ cm^3), respectively. A Mean Fluorescence Loss, which computes the difference in percentage ($\Delta F\%$) between the intensity of the green fluorescence of the sound enamel and the lesion, and the corresponding Area of the Lesion (mm^2) were assessed with the Inspektor QLF System (Inspektor™ Dental Care BV, Amsterdam, The Netherlands).

The MicroCT analysis was conducted for the MI Paste, F5000, and the MI Paste Plus groups. This chosen design permits that each untreated lesion measured at baseline acts as their own control, after the respective treatment period. Scan conditions were 70 kV with 200 ms integration time and 114 mA. Microtomographic slices were acquired at 1,000 projections at 360 degrees. The image matrix size was 1024X1024 pixels, and slice thickness of 30µm. For a detailed qualitative and quantitative 3-dimensional evaluation, the images were analyzed by the SCANCO µCT 40 software. The imaging of the specimen was done horizontally, “slicing” the laid down specimen containing ring from one pole to another.

The process of evaluation and analysis of the images was conducted once the specimens were scanned at baseline prior to treatment. To standardize the Lesion Volume analysis, we decided to use the same number of slices between two references for baseline and post-treatment assessments. These references would delimitate horizontally the top and the bottom of the lesion at baseline. Since each slice presents the same thickness, the specimen volume analyzed at baseline would be the same for post-treatment. To serve as the top reference, we established that the first baseline image presenting a defined morphology of the lesion would be set as the first lesion limit. In the same manner, the last image containing any trace of demineralization in the sound enamel would be the other lesion limit (bottom of the lesion). For this last evaluation, we used a software tool that examines the gray tones in each pixel and, based on a preset threshold limit, transforms them in black or white pixels. This way, the

visualization of the lesion is made clear. Then, the system tooth/ lesion was selected inside all individual slices between the two set lesion limits. After that, the selected images were iterated to reconstruct the piece of tooth containing the lesion, in order to be analyzed next, and the morphometric parameters – Lesion Volume and Lesion Density - were calculated, as well as the Enamel Density. Although not a variable in this present study, the density of the sound varnish-protected enamel was assessed at baseline and post-treatment periods to attest the reproducibility and consistency of the MicroCT measurements for density. In other words, if the density values obtained with the MicroCT for sound enamel were the same at baseline and post-treatment, it would imply that the test readings for that outcome measure are reliable.

After the baseline analysis, a post-treatment assessment was completed at 10th, 20th, and 30th days, on groups of specimens selected randomly removed at each of these time points. For the second round of evaluations, the same protocol previously described was used. The only difference being the definition of one of the lesion limits. The bottom of the lesion was set based on the number of analyzed images at baseline. If one given specimen had 15 analyzed images at baseline, for example, the second assessment would initiate at the first image presenting a defined morphology of the lesion, and end at the 15th subsequent slice. This approach was thought to be efficient in ensuring that the analyzed volumes, at baseline and post-treatment, would be as close to the same as possible for each specimen alone. Then, Lesion Volume and Lesion Density were calculated again.

Specimens' images acquired utilizing the Inspektor QLF System were analyzed with the software provided by the manufacturer (Inspektor™ Pro Software, version 2.0.0.32, Amsterdam, The Netherlands). The images were done before the demineralizing phase (sound tooth structure), and after the lesion was created, as baseline. Afterwards, new images were made every 5th day until the last day of treatment. The specimens randomly removed at each time point did not receive any further treatment. Mean Fluorescence Loss and Area of the Lesion were assessed after the 30 day period was completed, since all images are kept stored in a hard drive.

In order to insure that the images were captured from the same angle and in the same camera position, the video-repositioning tool of the software was used (correlation set to 0.90), with a polyvinyl siloxane matrix (3M ESPE, St. Paul, Minnesota, MN) that was previously confectioned for a pilot study. For this purpose, the software uses the baseline image as a reference when trying to correlate the subsequent images, based on their similar geometry of the fluorescence intensities [Romane et al., 2005]. The importance of this step is to permit a very precise comparison between the images captured on different moments in time [Mattousch et al., 2007; van der Veen et al., 2007]. Specimens were kept in deionized water all the time until just before analysis. For the capturing process, each lesion was dried for 5 seconds with an air syringe. Then, after waiting for focusing of the camera, the image was acquired under completely darkened laboratory conditions.

Once the images were captured, the detection of the baseline lesion was done visually. The decalcified area appears as dark areas surrounded by bright green fluorescing sound tissue [de Josselin de Jong et al., 1995]. After the lesion was identified, a patch was drawn around the lesion with its borders in sound enamel [van der Veen et al., 2007]. The software then virtually reconstructs the fluorescence levels of the lesion using the fluorescence radiance of the surrounding sound enamel as reference [van der Veen et al., 2007]. If any part of the border could not be placed in sound tooth structure, the instructions provided by the manufacturer were followed. This consists in selecting points contained in the border line in order to deactivate the part of the border that cannot be placed in sound enamel, allowing the software to discard it during the fluorescence reconstruction. The analyses patch and surface contours used in the baseline image are copied by the software for all the consequential images to ensure standardization of the analysis in each time point.

Eliminated Specimens

A total of 10 specimens (4 from MIPaste, 3 from MI+, and 3 from F5000) had to be discarded from the study after the baseline assessments were done with both methods of analysis, due to errors occurred during the image acquiring process.

Statistical Analysis

An ANOVA factorial analysis of variance (SAS 9.1) was used to assess whether the average difference between the baseline and treatment MicroCT values for Lesion Volume, Tooth Density, or Lesion Density differed depending on the group, the time of evaluation, or the interaction between the two fixed factors. Also, for the QLF assessment, a longitudinal design with unequal cluster sizes was performed to analyze the Mean Fluorescence Loss and Area of the Lesion. In order to assess the Mean Fluorescence Loss differences between the treatment groups over time, a general linear model for correlated data was used (SAS 9.1). Level of significance for both analyses was set at 0.05.

