PREVENTION OF WHITE SPOT LESIONS FORMATION WITH FLUORIDE VARNISH-IN-VITRO ASSESSMENTS

A Dissertation

by

MOUFIDA ABU FARWA

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Chair of Committee,	Peter H. Buschang
Committee Members,	Amal Noureldin
	Phillip M. Campbell
	Veun G. Varanasi
Head of Department,	Larry Bellinger

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ABSTRACT

The objectives of this study were first, to compare the effects of CPP-ACP fluoride varnish and CO_2 laser in preventing enamel demineralization, then to determine for how long the varnish prevents enamel demineralization, and finally to determine how often should it be reapplied to maintain the preventive effect. Human molars and premolars were halved. First experiment, teeth sections were randomly assigned to fluoride, laser, or control group. After pH-cycling enamel demineralization and mechanical properties were evaluated using the FluoreCam and CSMH, respectively. For the second experiment, teeth sections were randomly assigned to either fluoride or control groups, varnish was applied to one group, the specimens were placed in a tooth-brushing simulator, thermocycled, and subjected to pH cycling. Using the same procedures the third experiment compared one fluoride application, to multiple applications, either every 4 or 6-weeks. In the first experiment, the FluoreCam results significantly greater effects in the fluoride group than in the control and laser groups. Enamel microhardness was significantly greater in the fluoride than laser group, which was in turn harder than the control group. The second experiment showed significant and progressive demineralization in the control group at 2, 4, 8, and 12 weeks ($p<0.001$). The experimental group revealed no demineralization during the first 4 weeks and only limited demineralization after 12 weeks. PLM revealed typical WSLs in the control and more limited demineralization in the treated group. The third experiment showed significantly larger areas of demineralization in the control than the 6-week and 4-week

groups. There were no statistically significant differences between 4- and 6-week groups. In conclusion, CPP-ACP fluoride varnish is more effective in preventing enamel demineralization than CO_2 laser. It prevents demineralization completely for at least 4 weeks. Because the preventive effect starts to decline after 6 weeks, the varnish should be reapplied at least every 4 weeks.

DEDICATION

To my parents, husband, children, siblings and to the soul of my beloved mother in law, may her soul rest in peace, for their enduring love, prayers, and support.

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TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Dental Caries

Dental caries is a complex, multifactorial disease caused by the interaction of diet, dental plaque (cariogenic bacteria) and host factors such as saliva, tooth surface and acquired pellicle, acellular bacteria-free organic film deposited on the tooth surface.[1] Dental caries is a dynamic process involving cycles of demineralization and remineralization, especially for clinically undetected early lesions known as "incipient lesions". Demineralization is the dissolution of tooth minerals (calcium and phosphate) by organic acids. Following exposure to fermentable carbohydrates, plaque bacteria produce these organic acids, which diffuse into the acquired pellicle and through the pores of the enamel surface. When the pH reaches a critical level of approximately 5.5, dissolution of the enamel surface is initiated.[1,2] Some of the dissolved mineral ions (calcium and phosphate) precipitate into the outer surface layer of the enamel, whereas less soluble phases such as fluoridated hydroxyapatite precipitate on the enamel surface. At the same time, a portion of these minerals diffuse away from the enamel surface to the oral environment. Thus, the minerals leach out from the deeper enamel region to the surface layer and from the surface layer out to the surrounding environment. [1] This action results in the formation of a lesion composed of a highly mineralized, relatively intact surface layer with a considerably demineralized subsurface region. [1] If the equilibrium between the enamel and the oral environment is achieved, the

demineralization process can be arrested. However, if the process continues, the lesion will progress and ultimately result in a breakdown of the weakened enamel. [1,3]

Remineralization is the reverse process that acts as a natural repair response to demineralization. When the plaque pH rises above the critical acidic level, remineralization begins. With the help of fluoride, the salivary calcium and phosphate diffuse into the enamel to remineralize the crystalline structures in areas where demineralization took place. The newly formed crystals consisting of fluoroapatite and florohydroxyapaptite are more acid-resistant than the original structure.[1] Remineralization and demineralization normally alternate several times throughout the day and if balanced, no enamel carious lesion will result. However, after prolonged exposure to acidic conditions, the dynamic equilibrium tips toward demineralization, allowing the lesion to progress and eventually become a dental caries.[2]

Clinical Characteristics of White Spot Lesions

White spot lesions are the first clinical sign of dental caries. Sound enamel is a porous solid in which water in the inter-crystalline spaces serves as a channel for diffusion. The effective pore size of the sound enamel ranges from 1.4 to 2.4 nm^2 and represents 0.1% to 0.8% by volume.[1] Following an acid attack, enamel crystal dissolution begins with subsurface demineralization, creating pores between the enamel rods. As the porosity of the enamel increases, the surface roughness of the enamel increases and the loss of normal translucency of the enamel begins. The change in the translucency is explained by the change in the enamel reflective index, as porous enamel

scatters more light than sound enamel. This optical phenomenon gives the defective enamel a clinically opaque white appearance called “white spot lesions”. [4]

Although it is commonly referred to as “incipient lesion” by many clinicians, it is actually deeper and at a later stage than an incipient lesion. The lesion must be at least 300 μm to be clinically detected.[1] An early white spot lesion cannot be seen macroscopically when wet, but when dried, it appears chalky.[5] This phenomenon is related to increased enamel porosity in the enamel surface. In addition, white spot carious lesions can be either active or arrested. [5] Arrested white lesions have smooth surfaces and appear glossy white, whereas active lesions appear opaque and chalky and have a dull or matte surface. These lesions may also show a brownish discoloration with time. [5]

Histological Characteristics of a White Spot Lesion

As described by Silverstone [6-8], white spot lesion soaked in different media under polarized light microscopy has been classified into four distinct zones. Starting from the surface, the four zones are the surface zone, body of the lesion, dark zone and translucent zone.[7-9] The surface zone and body of the lesion are apparent when the section is immersed in water. However, the dark and translucent zone may be seen when the water is substituted with quinoline.[6] The surface zone has been shown to be an intact, relatively unaffected area of enamel overlaying the subsurface body of the lesion. The surface zone has a pore volume of less than 5%, whereas the body of the lesion has a pore volume from about 5% at the periphery to approximately 25% in the center of the lesion. The dark zone occurring in approximately 95% of the carious lesions examined

has a pore space of 2% to 4%. The translucent zone is the deepest zone and the advancing front of the lesion. It is seen in only 50% of carious lesions and shows minimal demineralization. The pore volume of the translucent zone is 1%, which is close to that of normal enamel (0.1-.8%).[7,8] It has been suggested that the surface zone remains intact and well mineralized because it is a remineralization site. Matter released by subsurface dissolution or from the saturated solution in plaque precipitates into the enamel surface. Although this relatively unaffected layer protects the underlying lesion from cavitation, it also impedes the progress of the lesion remineralization *in vivo* and *in vitro*.[9]

White Spot Lesions in Orthodontic Patients

In orthodontics, white spot lesions remain a serious problem. Patients undergoing orthodontic treatment are more susceptible to the formation of these lesions, which are most commonly seen on the labial-gingival surface of the anterior teeth.[10] Maxillary anterior teeth are affected more than mandibular teeth.[11] The maxillary lateral incisors and maxillary and mandibular canines are the most susceptible teeth, whereas, the maxillary posterior teeth are the least common site.[10,11] Although, there is no significant difference in white spot lesions between genders, males tend to be affected more compared to females.[10,11] It has been reported that white spot lesions can be seen in orthodontically treated patients as early as one month, which is less than the usual time period between consecutive orthodontic visits (4 to 6 weeks). The presence of lesions is explained by the increased difficulty of maintaining oral hygiene due to the fixed appliances plus the increase in plaque retention. Furthermore, the irregular surfaces of

brackets, bands, wires, and other attachments reduce the self-clearance provided by the salivary flow and tongue, lip and cheek friction.[12] Furthermore, studies have shown that the levels of S. mutans, the main cause of dental caries, can increase up to fivefold during orthodontic treatment.[4] Therefore, some extra preventative measures in these highly susceptible patients are necessary.

White spot lesions in orthodontic patients seem to behave differently from traditional caries formation.[13] The former have a superficial and more rapid characteristic, becoming visible within four weeks after the placement of fixed appliances. However, the formation of a 'normal' carious lesion is usually a slower process that can take at least six months to be evident.[14] The overall prevalence of white spot lesions in orthodontics varies considerably.[15,16] In 1971 Zachrisson et al. reported that 89% of patients developed white spot lesions, while Strateman and colleagues reported 58% prevalence in 1974.[17,18] Both Gorelick et al. (1982) and Mitchell et al. (1992) reported that the prevalence varied between 2 and 96%.[15,16] In 2005, Boersma et al. observed an even higher prevalence of white spot lesions, with 97% of their subjects displaying lesions after treatment.[19] More recently, Julien et al. reported that nearly 25% of their patients developed WSLs while in treatment. They also, reported that preexisting white spot lesions, declining oral hygiene during treatment, poor pretreatment hygiene, and prolonged treatment time (more than 36 months), are all risk factors and predictors of white spot lesions formation in anterior teeth.[11] The inconsistency in prevalence reports may be attributed to geographical location, unstandardized clinical examinations, the variety of detection and assessment methods,

and the presence of white spots before the start of orthodontic treatment as well as variations in the definition of a white spot lesion.[20] Remineralization of white spot lesions may occur, resulting in enamel with either normal or at least a visually acceptable appearance. However, in many cases, white spot lesions remain as permanent enamel scars, leading to unfavorable aesthetic results.[4] This issue is frustrating for both the orthodontist and the patient, as most of these patients seek orthodontic treatment to improve their esthetics.[21]

Prevention of White Spot Lesions

The risk of developing white spot lesions during fixed orthodontic treatment can be reduced by diet counseling and patient oral hygiene education, enhancing enamel resistance using fluorides, and other additional methods using different mechanisms (Figure 1).