RESULTS

For the MicroCT assessment, the mean Lesion Volume values and standard deviations are presented in Table 3. In the same manner, Lesion Density and Enamel Density are presented in Tables 4 and 5, and Figure 1. The overall model was not statistically significant for Lesion Volume ($p=0.185$) or Enamel Density ($p=0.226$). The overall model was statistically significant for Lesion Density ($p=0.048$). However, neither the interaction ($p=0.088$) nor the main effects (Group: $p=0.239$; Time: $p=0.096$) were statistically significant when the influence of one was examined controlling for the other two effects.

The QLF results for Mean Fluorescence Loss are presented in Tables 6 and 7, and Figure 2. The pattern of change in fluorescence was not the same for the four groups (Tx * Time: $p<0.0001$). Examining the pattern over time in the figure displayed, one notices that the F5000 treatment group had rapid change by day 10 compared to all other groups (Control: $p<0.0001$, MI Paste: $p=0.0001$ & MI Paste Plus: $p=0.003$). However, while the Control and MI Paste, and the MI Paste and MI Paste Plus groups were statistically similar, difference was found among MI Paste Plus and Control ($p=0.033$). On day 20, the pattern was very similar to day 10. The change was between the Control and MI Paste groups. While the Control group was statistically similar to MI Paste and MI Paste Plus, these last groups were different among themselves ($p=0.036$). The F5000,

Control, and MI Paste Plus groups were not statistically significantly different at 30 days ($p = 0.66$ & $p = 0.135$).

Also, the QLF results for Area of the Lesion are presented in Tables 7 and 8, and Figure 3. The pattern of change was different for the four groups (Tx * Time $P < 0.0001$). Evaluating the pattern over time in the figure displayed, one notices that the F5000 treatment group presented rapid change at day 10 compared to all other groups (Control: $p < 0.0001$, MI Paste: $p < 0.0001$ & MI Paste Plus: $p < 0.0001$). On the other hand, the Control, MI Paste, and MI Paste Plus groups were statistically similar. The same pattern was observed on day 20. The F5000 was still different from all other groups (Control: $p < 0.0001$, MI Paste: $p < 0.0001$ & MI Paste Plus: $p < 0.0001$), while Control, MI Paste, and MI Paste Plus were statistically similar. The only difference in this pattern was found on day 30. The Control and MI Paste groups were statistically different on that time point ($p = 0.004$).

DISCUSSION

In this present in vitro study, the two different methods of analysis found divergent paths of remineralizing ability overtime for the tested products, i.e. MI Paste, MI Paste Plus, and ControlRX. Although the MicroCT did not provide strong and conclusive results, the QLF analysis has demonstrated that the fluoride group was able to achieve an overall better remineralizing outcome overtime, when compared to control and the other treatment groups. Although it is difficult to establish comparisons with previously done studies due to a lack of standardization in methods, our findings are in accordance with the study of Pulido and colleagues, and Lennon and colleagues [Lennon et al., 2006; Pulido et al., 2008].

The efficacy of fluoride as a remineralizing agent is already well described and demonstrated in the literature, as previously stated, although the discussion about the relationship between dosage and effect still persists [ten Cate et al., 2008]. Presumably, the elevated fluoride product used in this study would cause some degree of remineralization over the period of 30 days. Indeed, this was first observed on the 10th day time point. Another fact is that fluoride manifested a unique behavior overtime. In both variable related graphics, one can perceive a steep line on the first 10 days of action, denoting a rapid remineralization process, tending to a plateau after that time point. Curiously, the same pattern

can also be noted on the MI Paste and MI Paste Plus groups, but in a smaller scale, especially on the graphic related to the Area of the Lesion. All the treatment groups, sooner or later, faster or slower, appeared to stagnate at some point over the 30 days period. However, the control group has also presented a unique behavior overtime. This untreated group – exposed only to low concentrations of calcium and phosphate - indicated a rather continuous and very effective remineralizing action that was even statistically significant to the fluoride group, for Mean Fluorescence Loss, after 30 days.

The explanation for the findings described above may rely on the different mineral concentrations in which all groups were exposed to. One could expect to see a higher rate of remineralization overtime due to the continuous exposure to remineralizing agents. Obviously, this was not found to be true. It has been previously hypothesized that the limitation of remineralization could happen due to the blocking of underlying pores in the carious lesion, caused by the presence of organic substances that attach to the enamel surface [Silverstone, 1982b], or by the precipitation of calcium mineral phases within the superficial aspect of the lesion [Hicks et al., 2004]. The artificial saliva formulation used in our study indeed contained porcine mucine, which is an organic substance [Wongkhantee et al., 2006]. Although it is very unlikely that the artificial saliva containing one single organic element would have the same effects over the enamel as whole human saliva, this thought cannot be discarded. Also, the theory about the effect of the concentration of calcium ions on the remineralization process is not new in the literature. According to that, a solution with high calcium content rapidly

precipitates on the superficial zones of the lesion, preventing the remineralization process of occurring at the body of the lesion [Silverstone et al., 1988b, a]. Thus, the addition of fluoride to calcium content solutions also affects the degree and extent of remineralization [Silverstone, 1982a]. Whereas the pattern of remineralization shown for all treatment groups overtime may suggest that interpretation, some points need to be considered. In the MI Paste and MI Paste Plus groups, the presence of CPP in those products would inherently prevent rapid precipitation of calcium and phosphate. In fact, this is the primary action of CPP. Additionally, the low concentration of calcium and phosphate in the artificial saliva were probably not enough to provoke this phenomenon either. The only group which the described effect is strongly related to the gathered data is the fluoride group. It has been reported that high fluoride levels on the external surface of the lesion would increase the gradient, pulling the fluoride deeper into the lesion. However, the fluoride diffusion rates would slowly drop due to adsorption onto and reaction with hydroxyapatite crystallites in the pore walls [ten Cate et al., 2008]. Although the word “slowly” does not provide objectiveness in terms of period of time, it seems to be possible the correlation between this theory and the behavior found in the fluoride group.