Diet counseling and patient oral hygiene education

The role of frequent consumption of fermentable carbohydrates in increasing caries risk has been well documented. Diet counseling can be beneficial to educate the patient about the need to reduce exposure to refined carbohydrates, especially sugared beverages including soft drinks, sports drinks, and juices.[22] These beverages not only serve as a source of fermentable sugars for cariogenic bacteria, but also have low "acidic " pH levels.[23] Brushing teeth twice a day is recommended by many clinicians and considered an important method for controlling plaque and attaining good oral hygiene.[4] Many manual and electric toothbrushes are available but there are conflicting reports about them. Their effectiveness among orthodontic populations seems to be equivalent.

However, it has been reported that patients with poor oral hygiene may achieve better results with use of an electric toothbrushes because plaque may be removed more easily with the use of active heads.[23] In addition to tooth brushing, dental flossing can be a helpful tool in removing interproximal plaque. Floss can also be used to clean areas under the main arch wire that are difficult to clean with a toothbrush.[10,24] Periodic reinforcement by the clinician of the importance of flossing may increase patient motivation; however, permanently changing patient behavior is reported to be a difficult task.[4]

Enhancing enamel resistance using fluorides

The carious lesion starts as a slight dissolution of the enamel surface. Consequently, any factor that decreases enamel solubility would be considered a cariostatic agent. Fluoride is one of the most well-known cariostatic agents and has been studied extensively.[3] To enhance enamel resistance to acid attacks and to avoid the development of white spot lesions, topical fluoride can be administered to the patient by means of home-applied topical fluorides, including topical (fluoridated toothpaste, mouth rinse, gel) and/or professionally applied fluoride products (Foam, gel and varnish) to help reduce white spot lesions formation.[23]

Mechanism of action of fluoride

Fluoride has several mechanisms of action to assist in the prevention of white spot lesions formation. Fluoride acts to enhance remineralization, inhibit bacterial metabolism, and inhibit bacterial colonization. The main mechanism of action of fluoride in enhancing enamel resistance to an acid challenge is through remineralization of the

demineralized enamel. After an acid attack, the fluoride ions react with the calcium and phosphate of the hydroxyapatite crystals, the structure forming the enamel. This reaction leads to the formation of fluorapatite crystals. For every two fluoride ions, ten calcium ions and six phosphate ions are required to form one fluorapatite unit [$Ca_{10}(PO_4)_6F_2$].[25]

Fluorapatite has a lower critical pH than hydroxyapatite; therefore, it provides less soluble and more acid-resistant enamel.[26] Moreover, fluoride within the plaque interferes with the bacterial metabolism of carbohydrates. It is effective at inhibiting enolase enzyme, a fermentation essential enzyme. Enolase is sensitive even to low levels of fluoride, thus leading to cariogenic bacteria with decreased-acid production.[27] A study by Takahashi showed that Fluoride (225 and 900 ppm F) inhibited lactate production from 10% glucose by 34% and 46%, respectively.[28] Additionally, fluoride can inhibit bacterial colonization of the tooth surface via competitive binding. Due to its electronegative properties, fluoride ions compete with bacteria for these binding sites, and therefore, bacterial adhesion is inhibited.[27] However, the major reaction product of the topical fluoride treatment is calcium fluoride (CaF_2). CaF_2 has been shown to play a significant role in the cariostatic mechanism of fluoride. It builds up in dental plaque as deposits on the enamel surface, which are protected from rapid dissolution by a phosphate-protein coating of salivary origin. Several studies have shown that CaF_2 is insoluble in saliva at neutral pH and may remain on the tooth surface for several weeks and months after topical application of fluoride, thus acting as a fluoride reservoir. At lower pH, the protective coating is lost, and an increased dissolution rate of CaF_2 occurs with the potential for the ions to be incorporated into the demineralized crystal lattice.

The CaF_2, therefore, acts as an efficient source of free fluoride ions during the cariogenic challenge. [26,29] In conclusion, fluoride decreases demineralization and enhances remineralization, making it an effective cariostatic agent and a proper tool to increase enamel resistance.

Home-applied topical fluorides

The regular use of fluoride toothpaste is a very common recommendation by orthodontists. Typically, fluoride dentifrices contain sodium fluoride (NaF), stannous fluoride, monofluorophosphate, amine fluoride, or a combination of these compounds. As orthodontic patients are at an increased caries risk, a fluoride concentration below 0.1% in dentifrices is not recommended.[30] In 2000 Alexsander and his colleagues suggested that, orthodontically treated patients should brush twice a day with a 5000 ppm fluoride dentifrice (prescription dentifrice). This regime was reported to provide much greater prevention than the daily use of 1000 ppm fluoride toothpaste (over the counter dentifrices) accompanied by daily use of a 500 ppm NaF rinse.[31] In 2010 a modified fluoride toothpaste technique was advocated to reduce the incidence of new white spot lesions in orthodontic patients. The technique involves brushing twice a day for 2 minutes followed by vigorous swishing of the toothpaste slurry for 30 seconds without rinsing with water, and avoidance of eating or drinking for 2 hours.[23] However, for less compliant orthodontic patients, the use of a fluoridated dentifrice alone is ineffective in preventing the development of carious lesions. Thus, other fluoride sources are often suggested, which is particularly important when these patients do not follow the suggested proper oral hygiene regimen.[30]

Many studies focused on the effect of Stannous fluoride gels (0.4%) during orthodontic treatment. [10,30,32,33] Boyd 1993 compared the use of 1100 ppm fluoride toothpaste alone or together with either a daily 0.05% NaF rinse or a 0.4% stannous fluoride gel applied twice daily using a toothbrush. He found that both the gel and rinse provided additional protection against decalcification compared with toothpaste alone, but neither was superior.[10] In addition to its antimicrobial activity, stannous fluoride also has an anti-plaque effect, especially against streptococcus mutans. It is believed that the tin atoms in stannous products interfere with the adsorption of plaque bacteria to the enamel surface. Also, by blocking the passage of sucrose into bacterial cells, tin inhibits acid production. [30] In 1994 Boyd et al. showed in their eighteen-month study that the use of 0.4% stannous fluoride gel was an effective adjunct to mechanical tooth cleaning at reducing plaque and gingivitis in patients with fixed appliances. [32] However, some mild-to-moderate discoloration of the teeth in 10% of the test patients was observed compared to 0% in the control group using sodium fluoride paste, which might be a concern for long-term use. Moreover, Moura reported in 2006 that the use of a fluoridated anti-plaque dentifrice reduced enamel demineralization around brackets more effectively than a fluoridated dentifrice alone. [33] Yet, for non-compliant orthodontic individuals, the use of a fluoridated dentifrice alone is not enough to prevent the demineralization of enamel, and supplemental sources of fluoride are often suggested. [30,34]

When used daily, fluoridated mouth rinses containing 0.05% NaF have been advocated to reduce the occurrence and severity of white spot lesions formation beneath the bands.[35] Studies have shown that daily rinsing with 0.05% or 0.2% NaF and/or

weekly with 1.2% acidulated phosphate fluoride rinse was useful at reducing the incidence of orthodontics-associated white spot lesions.[10] Geiger et al. reported a 25% reduction in the number of white spot lesions using a 0.05% NaF rinse.[36] He reported that 21% of the patients who rinsed regularly developed white spot lesions and that these lesions were seen in 49% of the non-compliant patients.[36] In a systemic review, Benson recommended that the best method to decrease enamel demineralization during fixed orthodontic treatment is daily use of 0.05% NaF mouth rinse.[35] It was also found after 2 weeks' use of NaF mouth rinse (one rinse per day) that the fluoride concentration in the saliva increased significantly.[37] However, it has been reported that only 13% of 206 orthodontic patients rinsed daily as instructed, despite the effort to educate patients to use the rinses, which were free of charge. Therefore, the conclusion reached is that orthodontic patients who do not comply with proper oral hygiene will probably not use fluoride rinses on a regular basis.[36] Furthermore, in an attempt to improve the cariostatic effect, mouth rinses have been combined with antibacterial agents such as chlorhexidene, triclosan, or zinc to improve their cariostatic properties.[30] While the proper use of these home products provides the patient with increased caries protection, compliance with their use can be difficult to obtain from some patients.[36]

Professionally applied topical fluorides

The use of home topical fluoride agents requires patient compliance. As a result, different topical fluoride delivery techniques that do not require this compliance have been implemented to prevent white spot lesions formation around orthodontic brackets.[10]

These products offer the combined benefit of delivering fluoride plus requiring less dependence on patient cooperation.[30]