Another possibility is the inability of the assessment methods used to detect and quantify small changes in remineralization. If one assumes that most of the remineralization process occurred in the first 10 days of study, the rates of mineral incorporation within the lesions for the 20th and 30th day time points

would be low. This fact itself could represent a “blind spot” for both systems, undetectable by the microCT and QLF machines.

Interestingly, both CPP-ACP based materials, MI Paste and MI Paste Plus, presented similar or even lower performances compared with the control group regarding Mean Fluorescence Loss and Area of the Lesion using QLF. For this observation several important factors may have played a role. First, since the artificial saliva formulation contained CaCl_2 and KH_2PO_4 , a certain degree of remineralization caused by exposure of the groups to this solution could have happened. This potential cofounder remineralizing effect was observed both in vitro and in vivo, causing regression of the lesions [Hicks et al., 2004; Pulido et al., 2008; van der Veen et al., 2007]. Yet, the formation of a biofilm as seen in vivo – directly influencing the ionic changes in the oral environment – may be very difficult to be done with an artificial substitute [Kautsky and Featherstone, 1993], which leads to a second consideration. The lack of biofilm and, consequently, the optimal environment for the CPP-ACP to create a state of supersaturation of calcium and phosphate ions could have prevented this system from properly exerting a remineralizing action.

In light of the issue stated above, it is logical to think that – due to the lack of a biofilm - all products tested were completely washed away by the deionized water immediately after the treatment times were completed. As a result, the treatment times may have been too short to provide any remineralizing benefits. Still, the time of exposure to treatment should not be a problem since we have strictly followed the manufacturer’s directions of use. Also, despite the acid

challenge in which the specimens were submitted after the treatments were completed, the acidic activation of CPP-ACP molecules separating ACP from casein, as previously suggested [Reynolds and Walsh, 2005], was very unlikely because of the absence of material on the tooth surface, i.e. biofilm. If this is true, the ACP would not precipitate and, consequently, promote crystal growth. Then, the remineralization would come primarily from the artificial saliva.

On the other hand, one can observe a slightly better performance overtime – for QLF outcome measures - coming from the MI Paste Plus group, when compared to the MI Paste group. It is even similar to the F5000 group on day 30, for Mean Fluorescence Loss. Considering that the main change between the Mi Paste and MI Paste Plus is the addition of 900 ppm of Fluoride, this subtle variance could be attributed to this modification.

It is also imperative to identify possible deficiencies of each assessment method utilized, in order to better understand the study results. The use of microCT to analyze *in-vitro* created caries lesions three dimensionally is a relatively new methodological approach. To date, only a few studies have used micro-computed tomography to evaluate enamel carious lesions [Clementino-Luedemann et al., 2006; Dowker et al., 2004; Huang et al., 2007; Lee et al., 2008; McErlain et al., 2004]. Notwithstanding, it has been found to be a useful tool in caries research and quantitative remineralization assessments [Huang et al., 2007]. The utility of microCT in caries research becomes more apparent when we think of enamel subsurface lesions as volumetric mineral changes (Figure 4). With this understanding and the increasing resolution of microCT for

quantitative volumetric analysis, it is evident that two dimensional planar analysis of enamel subsurface lesions, as microradiography and polarized light microscopy, will diminish overtime. Even so, it is evident in the literature that microCT based technology, from acquiring images to software design, is still in the process of improvement and calibration as well as validation in caries research [Huang et al., 2007; Zou et al., 2009].

The explanation for the weak statistical significance ($P=.048$) found in lesion density could result from the MicroCT threshold established by us (658), as a reference for the software to classify pixels on the image, could be too high. Each pixel of the electronic representation of the specimen is characterized by a tone inside the gray color scale (Figure 5). The threshold number delimits a line in this color scale in which a certain gray tone of each pixel is considered black or white, transforming the pixels with different gray tones in one of those two colors. For this experiment, we determined the threshold analyzing a histogram of one single specimen. The histogram is a graphic representation of the tonal distribution of gray pixels. If the image has two areas of different gray tones, corresponding to a structure of higher density (sound enamel) and another of lower density (carious lesion), one could select the threshold value looking for a number on the trough, between the two crests. Based on these concepts, the actual change in lesion density, whether overtime or product related, could have been camouflaged. The increase in mineralization may simply have not been sufficient to overpass the threshold. If we conceive that - in general - teeth present variability in enamel densities, from person to person, calculate a mean

of threshold numbers between all specimens may have helped to validate the choice of a threshold number and prevent this possible flaw. On the other hand, the Enamel Density was analyzed at baseline and after each time point. Since the readings were found to be very similar when compared to baseline, this fact tells us that the analysis of density by the microCT is consistent and reproducible.

The sequelae of the problem described above will directly involve the calculation of the Lesion Volume (Figure 6). Since the density of an object is defined as mass per unit volume, one would expect to see a parallelism of density and volume observations. In fact, despite the statistical significance found, we could fearlessly state that the overall lack of change in Lesion Density leads to maintenance of the Lesion Volume overtime and for each tested product.

Another major problem regarding volume calculation was the inherent presence of lesion like pixels on the borders of the tooth structure image. These pixels present gray tones around the same range found on those representing the demineralized tissue. This phenomenon happens due the gradual passage from one region of different mineral density to another, i.e. different gray tones, and from the region of interest to the radiolucent surroundings. Even though the software will automatically contour the tooth structure based on identification of pixels in the same tone range, this tool still presents flaws of accuracy. Thus, the direct implication on software interpretation cannot be neglected. These pixels are counted by the program as being part of the less dense tissue and, therefore,

a false augmentation in the Lesion Volume is observed. If a finer resolution is used during the acquirement of the images, one can expect increased precision on volume measurements. However, this would take more scanning time and, consequently, markedly increase the cost of the analysis.