Professionally applied fluoride foam and gel are products with neutral NaF or acidulated phosphate fluoride (APF). The most widely used concentration of fluoride foam and gel is 1.23 % APF (1.23% fluoride ion, or 12,300 ppm). The pH is typically in the range of 3-4.[38-40] The effectiveness of topical gels has been reported in several clinical trials. Reviews on the effectiveness of APF gels have indicated average caries reductions of 22% (95% CI=18–25%), showing good evidence of effectiveness.[38,41] Fluoride foams have not been assessed extensively in clinical trials compared to gels. Their characteristics are likely similar to gels, as the application method is the same, their fluoride concentrations are comparable, and the enamel fluoride uptake is equivalent.[40,41] Regarding orthodontically treated patients, Jiang conducted a randomized clinical trial and found that in the group treated with 1.23% APF foam every two months during the orthodontic treatment, the incidence of WSLs reduced by about 76% compared to the placebo group.[42] However, the high acidity of APF, which is believed to enhance the fluoride uptake by the tooth substance, can actually affect several types of restorative materials. [43] The disadvantage of gels and foams is that they are applied in styrofoam mouth trays for 4 minutes. A considerable amount of fluoride may be ingested following fluoride application, even if suction devices are used (on average 7.7 mg in children, 10.3 mg in adults). Therefore, the most common concern about these products is over-ingestion, which can lead to nausea and vomiting. [38,41]

Fluoride varnish has several benefits over other types of professionally applied fluoride vehicles. It has a high fluoride concentration that is slowly released in levels far below those considered toxic.[41] In addition to the fluoride mechanisms mentioned previously, the application of a fluoride varnish provides a protective layer on the tooth surface, thus decreasing enamel solubility.[4] Also, a thin layer of fluoride varnish adheres to the tooth surface for a longer time compared to other topical fluorides. The prolonged contact time between the fluoride and tooth surface enhances the enamel fluoride uptake into the surface layers of enamel.[41] It has been shown that fluoride varnish is superior to the use of daily NaF toothpastes, weekly APF gel application and daily NaF rinses.[44] Moreover, fluoride varnish does not compromise dental material bond strength and works in the presence of plaque; thus, thorough cleaning of the tooth surfaces prior to its application is not necessary. Fluoride varnish is a professionally applied preventive method requiring little or no patient compliance, only the patient's presence at the dental practice.[23] Several studies have tested the efficacy of fluoride varnish application for the reduction of white spot lesions formation in orthodontically treated patients (Table 1). The latest Cochrane Collaboration (2013) reported that fluoride varnish applied every six weeks was effective. However, this was based on only one study, which showed that fluoride varnish provided nearly 70% white spot lesions reduction.[45] Therefore, it can be inferred that periodic application of fluoride varnish may provide a clinically effective solution without depending on patient compliance; nevertheless, this material cannot completely prevent white spots.

Additional methods using different mechanisms

Fluoride-containing bonding agents have the potential to decrease enamel decalcification around brackets.[10] Glass-ionomer cement (GIC) has the benefit of releasing fluoride and bonding chemically to the tooth structure, thus eliminating the need to pretreat the enamel with phosphoric acid.[4] In a longitudinal study of 60 patients, an average of 16.5% reduction was achieved compared with the use of composite resin cements.[4] However, significant concerns regarding the reduction in bond strengths of traditional fluoride releasing cements, glass-ionomer cements and resin-modified GIC compared with composite resins limited their clinical use.[4,10]

Pit and fissure sealants have been successful at preventing occlusal surfaces caries. Therefore, the concept was transferred from occlusal surfaces to the labial surfaces of teeth with brackets. This approach has been relatively successful at reducing enamel demineralization in orthodontic treated individuals.[46,47] An in vitro study by Frazier showed that coating the labial enamel surface adjacent to the bonded orthodontic brackets with a light-cured unfilled sealant led to an 80% reduction in white spot lesions.[46] Moreover, a pilot clinical study by Benham et al. showed that the use of highly filled (58%) resin sealants when applied gingival to the bonded brackets significantly reduced the incidence of white spot lesions.[47] However, in another study, it was found that dual-cured lightly filled fluoride releasing sealant did not provide added protection to the enamel compared to the control. This finding might be due to the low mechanical and chemical wear resistance of the material applied.[48]

Although this approach seams relatively successful, some concerns still exist. Surface contamination during etching or sealant placement can interfere with the proper bonding of the resin, therefore white spot lesions underneath or around the sealant may occur. [46] Regular maintenance is required to ensure the sealant integrity. Breaks in the sealant layer due to mechanical and chemical assaults may lead to microleakage and subsequent enamel demineralization. [10] Dental plaque readily attaches to the resin surface thus; sealing the labial surface shifts the resin-enamel edge to the interproximal and labio-gingival surfaces of the teeth. This means, increasing the chance of developing interproximal caries and gingivitis. [49]

Casein phosphopeptide - Amorphous Calcium Phosphate (CPP-ACP)

Milk and dairy products are usually linked with good oral health. These products have anticariogenic properties that are attributed to the presence of calcium, phosphate, and casein. [50-53] Casein is the major protein found in milk and represents about 80% of the total protein. [54,55] It exists in micelles that can stabilize calcium and phosphate ions. This stabilizing property of the casein is explained by the presence of peptide sequences that can be released from protein as small peptides (casein phosphopeptides) when the casein is partially digested. [55-57] Casein phosphopeptides (CPP) contain the cluster sequences of multiple phosphoseryl residues from casein, by which CPP can remarkably stabilize calcium and phosphate ions.[55,58] This has led to the development of casein phosphopeptide-stabilized amorphous calcium phosphate complex (CPP-ACP), also known as Recaldent^{TM} technology. This product combines casein phosphopeptides (CPP) from bovine milk with nano-particles of amorphous calcium phosphate (ACP).

The precise ratio is 144 calcium ions, 96 phosphate ions, and 6 peptides of CPP.[59] The CPP-ACP complex was patented by Melbourne University, Australia, and the Victorian Dairy Industry Authority, Abbotsford, Australia, by Eric C. Reynolds in 1991. Exclusive manufacturing and marketing rights for CPP-ACP was retained, and the trademark (Recaldent) was owned by an Australian company named "Bonlac Foods Limited" The U.S. Food and Drug Administration (FDA) has approved products marketed in the United States containing the Recaldent™ technology. Interestingly, CPP-ACP has been added and tested as an additive not only to oral care products (glass-ionomer cements, dentifrices, mouthwashes, and MI Paste, MI Paste Plus and more recently, MI varnish), but also in a variety of food products such as chocolates, sugar-free gums, sports drinks, and lozenges.[58,59]

The anticariogenic potential of CPP-ACP has been demonstrated in several *in vitro* animal and clinical studies.[56,60-64] In an *in vitro* study, Reynolds et al. demonstrated that CPP-ACP solutions maintained high-concentration gradients of calcium and phosphate ions in subsurface carious lesions in the enamel of human third molars. This elemental composition led to a greater remineralizing capacity for solutions with higher levels of CPP-ACP, with 1.0% CPP-ACP solution replacing 63.9 ± 20.1% of mineral lost.[63] Enamel remineralized by the effect of CPP-ACP was found to be more resistant to acid challenges, which is due to the high concentration of calcium and phosphate ions within the reformed hydroxyapatite.[64] A study by Ramalingam et al. demonstrated that various concentrations of CPP-ACP added to Powerade sports drink (Coca-Cola, Atlanta) showed a significant reduction in the erosive effect of the beverage. Human

enamel specimens were immersed into Powerade alone, Powerade with four concentrations of CPP-ACP (0.063%, 0.09%, 0.125%, and 0.25%), and double-deionized water as the placebo. scanning electron microscopy was used to examine the specimens. The erosion that developed in specimens immersed in Powerade was not seen in those immersed in CPP-ACP added Powerade at all concentrations except 0.063%.[62] In an animal study, Reynolds et.al applied CPP-ACP solution twice a day to molar teeth of specific-pathogen-free rats inoculated with Streptococcus sobrinus. The application of CPP-ACP significantly reduced caries activity in a dose-response fashion, with 1.0% CPP-CP producing 55% and 46% reductions in smooth surface and fissure caries activity, respectively, being similar to the effect of 500 ppm fluoride.[56]

In a two-year randomized clinical trial, 2% CPP with calcium carbonate dentifrice significantly reduced caries compared to the placebo, with a slightly better efficacy than the 1190-ppm fluoride dentifrice. Of the children using the CPP/calcium carbonate dentifrice, 72.3% remained caries-free compared with 53.2% using the fluoride paste and 31.1% using the placebo.[61] A randomized controlled clinical trial assessed the impact of CPP-ACP in sugar-free gum relative to a control sugar-free gum. In this study, 2720 school children were randomly assigned to each group and all children were instructed to chew their assigned gum for 10 min three times per day. Standardized digital radiographs were taken at baseline and at the completion of the trial. The effect was shown as 18% reduction in caries progression after 24 months and a 53% greater regression (remineralization) of baseline lesions compared with the control group. The authors concluded that the addition of CPP-ACP in a sugar-free gum significantly

slowed the progression and enhanced the regression of caries compared with the control sugar-free gum.[60]

Several studies have been conducted to examine the effect of CPP-ACP technology in patients with orthodontic appliances.[65-67] He et al. examined the effect of CPP-ACP on the progression and regression of white spot lesions during orthodontic treatment in a randomized trial. The daily application of CPP-ACP paste was compared with a positive control at every three-month fluoride varnish treatment (0.9% difluorosilane, Fluor protector) and a 'no treatment' control. Seventy-five patients were included in the study, and visual assessment of the enamel was done by means of digital photographs. The incidence of white spot lesions were 32%, 35% and 60% in CPP-ACP, fluoride, and the control group, respectively. Therefore, CPP-ACP treatment was as effective as the fluoride treatment, and significantly more effective than no treatment at reducing the incidence of enamel decalcification.[65,66] Another randomized – but single blinded – study by Uysal et al. showed similar results. This study was conducted on 21 patients undergoing orthodontic treatment for a 60-day period. The effect of 5-minute application of CPP-ACP paste was compared to a 5-minute application of 5% NaF gel by means of cross-sectional microhardness. The effect of both treatments was similar and significantly higher than that of the control ($p<0.001$).[67]

CPP-ACP Mechanism of Action

The CPP-ACP complex consists of two chemical structures that complement each other, amorphous calcium phosphate (ACP) and casein phosphopeptide (CPP). ACP possesses the advantage of having both calcium and phosphate together in an

amorphous phase. However, ACP alone favors the formation of clusters and the precipitation in the form of calculus. This problem is resolved by the CPP, which adheres to the enamel surface and has the ability to attract and bind to calcium and phosphate, making complexes in the form of amorphous calcium phosphate.[58,59] This complex is a nanocluster of ACP with four multiphosphoserylated peptides that prevent its growth to the critical size required for nucleation and precipitation, plus creates a supersaturated state of calcium and phosphate in close proximity with the tooth surface.[58,59] The release of calcium and phosphate ions by the CPP localized in the plaque is promoted by low pH. As the acidic by-products of cariogenic bacteria increase, creating an acidic environment, this would facilitate the release of calcium and phosphate ions from the complex. When the plaque pH rises, CPP can act as a sink for calcium and phosphate thus increasing the ionic content of the plaque again. Enzymes within the plaque, such as plaque peptidases and phosphatases, can degrade phosphopeptides in the CPP, thereby reducing the ability of the peptides to bind calcium and phosphate ions. From the immunolocalization time-course study of CPP in plaque, Reynolds et al. calculated the half-life of CPP in plaque and reported it to be 124.8 min. [55,68] Nevertheless, the enzymatic breakdown of the CPP in plaque has been shown to lead to the production of ammonia, thus increasing the plaque pH.[55]

Moreover, a study by Rose in 2000 showed that CPP-ACP binds well to dental plaque, providing a large calcium reservoir that may inhibit demineralization and assist in subsequent remineralization.[69] In another study, Rose showed that in streptococcal model plaques, 0.1% CPP-ACP provides a large number of possible binding sites for

calcium and reduces the free calcium diffusion coefficient by about 65% and 35% at pH 7 and 5, respectively.[70] During an acid challenge, 0.1% CPP-ACP decreased the mineral loss and provided a potential source of calcium and phosphate for subsequent remineralization.[58] Furthermore, Schüpbach et al. showed that the incorporation of CPP into enamel pellicle inhibits adherence of mutans streptococci to enamel in vitro and in animal studies. These authors suggested that CPP-ACP incorporates into enamel pellicle and dental plaque. This activity may lead to an ecological disturbance in the bacterial environment, which, together with the remineralizing capacity of the CPP-ACP, modifies the plaque's cariogenic potential.[55,71] To summarize, the proposed anticariogenic mechanism of CPP-ACP is by localizing amorphous calcium phosphate within dental plaque at the tooth surface, buffering the acidogenic pH and maintaining a state of supersaturation of calcium and phosphate ions on the enamel surface.

Casein phosphopeptide - Amorphous Calcium Fluorophosphate

CPP-ACP has a synergistic remineralization effect with fluoride. Studies have shown that the CPP-ACP complex is stable in the presence of fluoride and binds to calcium and phosphate as well as to fluoride.[66,72,73] This led to the production of casein phosphopeptide stabilized amorphous calcium fluoride phosphate complexes, also known as (CPP-ACFP).[72-74] Products combining CPP-ACP technology with fluoride have recently been developed. Examples of these products are MI Paste Plus™ (GC America Inc., Alsip, IL) and MI varnish (GC America Inc., Alsip, IL). MI Paste Plus, for example, was primarily used as an abrasive prophylaxis paste and secondarily a treatment for tooth sensitivity after bleaching procedures, ultrasonic scaling, hand

scaling, or root planning. However, it was found that MI Paste Plus is effective for enamel and dentine remineralization and caries prevention, and thus was used as an off-label application. [58] More recently, MI Varnish was developed to combine the effect of CCP-ACP complex with the retention and prevention effect of fluoride varnish. Cochrane et al. reported that CPP stabilizes high concentrations of calcium, phosphate, and fluoride ions at all pH values ranging from 4.5 to 7.0. Remineralization of the enamel carious lesions was observed at all pH values within this range, with a maximal effect at pH 5.5.[73] Remineralization of enamel subsurface lesions with CCP-ACP and with the absence of environmental fluoride predominantly leads to the formation of hydroxyapatite, which is less resistant to acid dissolution than fluorapatite.[59] CCP-ACFP at pH values of 5.5 and below produces greater remineralization effect because the major product is fluorapatite.[59,73] Iijima and colleagues showed that the acid challenge of enamel lesions in situ after remineralization with sugar-free CPP-ACP containing gum associated with the daily use of fluoride toothpaste led to demineralization underneath the remineralized zone, which indicates that the remineralized mineral was more resistant to subsequent acid challenge.[75] Furthermore, the delivery of simultaneous calcium, fluoride and phosphate provides an effective means of controlling the fluoride levels in the dental biofilm. These levels influence the behavior of cariogenic bacteria, as well as contribute to remineralization.[59]

The CPP-ACP and fluoride combination has been shown to impair demineralization and promote remineralization in a range of clinical trials, in situ models, and in vitro studies.[64,66,74,76,77] Clinical studies combining CCP-ACP and

fluoride in mouth rinses and dentifrices have provided interesting insights into the synergistic effect between these components. Reynolds and coworkers showed that the incorporation of fluoride into dental plaque increased by the addition of CCP-ACP to a fluoride mouth rinse. In addition, dentifrice-containing CPP-ACP with fluoride provides superior remineralization compared to CPP-ACP alone and to both conventional and high-fluoride dentifrices alone.[74] Moreover, the synergistic effect of CPP-ACP and fluoride has also been identified in laboratory studies using Recaldent™ creams. Kumar et al. demonstrated that CPP-ACP containing cream without fluoride remineralized initial enamel lesions and showed a higher effect when applied as a topical coating after the use of fluoride dentifrice.[76] Furthermore, Robertson et al. compared nightly tray application of a 10% CPP-ACP paste and 900 ppm fluoride with a placebo paste applied after brushing with toothpaste. It was found that individuals using CPP-ACP and fluoride-containing paste developed fewer lesions and showed more lesion regression compared with those using the placebo paste after brushing with toothpaste.[66]

Recently, a new product named MI Varnish (GC America Inc., Alsip, IL) Recaldent (CPP–ACP) was added to enhance the effectiveness of a 5% sodium fluoride varnish. An *in vitro* study by Conchrane et al. was conducted over a time period of 1 to 168 hours to measure the ion release of four fluoride varnishes with different forms of calcium-phosphate. MI varnish contained calcium phosphate in the form of CPP-ACP, whereas the other three varnishes, Clinpro White, Enamel Pro and Bifluorid 5, contained functionalized tricalcium phosphate, amorphous calcium phosphate and calcium fluoride, respectively. Durophat, a fluoride varnish (no calcium added) was included as a

positive control. It was shown that all calcium-containing varnishes had a cumulative release of fluoride ions at 24 hours that was similar or better than that of the control varnish. The percentage of cumulative fluoride release from MI Varnish was 96%-103% after 24-168 hours. Moreover, MI varnish was significantly capable of releasing calcium and inorganic phosphate ions, which is in agreement with the CPP-ACP bioavailability nature of the varnish. It was concluded that, compared to other varnishes, MI varnish exhibited the best fluoride and calcium ion release over time and was considered to be the most promising.[77] Furthermore, Pithon and colleagues evaluated the efficiency of varnish with CPP–ACP (MI Varnish) at preventing white spot lesions around orthodontic devices in vitro. They also tested whether MI Varnish is more effective than the conventional varnishes with or without brushing and/or rinsing. Eight groups were compared: (1) brushing (control), (2) brushing/fluoridated mouth wash (control), (3) fluoride varnish, (4) fluoride varnish/brushing, (5) fluoride varnish/brushing/mouth wash, (6) MI varnish, (7) MI varnish/brushing, and (8) MI varnish/brushing/mouth wash. The groups were challenged with 28-days pH cycling. Brushing with fluoridated dentifrice (1450 ppm F^+) and immersion in mouthwash (225 ppm F^+) were performed for 1 minute, followed by washing in deionized water three times per day. The application of varnish with CPP–ACP was shown to be more effective than fluoride varnish and significantly reduced the depths of WSLs around orthodontic brackets, irrespective of brushing and mouth wash. [64] Therefore, based on the literature, this novel varnish may have the potential to further improve caries prevention.