The other assessment method used in this study is the Inspektor QLF System (Inspektor™ Dental Care BV, Amsterdam, The Netherlands). The acronym stands for quantitative light-induced fluorescence, and the basic idea of this diagnostic device is to use fluorescence laser to detect carious lesions. In fact, factors such as mineral loss, caries lesion depth, and area of the lesion can be quantified by this machine. Its *modus operandi* involves illumination of the tooth structure by a broad beam of a blue–green light from an argon ion laser [Bjerkhagen et al., 1982]. Then, an intra-oral video camera and a frame grabber capture the image of the illuminated tooth, which is stored and analyzed by the custom-made Inspektor™ Software (Inspektor™ Pro Software, version 2.0.0.32, Amsterdam, The Netherlands) [Whelton, 2004]. The demineralized carious lesion (white spot) appears as dark spots due to the higher scattering of the fluorescent light, when compared to sound enamel [Angmar-Mansson and ten Bosch, 1987; de Josselin de Jong et al., 1995; Wenzel, 2004] (Figure 7 and 8).

In spite of the fact that the QLF machine was suggested to be an useful diagnostic device either *in vitro* or *in vivo* [Al-Khateeb et al., 1998; de Josselin de Jong et al., 1995; Heinrich-Weltzien et al., 2005; Pretty et al., 2004], the method is subjected to confounding factors such as extrinsic stain, presence of plaque, underlying dentine thickness, focal distance, ambient light, and extent of lesion

hydration [Al-Khateeb et al., 1998; Amaechi and Higham, 2002; Angmar-Mansson and ten Bosch, 1987]. Although none of the teeth used for this study was cleaned besides the ground area beforehand, we have tried to minimize the influence of the other cofounders. For this purpose, all images were captured in a completely dark room and with the help of a custom-made putty device, as described above. Since all pieces of enamel were embedded in phenolic rings with equal dimensions, the device fit perfectly all mounted specimens. Its use, combined with the software positioning tool, assured that all images were made from the exactly same position. Another adopted measure was the standardization of drying time for 5 seconds. The influence of dehydration on enamel fluorescence had been previously reported in the literature [Al-Khateeb et al., 2002; Pretty et al., 2004; van der Veen and de Josselin de Jong, 2000b]. The major concern regards the difference in the refraction index of air ($n=1$) and water ($n=1.33$) [Al-Khateeb et al., 2002]. This fact can lead to inconsistent fluorescence readings since air is more scattering than water [Al-Khateeb et al., 2002]. Less fluorescence is reflected back to the sensor and, consequently, a darker representation of the same lesion is observed, resulting in an aggravated assessment. In light of this, we have chosen to standardize the drying procedure to a period of time previously utilized [Kuhnisch et al., 2006], which also seems to be reasonable in reproducing a clinical diagnosis scenario. Porosity of lesions could also interfere in the drying process [Al-Khateeb et al., 2002], but this factor was certainly minimized since all specimens were submitted to the same demineralizing procedures.

CONCLUSION

In light of the findings presented in this study and within its limitations, it is possible to conclude that ControlRX demonstrated better remineralization ability than MI Paste and MI Paste Plus. Moreover, the period of 10 days may be enough to remineralize white spot lesions when a toothpaste containing 5000 ppm of fluoride is used as therapeutic agent.

The presence of calcium and phosphate ions in low concentrations may also represent an effective approach to treat white spot lesion overtime, which means that whole human saliva – in favorable oral conditions - may also act as a slow but powerful remineralizing agent, since these two ions are known to be part of it.

Other factors such as lack of biofilm and period of exposure to the CPP-ACP therapeutic agents may exert a negative influence over the remineralizing ability of these products, when tested in *in vitro* conditions.

In regards to the assessment methods used in this study, we extend the potential utility of MicroCT as a new, promising, and helpful tool to be used in white spot lesions studies done in vitro. Nevertheless, the development of a proper protocol to standardize the scanning process and software analysis of the specimens is axiomatic and, hopefully, imminent. On the other hand, the QLF has demonstrated to be a useful method to assess white spot lesions in vitro. Although

relatively easy to operate, it requires a high level of attention in order to minimize possible cofounders, especially the presence of water/ saliva in the lesion, which could unfavorably modify the image capturing process and, therefore, any conclusions drawn from its analysis.

The need for unbiased, well designed *in vivo* studies to assess the remineralization ability of products containing CPP-ACP is apparent, due to the inherent limitations of *in vitro* studies. However, it is also clear that the results of *in vitro* tests cannot be ignored.

Table 1. Products tested and the respective composition

Product	Composition
MI Paste	Recaldent™ (CPP-ACP) and vehicle
ControlRX	Sodium Fluoride 1.1% w/w, water, Sorbitol, Hydrated Silica, Glycerin, MICRODENT® 2.0% w/v – a patented ULTRAMULSION® of Dimethicone and Poloxamer 407, PEG 12, Flavor, Cellulose Gum, Sodium Lauryl Sulfate, Titanium Dioxide, Sodium Saccharin
MI Paste Plus	Recaldent™ (CPP-ACP), 0.2% w/w (900ppm) of Fluoride, and vehicle.

Table 2. Products tested and the correspondent application procedures

Product	Directions for use
Control	No treatment applied.
MI Paste	Applied twice every 24 hours, in a form of slurry, made with 1 part of toothpaste to 3 parts of artificial saliva. Product was applied for 3 minutes and rinsed with deionized water for 5 seconds. No application of fluoride was done prior to this treatment.
ControlRX	Applied once every 24 hours, in a form of slurry, made with 1 part of toothpaste to 3 parts of artificial saliva. Product was applied for 2 minutes and rinsed with deionized water for 5 seconds.
MI Paste Plus	Applied twice every 24 hours, in a form of slurry, made with 1 part of toothpaste to 3 parts of artificial saliva. Product was applied for 3 minutes and rinsed with deionized water for 5 seconds.