LASERs

The term LASER is an acronym for 'Light Amplification by the Stimulated Emission of Radiation'. Albert Einstein proposed the theory for the invention of the laser in 1917.[78] In 1960, Maiman constructed the first working laser, the ruby crystal laser.[79] Many researchers used ruby laser to investigate the laser tissue interaction with enamel and dentin.[80] Many other lasers were created rapidly thereafter, including the neodymium-doped yttrium aluminium garnet laser (Nd-YAG), diode laser, argon laser, carbon dioxide laser (CO_2), erbium-yttrium-aluminum-garnet laser (Er:YAG), erbium-chromium-yttrium-scallium-gallium-garnet laser (Er Cr:YSGG).The Nd: YAG was the first laser to be approved for dental application by the FDA.[78,81]

Laser therapy has been applied for numerous applications in the field of dentistry including soft and hard tissue procedures.[81] Some of the soft tissue applications include aesthetic gingival re-contouring, crown lengthening, frenectomies, hyperplastic tissue removal, impacted tooth uncovering, wound healing, and photodynamic therapy for malignancies. For hard tissue application, the laser is used for bleaching, enamel etching, caries prevention, restorative removal and curing, cavity preparation, dentin desensitization, osteoplasty, osteoctomy and diagnostic purposes. The common laser types used in dentistry, their wavelengths and some of their oral applications are listed in Table (2).[78,82,83]

Laser Emission Mode

Dental lasers can emit beams in either continuous-wave mode or discrete-pulsed mode. Continuous-wave mode refers to a laser that is emitted (laser-on) continuously; its

beam is not interrupted and its power is constant over time. Pulsed mode means that the laser beam appears in pulses of some duration at some repetition rate. There are two types of pulsed modes: gated wave and free-running pulse. Gated wave pulse beam is a continuous beam interrupted by a shutter (gate) leading to a periodic alteration of the laser energy. The time between two pulses is measured in milliseconds, whereas a free-running pulse is a non-continuous beam that is emitted in powerful peaks of laser energy. The laser is emitted for a short time measured in microseconds followed by a relatively long (laser-off) time.[81,84]

The main principle of any laser mode of emission is that the laser energy strikes the tissues for a certain amount of time, producing a thermal interaction. The thermal implications of both pulse modes are profound. Thermal relaxation refers to the ability of the irradiated tissue to absorb heat produced by laser interaction. In continuous mode, the tissue does not have a thermal relaxation and, therefore, the temperature of the target can build up quickly, leading to potential thermal damage. In contrast, the pulse mode laser allows an interval between each pulse so that the tissue can absorb and dissipate the heat, thus minimizing thermal damage.[84]

Laser-Tissue Interaction

When a laser beam is aimed at a tissue, there are four ways in which the laser interacts with the tissue: reflection, transmission, absorption and scattering of the laser beam. The laser beam may reflect off the surface without penetrating or interacting with the tissue. A portion of the light may be transmitted through the tissue with no effect. Some of the light may be absorbed by the element of the tissue. The remaining light may

penetrate and scatter with unnoticeable effect on the tissue.[78,81] The extent of laser-tissue interaction generally depends on the laser-specific wavelength and target tissue optical characteristics. The optical properties of the tissue determine the nature and extent of the tissue response**.**

The extent of laser interaction is generally proportional to the absorption level of a particular wavelength by tissue.[81] Laser-tissue absorption requires a light absorber termed "chromophores". A chromophore is a specific substance with a certain affinity for specific light wavelengths. Examples of chromophores include melanin and hemoglobin in soft tissue, and water and hydroxy apatite in dental hard tissue. When the laser light hits the tissue, chromophores absorb the energy and convert it into thermal or mechanical energy. Different laser wavelengths have different absorption coefficients with respect to the tissue components, which makes the laser selection procedure-dependent.[78]

Laser and Caries Prevention

In the 1960s, Sten and Sognnaes were the first to demonstrate the role of laser irradiation in the prevention of caries. They demonstrated that exposure to ruby laser irradiation increased the acid resistance of tooth enamel.[80] Since then, many investigators have explored the application of lasers in the area of preventive dentistry. In 1980 Yamamoto and Sato showed the potential of a Nd:YAG laser to increase enamel acid-resistance. They found that Nd:YAG laser-irradiated enamel is more resistant to acid decalcification than unlased enamel.[85] However the wavelength of Nd:YAG laser failed to be absorbed effectively by human enamel; therefore, Nd:YAG lasers were the least

effective for caries prevention.[86] Argon laser was also found to decrease enamel demineralization. Hicks et al. (1993) reported that argon laser irradiation of sound enamel surfaces enhances the ability of lased enamel to resist a constant cariogenic challenge in vitro. They showed that enamel surfaces irradiated with argon laser underwent a significant reduction in lesion depths after an acidic challenge.[87] Moreover, Taveres et al. (2012) compared the effect of argon and Nd:YAG laser on enamel demineralization using two different models to induce artificial caries. They compared lesion depths using polarized light microscopy, concluding that the argon laser was more effective for caries prevention than Nd:YAG laser.[88] However, argon laser is also poorly absorbed by the tooth structure. [83] Erbium lasers and CO_2 lasers are highly absorbed by hydroxyapatite.[83] Regarding erbium laser, there are contradictory reports about its effect in caries prevention. Hossain and colleagues found an increase in the calcium-to-phosphorus ratio during Er:YAG laser irradiation, supporting the caries inhibition effect.[89] Moreover, Kim et al. showed that enamel treated with Er:YAG laser improved in crystalline structure and had the lowest mineral dissolution compared to the control and to specimens etched with phosphoric acid, thus providing a caries-preventive effect on enamel.[90] However, other researchers did not find any significant effect in the use of Er:YAG laser with respect to the enamel demineralization prevention.[91,92] Apel et al. found that Er:YAG laser was unable to achieve any notable reduction in the acid solubility of dental enamel.[92] Ahrari et al. showed that Er:YAG laser may be useful for enamel etching before bonding orthodontic brackets; however, it did not decrease enamel demineralization when exposed to cariogenic challenge. [91] CO_2 laser has an

absorption coefficient greater in enamel than does the erbium laser. Therefore, CO_2 has the greatest potential to interact with enamel and dentin compared to other lasers.[83]

CO_2 laser was studied extensively in the field of caries prevention. Several researchers have shown that CO_2 provide a very efficient interaction with the enamel, and good results related to the inhibition of incipient caries (Table 3). CO_2 laser is emitted in several wavelengths ranging from 9 to 11μm (9.3, 9.6, 10.3, 10.6μm). They are highly absorbed by dental enamel and thus, have a greater potential to interact with enamel. Among the CO_2 laser wavelengths, the two shorter (9.3 and 9.6μm) have greater absorption while the two longest wavelengths (10.3 and 10.6μm) the absorption is smaller.[93] Several researchers investigated the effect of the CO_2 laser in caries prevention.[94] CO_2 laser with a 9.3μm wavelength was used by Nelson et al. to pretreat human enamel exposed to an acid challenge. They reported that laser-treated enamel produced lesions that were 50% less demineralized than the controls.[95,96] Featherstone et al. tested enamel irradiated with 9.6μm CO_2 laser and demonstrated a 70% reduction in enamel demineralization.[97] Young et al. applied a CO_2 (9.6μm) wavelength to occlusal pits and fissures of extracted human teeth. Using polarized light microscopy and transverse microradiography, they revealed a 50% inhibition of caries progression in enamel of the occlusal pits and fissures. [98] Kantorowitz et al. showed that CO_2 laser (10.6μm) preventive treatment inhibited caries-like lesion progression by up to 87%.[99] Moreover, Oliveria et al. reported that CO_2 10.6μm laser irradiation resulted in 81% reduction in mineral loss.[100] Furthermore, Hsu and colleagues showed that CO_2 laser treatment led to almost complete inhibition of enamel demineralization (98%).[101]

The high absorption of CO_2 laser by enamel structure also means that the initial temperature rise occurs in a thin layer (10-12μm) of the enamel surface, decreasing the risk of damaging the dentin or the dental pulp.[102,103] Although the 9.6 μm wavelength has 10 times higher absorption in enamel (8,000 cm^{-1}) than the 10.6 μm wavelength (825 cm^{-1}) and causes the greatest temperature increase, the penetration depth of 9.6μm wavelength is only 1μm from the outer surface of enamel. On the other hand, with the 10.6μm wavelength, the lower absorption will cause a smaller increase in temperature compared to that of 9.6 μm but, result in a higher penetration depth (12 μm) and, therefore, can affect a thicker enamel layer. For this reason, it has been suggested that it may cause a longer-lasting caries-preventive effect.[104]

The preventive effect of tooth demineralization can be achieved either with the CO_2 laser operating in continuous or pulsed mode. However, it is known that continuous mode irradiation significantly increases the chances of thermal damage to the enamel surface, dentin and can spread widely to damage the dental pulp tissue.[105,106] Considering that an increase in pulp temperature of only 5.5°C could result in irreversible damage to the pulp, the continuous mode presents greater risks for clinical use.[107] On the other hand, in the pulsed emission, the interval between the consecutive pulses allows the enamel thermal relaxation to occur and therefore, the spread of heat to the inner layers can be reduced.[108] Fried et al. (1996) found that shorter pulses led to a higher surface temperature and less heat propagation to the deeper layers. In addition, low energy densities and short pulse duration caused less thermal damage to the enamel surface and lower risk of damaging the dental pulp.[109] For this reason, the most recent

studies, testing the effects of CO_2 in prevention of demineralization, have been conducted using the pulsed emission.

Another fact that supports the localized heating of the surface is the use of short pulse duration, which is lower than the thermal relaxation time of enamel 90 μs. This decreases the possibility of enamel surface damage and heat propagation into the tooth.[93] Esteves-Oliveira et al. explored different low-fluence CO_2 laser (10.6μm) looking for parameters resulting in maximum caries preventive effect with the least thermal damage. It was demonstrated that pulsed CO_2 laser (10.6 μm) using short pulses (0.3 J/cm^2, 5 μs, 226 Hz) significantly (81%) enhanced the acid-resistance of human enamel samples compared to fluoride application (25%) and the control with no evidence of any damage to the enamel surface.[100] They also showed in another study that pulsed CO_2 laser (0.3 J/cm^2, 5 μs, 226 Hz) increased the enamel resistance to tooth-brushing abrasion following an acid challenge.[103] In 2011 the same group evaluated the effect of pulsed CO_2 (10.6 μm) laser irradiation (11 J/cm^2, 540 mJ and 10 Hz) in raising the temperature inside the pulp chamber of freshly extracted human third molars (n=20). They inserted measuring sensors into the pulp chamber through the root apex, and the entire root canal system was filled with a nano thermo-conductive paste to ensure good contact between the sensor and the tooth. It was shown that CO_2 laser irradiation caused only a slight increase in intra pulpal temperature, which was below 2°C and considered to be safe to the dental pulp tissue.[102] In a short-term clinical pilot trial, Rechmann and colleagues showed that short-pulsed 9.6 μm CO_2 laser irradiation significantly inhibited demineralization around orthodontic brackets.[110] Brackets were placed on bicuspids and

an area next to the bracket was irradiated with a pulsed CO_2 laser. An adjacent nonirradiated area served as control. Bicuspids were extracted after four and twelve weeks. Cross-sectional microhardness testing revealed a 46% demineralization inhibition for the 4-week period and a marked 87% inhibition for 12 weeks. This study showed for the first time in vivo that 9.6 μm CO_2-laser irradiation successfully inhibits demineralization of tooth enamel for a period of 12 weeks.[110] The smaller mineral loss for the four weeks was thought to be due to the enhanced remineralization over a longer period of time. However, no studies have studied the long-term effect of 10.6um. Since it penetrated deeper than 9.6 μm CO_2, it is expected to have a longer lasting effect, rendering this area a fertile area for further investigation.

Mechanism of Action

The reduced acid solubility of dental enamel after irradiation with laser light is related to physical and chemical changes caused by the photo thermal chemical effects. An even better improvement was seen when laser was combined with fluoride application. Various explanations have been given for the increased caries resistance after laser irradiation of the enamel (Figure 2). The most discussed mechanisms in the literature are:

Purification of enamel hydroxyapatite (HAP)

This mechanism suggests that the heat produced by the laser irradiation of dental enamel leads to some compositional changes that purify its hydroxyapatite crystalline structure. This mechanism includes a reduction in carbonate content, conversion of acid

phosphate to pyrophosphate and loss of structurally incorporated water during specific laser irradiation.[94,111]

Apatite crystals that contains carbonate, which is a soluble mineral, is called "carbonated apatite". Carbonate does not fit perfectly in the apatite lattice, leading to a less stable and more acid-soluble apatite phase. Fowler and Kuroda found that the laser treatment at temperatures ranging from 100 to 650°C led to an overall reduction in total carbonate content.[94,112] It was reported that 10.6 μm laser resulted in a 98% reduction of carbonate content in 1μm and 60% in 4 μm of the enamel surface.[93] Christoffersen studied the kinetics of calcium hydroxyapatite dissolution and reported that pyrophosphate reduced the hydroxyapatite dissolution rate in aqueous solution.[113] Others suggested that the heat generated from the laser light has the potential to convert acid phosphate to pyrophosphate. An increase in pyrophosphate caused by laser thermal effect (200-400°C) strongly reduced hydroxyapatite dissolution rate.[112] Also, purification of enamel hydroxyapatite may be partially explained by the contraction of the a-axis length of hydroxyapatite crystals, which is attributed to the loss of crystalline water from the apatite lattice.[114] Based on Sakae's findings, there was a nearly linear association between the temperature rise and the contraction of the a-axis.[115]

Reduction of enamel diffusion

This mechanism suggests that the photothermal effect of the laser either narrows or physically blocks the diffusion pathways of the enamel microstructure. The cariostatic effect of enamel laser treatment may be explained by the reduction in enamel permeability; however, some controversy exists. A study by Ying showed that the pore

volume and surface area of the laser-treated enamel decreased significantly by 16.7% and 11.9%, respectively.[116] A similar study demonstrated a 36 to 62% reduction in enamel diffusion.[117] In contrast, Borggreven et al. found that laser irradiation increased the permeability of enamel instead of decreasing it. These authors suggested that the enhanced resistance of laser-irradiated enamel is likely due to chemical changes rather than a reduction in enamel permeability.[118]

Another hypothesis known as (the Inorganic theory) states that the enhanced enamel resistant is due to melting, fusion and recrystallization of enamel crystallites. This physical sealing of the enamel surface retards the diffusion of acids and ions through enamel microspaces.[95] Furthermore, in addition to the reduced carbonate content of the melting surface, tetracalcium diphosphate monoxide, a new less soluble product, was identified as a component of this melted layer.[119] However, a study using a cross-sectional transmission electron microscope revealed that the melting of the enamel surface was not homogeneous and usually occurred in limited areas. Moreover, a significant increase in inter-and intra-crystalline spaces was seen beneath the melted surfaces.[94] Therefore, it seems that there is no conclusive evidence supporting this theory although it is frequently mentioned in the literature.

In addition to enamel solubility, caries formation also involves efficient diffusion of ions into and out of the enamel microgaps. It has been suggested that the organic matrix, which fills the inter- and intra-prismatic spaces, governs that diffusion pathway. The smallest level of acid dissolution of enamel is achieved at temperature 300-350°C, which is high enough to partially decompose the organic matrix of enamel.[120] It was

suggested that the photothermal effect of the laser result in denaturation and swelling of the organic matter, thus blocking the diffusion pathway. Therefore, the partial decomposition of the organic matrix may lead to retardation of enamel demineralization.[114] Hus et al. demonstrated that enamel-laser treatment caused a great reduction in enamel mineral loss (> 98%); however, the effectiveness of the laser treatment dropped to about 70% when the organic matrix was removed.[101] Interestingly, this theory disagrees with the inorganic block theory, which advocates sealing of enamel diffusion pathways by melting hydroxyapatite.

Increased enamel fluoride uptake

Various authors have reported more effective fluoridation from the use of lasers combined with topical fluoride than from conventional topical fluoridation or laser alone. Along with the beneficial effect of lasers in reducing surface enamel solubility in low pH, laser CO_2 can also increase the uptake of fluoride. Importantly, the use of laser favors the incorporation of the fluoride into the enamel, not only through surface deposition of calcium fluoride (CaF_2) but also by the formation of fluoridated hydroxyapatite enhancing its crystalline structure. CaF_2 acts as a surface reservoir released during acid attack to reduce demineralization and enhance remineralization.[111] Hsu et al. showed that fluoride uptake on the enamel surface increased by 37% more than the control after CO_2 laser irradiation. Fluoridated hydroxyapatite is more acid-resistant than natural hydroxyapatite. CO_2 laser irradiation may transform synthetic hydroxyapatite (HA) into “fluorapatite” in the presence of fluoride.[121] The fluorapatite

concentration in the enamel may reach 400% of the original concentration after fluoride-laser application.[122]

It has been reported that the parameters of the a-axis in human enamel change linearly with fluoridation concentration in the hydroxyapatite lattice.[123] Y liu showed an additional contraction in the a-axis of the HA crystal in the fluoride-laser group compared to that of the laser group.[111] There are two possible mechanisms that explain the laser-induced increase of fluoride uptake. First, the heat generated from laser irradiation was found to enhance the enamel fluoride uptake; therefore, the thermal effect of the laser could be the main factor in promoting fluoride uptake. Secondly, alterations in enamel surface following laser irradiation, such as an increase in cracks and roughness may also play a role in increasing fluoride uptake and facilitating the penetration of fluoride into enamel.[122]

Parameters of CO_2 Laser

Many past studies explored the effect of the CO_2 laser on the enamel surface and showed that with CO_2 laser, pretreatment inhibited enamel demineralization by 17-98%, depending upon the laser irradiation conditions.[94,124-126] There are several variables involved in determining the potential optimum laser irradiation conditions that would be likely to inhibit caries. These parameters include: wavelength, energy level, duration of pulse, number of pulse, beam size and repetition rate.[127] The demineralization reduction effect has been observed with very different parameters and different conditions of irradiation[97,101,124] Featherstone et al. conducted a systematic evaluation for 10.6 μm CO_2 lasers irradiation using a parameters (duration pulse, energy density, repetition rate) as

shown in Table 4, the highest percentages of inhibition of mineral loss were observed in energy density around 12 J/cm2 [97,99,128] More recent studies by Hsu et.al showed a quite high percentage of reduction (approximately 98%) in enamel mineral loss after irradiation with lower energy density (3.4 J/cm^2), a doubled repetition rate of (20 Hz) and a 50 times bigger pulse duration than that of Featherstone's parameters set.[101,121]

Although these parameters seem inspiring, most of the parameters that showed a reduction of mineral loss around 90% have also shown to cause excessive heat damage to the enamel surface and the dental pulp.[101,121,128] The reason behind this finding was probably due to the high pulse duration.[109] Esteves-Oliveira et al. conducted an tested low fluence CO_2 (10.6 μm) parameters in the caries preventive effect with the least thermal damage. The results of the study revealed that 0.3J/cm2; 5μs; 226Hz for 9 seconds increased enamel dissolution resistance up to 81% compared to the control. The effect of CO_2 was even significantly better than fluoride application (25%). The scanning electron microscopy examination did not show any enamel damage following the laser application. Furthermore, the set of parameters used in the study seems to provide a temperature increase very close to the ideal range (600-900 °C) for making the dental enamel more resistant to acid attack.[100]

Laser-induced dental caries prevention has been studied extensively, providing evidence of its efficiency as another valuable tool for rendering the enamel surface more resistant to caries attacks.[91,129-131] Carbon dioxide laser is reported to be more promising and has more potential than other types of lasers.[97,99,100,132] The available data suggest that lasers combined with fluoride are a promising treatment in caries prevention.