Table 3. Differences in the mean Lesion Volume overtime for each product tested, measured with MicroCT, in cubic millimeters (mm³)*

		n	Baseline	Treatment	Difference
MI	10day	12	3.0 (±0.8)	3.0 (±0.8)	0.0 (±0.4)
	20day	10	2.9 (±0.5)	2.7 (±0.6)	0.2 (±0.4)
	30day	8	2.8 (±0.8)	2.2 (±0.5)	0.3 (±0.2)
F5000	10day	12	2.8 (±0.6)	2.8 (±0.6)	0.0 (±0.2)
	20day	11	3.5 (±0.7)	3.3 (±0.6)	0.2 (±0.4)
	30day	8	2.7 (±0.7)	2.6 (±0.6)	0.1 (±0.2)
MI +	10day	10	3.2 (±0.8)	3.2 (±1.0)	-0.1 (±0.3)
	20day	11	2.9 (±0.5)	2.8 (±0.5)	0.1 (±0.2)
	30day	10	2.8 (±0.8)	2.7 (±0.7)	0.1 (±0.2)

*The overall model was not statistically significant for Lesion Volume (p=0.185).

Table 4. Differences in the mean Lesion Density overtime for each product tested, measured with MicroCT, in milligrams of hydroxyapatite per cubic centimeter (mg HA/ cm³)*

		n	Baseline	Treatment	Difference
MI	10day	12	1398.9 (±57.2)	1432.5 (±61.2)	-33.6 (±66.7)
	20day	10	1422.7 (±59.3)	1453.4 (±56.2)	-30.6 (±40.0)
	30day	8	1417.9 (±56.4)	1465.4 (±60.4)	-47.5 (±39.7)
F5000	10day	12	1392.9 (±32.8)	1472.9 (±39.9)	-80.0 (±25.7)
	20day	11	1409.6 (±57.5)	1425.5 (±66.6)	-15.9 (±37.3)
	30day	8	1440.0 (±77.1)	1500.2 (±72.5)	-60.1 (±60.6)
MI +	10day	10	1409.6 (±47.6)	1442.9 (±35.5)	-33.4 (±33.4)
	20day	11	1398.8 (±57.7)	1433.8 (±71.5)	-35.0 (±31.8)
	30day	10	1418.1 (±65.0)	1451.8 (±59.6)	-33.7 (±43.5)

* The overall model was statistically significant for Lesion Density (p=0.048), but similar for the interaction (p=0.088) or controlled main effects (Group:p=0.239; Time: p=0.096).

Table 5. Differences in the mean Enamel Density overtime for each product tested, measured with MicroCT, in milligrams of hydroxyapatite per cubic centimeter (mg HA/ cm³) *

		n	Baseline	Treatment	Difference
MI	10day	12	1739.7 (±94.8)	1768.8 (±81.4)	-29.0 (±83.8)
	20day	10	1762.0 (±64.6)	1785.6 (±102.5)	-23.6 (±74.8)
	30day	8	1658.7 (±104.1)	1717.8 (±98.1)	-59.0 (±21.5)
F5000	10day	12	1719.1 (±91.4)	1785.5 (±79.9)	-66.4 (±30.2)
	20day	11	1769.9 (±64.5)	1780.1 (±87.8)	-10.2 (±36.3)
	30day	8	1798.3 (±103.0)	1837.1 (±100.7)	-38.9 (±27.0)
MI +	10day	10	1732.7 (±49.4)	1778.9 (±64.5)	-46.2 (±58.5)
	20day	11	1757.4 (±120.1)	1776.5 (±127.7)	-19.1 (±47.8)
	30day	10	1749.2 (±79.4)	1773.2 (±92.2)	-24.0 (±44.6)

Table 6. Test of fixed effects for Mean Fluorescence Loss

Test	Degrees of Freedom	F-Value	P-Value
Treatment	3, 135	3.32	0.0217
Time	3, 135	15.50	< 0.0001
Treatment * Time	9, 135	6.33	< 0.0001

Table 7. Contrasts between treatments at different time points for Mean Fluorescence Loss (ANOVA)

Test	Degrees of Freedom	F-Value	P-Value
Control Vs. F5000 Day 10	1, 135	26.42	< 0.0001
Control Vs. F5000 Day 20	1, 135	13.67	0.0003
Control Vs. F5000 Day 30	1, 135	0.19	0.6615
Control Vs. MI + Day 10	1, 135	4.62	0.0334
Control Vs. MI + Day 20	1, 135	1.28	0.2603
Control Vs. MI + Day 30	1, 135	1.14	0.2881
Control Vs. MI Paste Day 10	1, 135	1.23	0.2690
Control Vs. MI Paste Day 20	1, 135	1.03	0.3125
Control Vs. MI Paste Day 30	1, 135	9.37	0.0027
F5000 Vs. MI + Day 10	1, 135	8.95	0.0033
F5000 Vs. MI + Day 20	1, 135	6.42	0.0124
F5000 Vs. MI + Day 30	1, 135	2.26	0.1350
F5000 Vs. MI Paste Day 10	1, 135	15.94	0.0001
F5000 Vs. MI Paste Day 20	1, 135	21.59	< 0.0001
F5000 Vs. MI Paste Day 30	1, 135	12.22	0.0006
MI + Vs. MI Paste Day 10	1, 135	1.05	0.3080
MI + Vs. MI Paste Day 20	1, 135	4.48	0.0362
MI + Vs. MI Paste Day 30	1, 135	3.98	0.0480

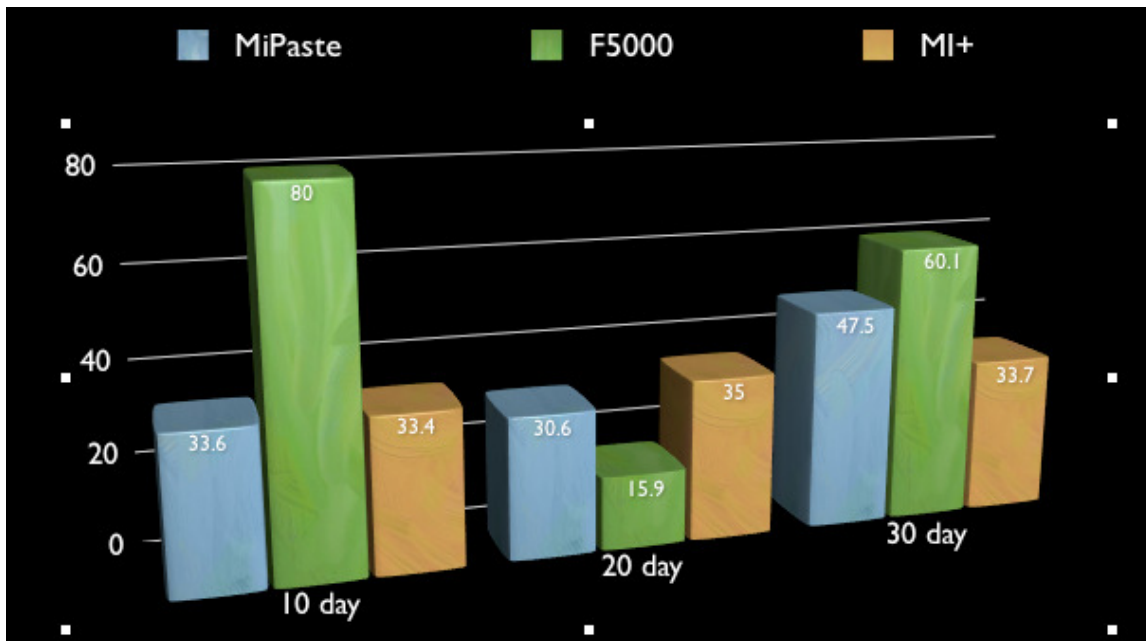
Table 8. Test of fixed effects for mean Area of the Lesion

Test	Degrees of Freedom	F-Value	P-Value
Treatment	3, 135	8.55	< 0.0001
Time	3, 135	252.34	< 0.0001
Treatment * Time	9, 135	9.74	< 0.0001

Table 9. Contrasts between treatments at different time points for mean Area of the Lesion (ANOVA)

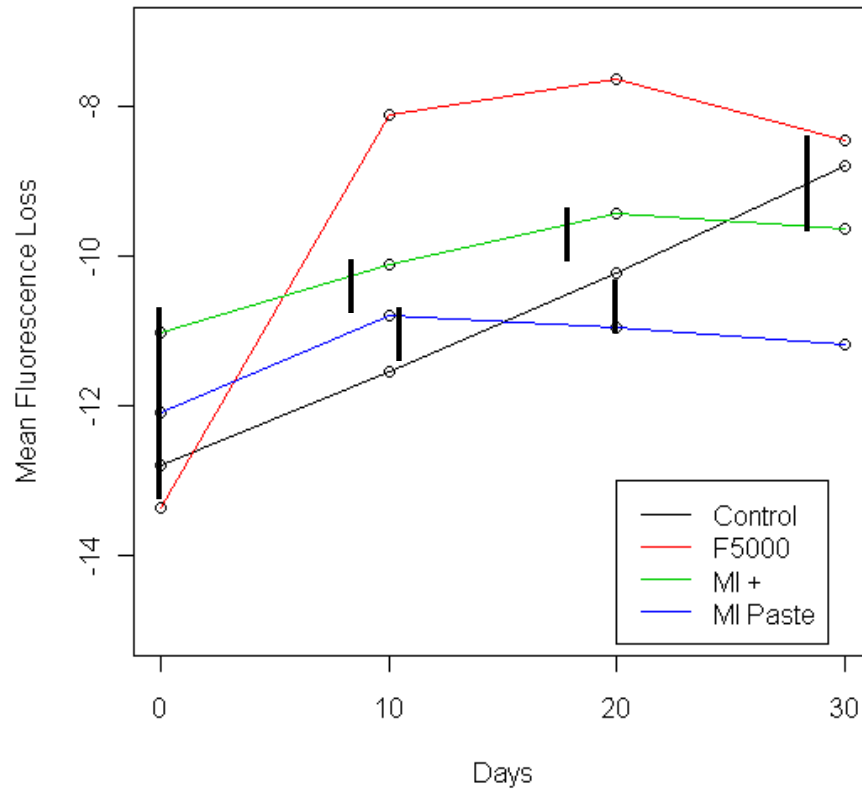
Test	Degrees of Freedom	F-Value	P-Value
Control Vs. F5000 Day 10	1, 135	45.26	< 0.0001
Control Vs. F5000 Day 20	1, 135	35.93	< 0.0001
Control Vs. F5000 Day 30	1, 135	5.38	0.0218
Control Vs. MI + Day 10	1, 135	3.67	0.0576
Control Vs. MI + Day 20	1, 135	3.75	0.0549
Control Vs. MI + Day 30	1, 135	1.43	0.2346
Control Vs. MI Paste Day 10	1, 135	2.44	0.1209
Control Vs. MI Paste Day 20	1, 135	0.00	0.9488
Control Vs. MI Paste Day 30	1, 135	8.46	0.0042
F5000 Vs. MI + Day 10	1, 135	23.16	< 0.0001
F5000 Vs. MI + Day 20	1, 135	16.16	< 0.0001
F5000 Vs. MI + Day 30	1, 135	12.27	0.0006
F5000 Vs. MI Paste Day 10	1, 135	26.19	< 0.0001
F5000 Vs. MI Paste Day 20	1, 135	35.52	< 0.0001
F5000 Vs. MI Paste Day 30	1, 135	26.80	< 0.0001
MI + Vs. MI Paste Day 10	1, 135	0.12	0.7341
MI + Vs. MI Paste Day 20	1, 135	3.88	0.0510
MI + Vs. MI Paste Day 30	1, 135	2.99	0.0862