Applying fluoride followed by laser, or laser followed by fluoride produced a better effect in reducing WSLs than each treatment alone.[94,122,133-135] However, few studies have tested the sequence of their application and the longevity of the effect, which makes it an area needing further research.

Therefore, this study is mainly designed to answer the following questions:

Research Questions

1. Does CPP-ACP fluoride varnish and laser CO_2, prevent WSLs formation.
2. For How Long Does CPP-ACP fluoride varnish prevent WSLs Formation?
3. What is the effect of CCP-ACP fluoride varnish repeated applications on smooth surface enamel demineralization.

Specific Aims

Aim 1: To compare the ability of CO_2 laser and fluoride varnish containing CCP-ACP in preventing enamel demineralization.

Aim 2: To evaluated the longevity of the CPP-ACP fluoride-varnish preventive effect on enamel demineralization.

Aim 3: To evaluate the preventive effect of repeated application of CPP-ACP fluoride varnish (every 4 weeks, and every 6 weeks) on enamel demineralization.

CHAPTER II

COMPARING THE EFFECT OF LASER OR FLUORIDE APPLICATIONS ON WHITE SPOT LESIONS PREVENTION

Synopsis

The objective of this study was to compare the ability of CO_2 laser and fluoride varnish containing CCP-ACP in preventing enamel demineralization. Human teeth were halved and randomly assigned to three groups, fluoride, laser, and control (N= 21 per group). Baseline images of the enamel surfaces were obtained under standardized conditions using the FluoreCam. The 2x4 mm windows of exposed enamel were treated with fluoride varnish, CO_2 laser, or no treatment (control). All specimens were subjected to 9-days of pH-cycling, after which final FluoreCam images were obtained. Enamel demineralization and mechanical properties were evaluated using the FluoreCam and cross-sectional microhardness, respectively. Within-group analyses showed statistically significant ($p< 0.05$) changes of FluoreCam area, intensity and impact in the control and laser groups, but not the fluoride group. ANOVA showed statistically significant between-group differences in the changes that occurred for area ($P< 0.001$) and impact ($P= 0.002$), but not for intensity ($p= 0.07$). The control group showed significantly greater area and impact of enamel demineralization than the fluoride group. Compared to the fluoride group, the area of enamel demineralization in the laser group was significantly greater. Enamel demineralization of the laser and control groups were comparable. ANOCVAR showed statistically significant between-group differences in

enamel microhardness at 20 µm, 40 µm, and 60 µm depths, but no differences at the 80 µm, 100 µm and 120 µm depths. The fluoride group showed statistically significant harder enamel than the control group at 20 µm, 40 µm, and 60 µm depths; the laser group enamel was significantly harder than control enamel at 20 µm, 40 µm depths. The fluoride group showed statistically significant harder enamel than the laser group at 20 µm depth. In conclusion, fluoride varnish containing CCP-ACP is more effective than CO_2 in increasing the enamel resistant to demineralization.

Introduction

Smooth surface enamel demineralization, known as "white spot lesions" (WSLs), is a common risk associated with fixed orthodontic treatment. WSLs, which appear as chalky white patches on the buccal and labial surface of the teeth, jeopardize the esthetic benefits of the treatment.[47,136] The prevalence of WSLs among orthodontic patients in university and private practice settings ranges from 25-28%.[11,137] The development of these lesions has been attributed to prolonged plaque accumulation around the brackets, as well as shifts in the bacterial flora.[41,138] Because cariogentic (acidogenic) bacteria decrease plaque pH to a greater extent in orthodontic patients than in nonorthodontic patients, the progression of enamel demineralization is faster.[139] Although the formation of regular caries usually takes at least 6 months, WSLs in orthodontic patients can be evident as early as four weeks following the appliance insertion.[47,136,139] This is often shorter than the period between consecutive orthodontic visits.

Fluoride therapy has become the gold standard for caries prevention. Among the various fluoride vehicles, varnish has proven to be the most successful method for

reducing the incidence of WSLs in orthodontic patients.[45,140,141] The high amount of fluoride (22,000 ppm) in varnish and its resin base provide prolonged enamel-fluoride contact. However, fluoride application alone is insufficient for the prevention of enamel demineralization. To prevent WSLs, enamel demineralization must be reversed. Although fluoride ions decrease enamel demineralization and enhances remineralization, the bioavailability of calcium and phosphate ions is necessary for remineralization to occur. Recently, an enhanced form of dental varnish, MI Varnish (GC America Inc., Alsip, IL), has been developed that combines the preventive effects of fluoride and the CCP-ACP complex. CPP-ACP is a milk-derived product that provides bioavailable calcium and phosphate to the tooth surface when oral pH falls below the critical level (pH=5.0.) [64,66,74,76,77] Several studies have demonistrated the anticariogenic activity of CPP-ACP, [56,68,142-144] as well as its synergistic effect with fluoride.[72,74] MI varnish has been shown to be more effective in enhancing enamel resistance against acidity[145] and re-hardening early caries lesions.[146] Furthermore, it has a higher fluoride, calcium, and phosphate ions release than other varnishes.[77,146]

Lasers also potentially inhibit enamel demineralization.[91,129-131] Carbon dioxide lasers (CO_2) are by far the most promising.[97,99,100,132] Because CO_2 lasers have wave lengths that can be absorbed by the enamel, higher preventive effects with minimum harm to dental tissues have been reported.[97,99,100,132] Studies testing the preventive effect of different CO_2 laser wave lengths suggest that the 10.6 μm laser is the most applicable.[93,100,102] The possible mechanisms of the cariostatic effect produced by laser include purification of enamel hydroxyapatite, reduction of enamel permeability,

increased enamel fluoride deposition and uptake.[111] To assess and accurately measure changes in enamel WSLs, a variety of detection and quantification analysis techniques are now available. One of the common devices is Quantitative Light-induced Fluorescence system (QLF), which has been validated for its high sensitivity and reliability.[147-149]

Based on the same principle as the QLF system (Inspektor Research Systems BV, Amsterdam, Netherlands), the FluoreCam (DARZA, corporate Headquarters, Noblesville, IN) was introduced as another early-caries detection device for detecting, quantifying and monitoring minute changes in enamel.[150-153] The FluoreCam (DARZA, corporate Headquarters, Noblesville, IN) is based on the semi-translucency and the auto-fluorescence properties of the enamel. It excites the surface of a tooth with an intense light and the computer software receives the resulting fluorescent image and analyzes it.[150-153] The numerical outputs of the FluoreCam are size, intensity, and impact of the demineralized enamel lesion. The FluoreCam has previously been shown to be as reliable as the QLF for detecting enamel demineralization.[154] In contrast to QLF, the FluoreCam is a portable hand-held system, analyze data automatically, thus it is simpler to use.[150-153]

The aim of the present study was to compare the effect of fluoride varnish alone and the CO_2 laser alone on the prevention of enamel demineralization.

Materials and Methods

Sample Size and Power Analysis

Thirty-five sound human molars and premolars were collected from the surgery clinic at Texas A&M University Baylor Collage of Dentistry. The teeth were free of

cracks, restorations, caries, fluorosis, or any other developmental defects. They were stored in 0.1% (wt. /vol) thymol solution at 4°C for approximately 1 month. The sample size and power were determined from estimates of our previous study.[155] Assuming an effect size of 1.3, a sample of 21 per group was adequate to ensure an acceptable type I error rate (α=0.008) and adequate power (90%).[156] The teeth were sectioned mesiodistally into halves and randomly assigned to three groups according to the surface treatment: Fluoride varnish (F group), CO_2 laser (L group) and untreated control (Figure 3). Two additional specimens were added to each group in case of loss or damage.

FluoreCam Baseline Record

Baseline images were obtained using the FluoreCam under standardized conditions. [154,155] A laboratory stand with clamps was used to position the FluoreCam device and a small mounting table was placed at a fixed distance from the stand. A mold was made for each specimen using an impression material (Exaflex® Putty, GC America, Inc. IL). The root part of each specimen was horizontally inserted in the mold to the level of the CEJ. The FluoreCam tip was positioned so that it made contact with the enamel surface and the impression material was molded around the tip, providing a reference indentation for later FluoreCam repositioning. After image capture, the FluoreCam computer software recorded the area (mm^2), intensity (pixel) and impact ($pixel.mm^2$) for each image.

Surface Treatment

The specimens were removed from their molds and covered with a double layer of an acid-resistant nail polish (Revlon Cherry color), leaving a 2x4 mm enamel window

exposed. Photographs of the specimens were taken to record the location of the windows so that it could be demarcated later.

Enamel windows of the F group were treated with a one layer of MI varnish (GC America Inc., Alsip, IL) using a fluoride varnish applicator (microbrush) and immediately immersed in 5 mL artificial saliva for 24 hours (1.5mmol/L Ca, 0.9mmol/L P, 150mmol/L KCl, 0.05µg F/mL in 0.1mol/L Tris buffer, pH 7.0).[157] After 24 hours, the specimens were washed and the fluoride varnish layers were gently removed, uncovering the enamel windows.