Figure 1. Distribution of mean Lesion Density differences overtime for each product, in milligrams of hydroxyapatite per cubic centimeter (mg HA/ cm³)*



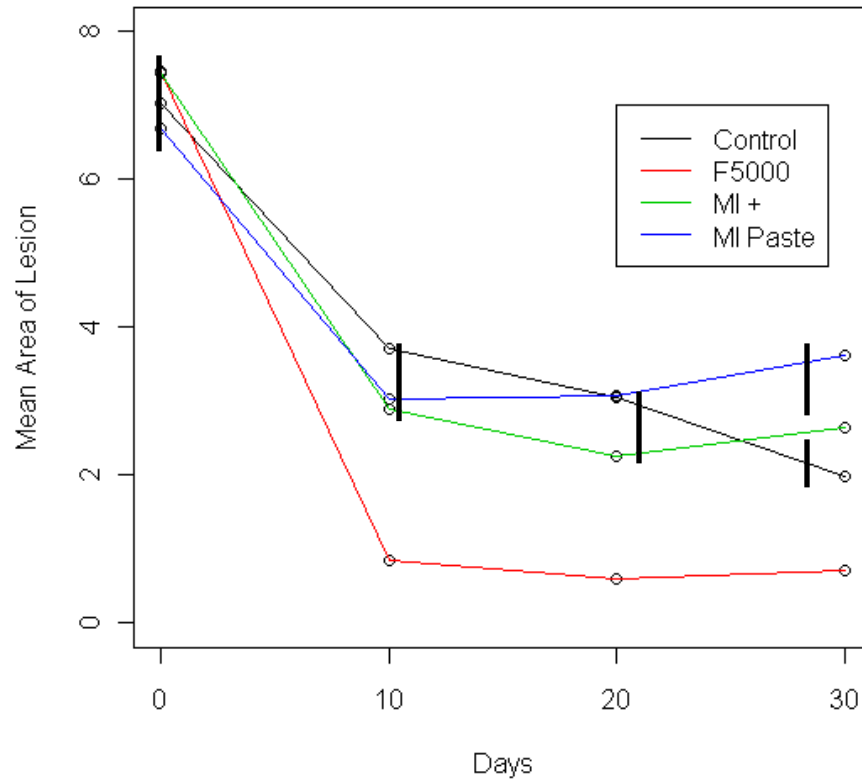
* The overall model was statistically significant for Lesion Density ($p=0.048$), but similar for the interaction ($p=0.088$) or controlled main effects (Group: $p=0.239$; Time: $p=0.096$).

Figure 2. Mean Fluorescence Loss by treatment group and time as estimated from the general linear model including treatment, time, and the treatment by group interactions (model predicted curves)*



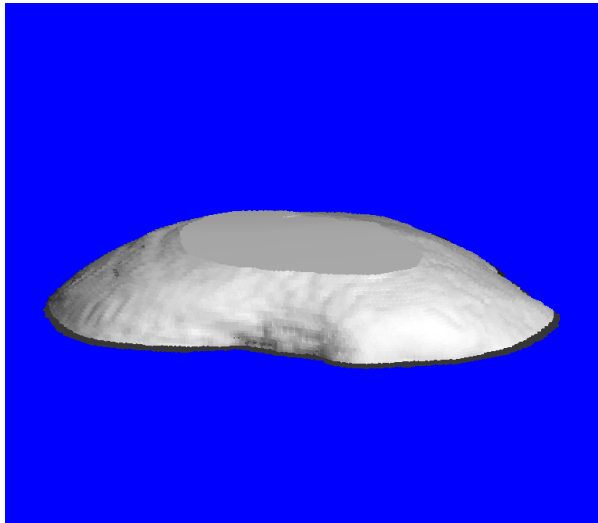
* Groups connected by the black bars in different time points were statistically similar.

Figure 3. Mean Area of the Lesion by treatment group and time as estimated from the general linear model including treatment, time, and the treatment by group interactions (model predicted curves)*



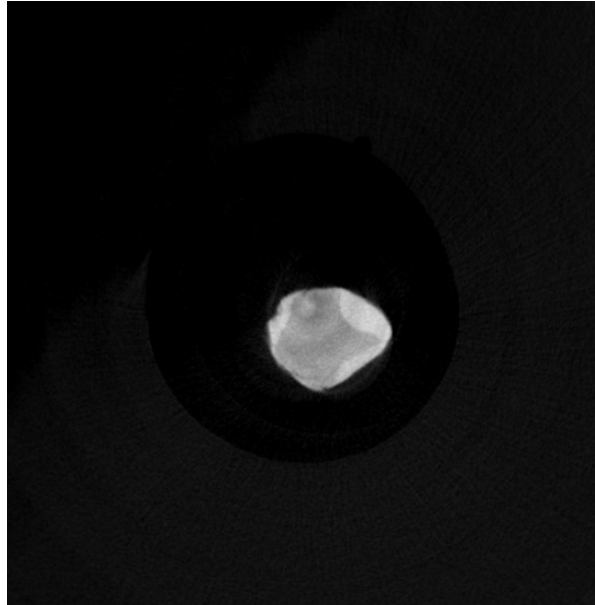
* Groups connected by the black bars in different time points were statistically similar.

Figure 4. MicroCT obtained 3-dimensional representation of a specimen*



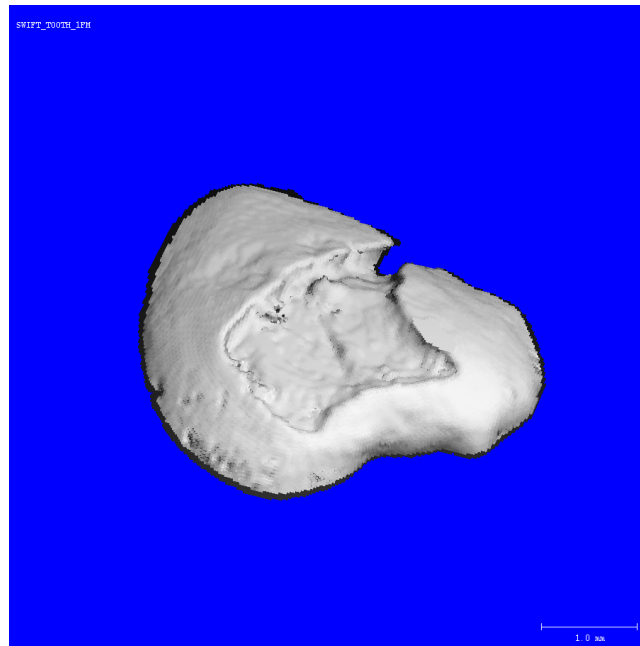
* Note the ground sound enamel where the white spot lesion was created.

Figure 5. MicroCT obtained “slice” image of a specimen*



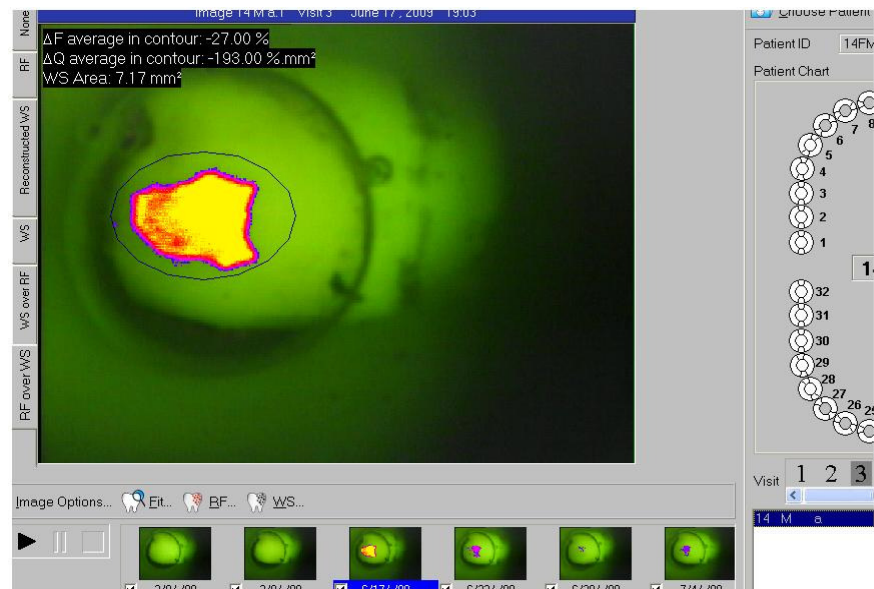
* Note the brighter segment indicating an area of higher density, and the darker indicating an area of lower density, corresponding to the white spot lesion.

Figure 6. MicroCT obtained 3-dimensional representation of a specimen*



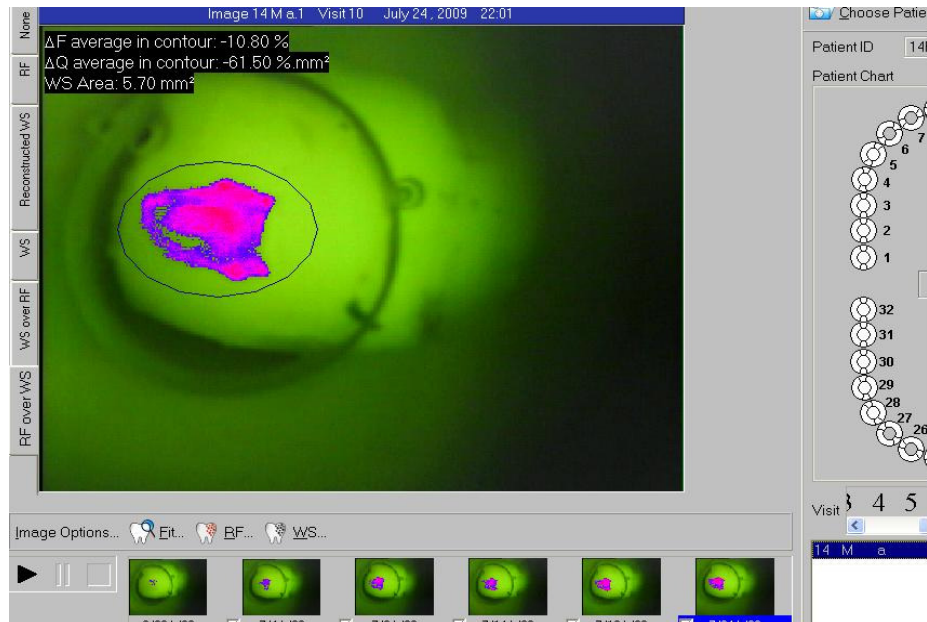
* Note that image represents the higher density segment (sound enamel). The lower density segment (white spot lesion) was removed creating an appearance of crater, which corresponds to the volume of the lesion

Figure 7. QLF screen image representing the analysis process of the circumscribed white spot lesion at baseline*



* Note the yellow color on the body of the lesion denoting area of higher scattering of light and, consequently, more demineralized.

Figure 8. QLF screen image representing the analysis process of the circumscribed white spot lesion after treatment was applied overtime*



* Note the purple tone in the body of the lesion indicating less scattering of light and, consequently, a more remineralized tissue. Also, the Area of the Lesion is reduced, especially on the borders of the lesion.

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