Enamel windows of the L group were irradiated with 10.6 µm CO_2 laser (2- watt pulsed mode) using a 0.8 mm diameter ceramic tip (LX-20SP Novapulse CO_2 laser, Luxar Ltd. WA, USA). The laser parameters were set to an average power, 0.3 watt; irradiation time, 0.2 sec; pulse width, 10 m seconds; frequency, 15 Hz; energy density: 15J/cm^2 .[158] The distance from the ceramic tip to the enamel surface was approximately 2 mm. The handpiece was moved longitudinally and uniformly over the enamel window (scanning mode). The CO_2 laser has a cooling system, which blows air while the laser is emitted. Following irradiation, the specimens where soaked in artificial saliva for 24 hours.

pH Cycling

The specimens were subjected to 9 days of pH-cycling.[157] First, each specimen was suspended in a Falcon tube and immersed in 50ml demineralizing solution for 4 hours (1.28 m M calcium nitrate, 0.74 mM sodium dihydrogen phosphate, 0.05 M acetate buffer, 0.03 g F/ml, and pH 5.0). They were then rinsed with distilled water,

dried with absorbent paper, and immersed in 25 ml remineralizing solution for 20 hours (1.5mmol/L Ca, 0.9mmol/L P, 150mmol/L KCl, 0.05µg F/mL in 0.1mol/L Tris buffer, pH 7.0.). During the cycling procedure, the falcon tubes were kept in an incubator (Excella E24 Incubator Shaker Series, New Brunswick Scientific Co., Inc. Enfield, CT) at 37 °C under constant agitation (50 rpm). The pH was checked daily and every fourth day the solutions were replaced. On day 9, the specimens were kept in the remineralizing solution for 24 hours. The proportion of solutions per exposed enamel area was 6.25 and 3.12 mL/mm^2 for the de- and remineralizing solutions, respectively.[100,157]

FluoreCam System Assessment

The nail polish was carefully removed from the entire enamel surfaces and the specimens were reinserted in their prefabricated molds. Under the same baseline settings, the final FluoreCam images were captured.

Cross Sectional Microhardness (CSMH) Assessment

Ten specimens from each group were randomly selected for microhardness testing. The enamel lesions "exposed enamel" were demarcated and the roots were cut off. The crowns were cross-sectioned perpendicular to the enamel windows using low-speed (100 rpm) diamond-saw (Isomet, Buehler1000, IL). One of the halves was randomly selected and embedded in self-curing epoxy resin, leaving the cross-section surfaces exposed. The surface of the resin blocks were polished (Ecomet, Buehler Lake Bluff, IL) with silicon carbide discs of diminishing grit area (250, 320, 400, 600, 800 and 1200).[159] The mechanical properties of enamel were assessed using cross-section microhardness tester (FM-le Digital Microhardness Tester, Future-Tech Corp,

Novi, MI), with a Knoop indenter [load (50 g), dwell-time (5 seconds), room temperature (23±1°C)].[160,161] Three sets of 6 indentations were made; one set at the central region of the (exposed) enamel window, and another two sets approximately 100 µm distant from the central row (Figure 4). The indentations were 20, 40, 60, 80, 100 and 120 µm from the outer enamel surface. The first indentations were made 20 µm away from the outer enamel surface to avoid surface cracking.[161] To evaluate the (unexposed) sound enamel, another additional sets were made 1 mm away from each side of the window (Figure 4). The Knoop-hardness number (KNH) was obtained from the relationship KNH (Kg/mm^2) = $14230K/L^2$ where K is applied force (grams), and L is observed indentation length (µm).

Statistical Analysis

The FluoreCam data were normally distributed (SPSS version 22, Chicago, IL, USA). The baseline data were evaluated using Analysis of Variance (ANOVA). Within each group, the differences in the FluoreCam measures, *Δ area*, *Δ intensity* and *Δ impact* (*Δ= Final -baseline*), were analyzed using one-sample t tests. Between-group differences in the changes that occurred were compared using ANOVA followed by a set of Bonferroni post-hoc tests.

The CSMH data also were normally distributed. The hardness of the unexposed enamel was significantly different among the groups. Therefore, the differences in enamel hardness *(Δ hardness = exposed – unexposed)* at each depth were evaluated using Analysis of Covariance (ANCOVA), followed by a set of pair wise simple contrasts of the groups.

Results

At baseline, the FluoreCam data showed no statistically significant between-group differences for area ($p=0.09$), intensity ($p=0.63$) or impact ($p=0.17$) (Figure 5). After pH cycling, within group analysis showed a significant increase in area of demineralization ($p< 0.001$) and a significant loss of the fluorescent light intensity ($p= 0.012$) and impact ($p< 0.001$) (i.e. increase in enamel demineralization) for the control group. The L group also showed statistical significant within group differences for area ($p< 0.001$), intensity ($p= 0.011$) and impact ($p< 0.001$). In contrast, the F group showed no statistically significant differences for any of the FluoreCam measurements ($p> 0.05$). There were also statistically significant between-group differences in the changes that occurred for area ($P< 0.001$) and impact ($P= 0.002$), but not for intensity ($p= 0.07$) (Figure 6). The F group showed significantly less area and impact of enamel demineralization than the control group. Compared to the L group, the area of enamel demineralization in the F group was significantly less (Table 5). Enamel demineralization of the L group and the control were comparable.

There were statistically significant between-group differences in enamel hardness at 20 µm, 40 µm, and 60 µm, but there were no significant differences at the 80 µm, 100 µm and 120 µm depths (Figure 7). The F group showed statistically significant hardness differences at 20 µm, 40 µm, and 60 µm compared to control, whereas the L group was significantly different at only 20 µm, 40 µm depths compared to control. The F group showed statistically significant higher enamel hardness compared to L group at 20 µm depth (Table 6).

Discussion

Fluoride varnish containing CPP-ACP prevents enamel demineralization. Enamel treated with MI varnish showed no significant enamel demineralization, whereas the laser and control groups showed significant demineralization when challenged by pH cycling. Because the MI varnish has only recently become available, clinical trials have not yet been conducted comparing its preventive effect to other fluoride varnishes. Two *in vitro* studies have shown that MI varnish releases greater amount of calcium, phosphate and fluoride than other varnishes. [77,146] This suggests that the MI varnish, which has 22,000 ppm F, may provide better protection than MI Paste Plus, which has only 900 ppm F.[162,163] Clinically, MI Paste Plus has been shown to decrease the development of WSLs during orthodontic treatment and reduce the number of pre-existing WSLs.[162] It has also been shown to remineralize smooth surface WSLs to a greater extent than fluoride varnish.[163] In the present study, the preventive effect to demineralization could be attributed to the dual effects of the CPP-ACP and the fluoride. CPP-ACP has an anticariogenic effect by promoting remineralization of enamel.[56,68,142-144] The casein phosphopeptides (CPPs) at neutral pH stabilizes the calcium and phosphate ions, creating the CPP-ACP complex. When the pH drops to acidic levels, the complex releases the calcium and phosphate, thereby promoting remineralization.[64,66,74,76,77,164] Furthermore, CPP-ACP interacts well with fluoride ions to produce amorphous calcium fluoride phosphate, which can also be stabilized by CPP at the enamel surface.[57,72,74] The fluoride component of the varnish further inhibits

demineralization and enhances remineralization, providing the necessary elements for fluorapatite crystal formation, which is more resistant to further acid attacks.[57,72,74]

Fluoride varnish containing CPP-ACP increases enamel microhardness. In the present study, enamel treated with MI varnish was significantly harder than control enamel, suggesting that there was an increase in mineral content. A linear relationship between enamel hardness and enamel mineral content has been demonstrated.[165,166] MI varnish has previously been shown to increase enamel hardness of early caries lesions more than the other varnishes.[146] Increased hardness can be attributed to the synergistic effect of the fluoride and CPP-ACP complex. During the pH cycling the fluoride ions interact with partially demineralized enamel and form a less soluble and harder form of the enamel crystals (fluorapatite).[26]

Importantly, the present study showed that MI varnish increased enamel hardness to a depth of at least 60 μm in depth. This indicates that the remineralizing ions of the varnish, (i.e. calcium, phosphate, and fluoride) penetrated deep into the enamel and were not restricted to the surface. Previous studies have reported that fluoride uptake localized in the outermost surface layer (approximately 10 μm).[167,168] The CPP-ACP complex is thought to be responsible for the penetration of the fluoride ions into the deeper layers of enamel.[73] Fluoride incorporation has been shown to be significantly higher in enamel treated with CPP-ACFP than with fluoride alone, indicating that the fluoride incorporation is calcium phosphate limited.[73] At low pH, fluoride with the CPP-ACP complex develops a neutral form of fluoride, calcium, and phosphate, that does not precipitate onto the enamel surface. This inactive form allows for the diffusion of

fluoride, together with calcium and phosphate ions, deep into the enamel, and thus accelerates remineralization of enamel subsurface lesions.[73]

CO_2 lasers potentially enhance enamel's resistance to demineralization. Although there were no significant differences in the enamel demineralization between the laser and control groups in the present study, the laser group showed less demineralization than the control group. Post hoc power analysis revealed that there was insufficient power to detect between-group differences. Furthermore, enamel microhadness testing showed significant increase in enamel hardness to a depth of 40 µm. Previous studies have shown that CO_2 lasers increase hardness and decrease the demineralization of teeth.[133,169] The enamel hardness in the laser group also showed a tendency to being higher at deeper layers than at surface enamel. Paulos et al. have shown that the CO_2 (10.6 µm) wavelength laser penetrates deep and affects a thick enamel layer.[170] However, the present study did not show significant decreases in enamel demineralization at the surface enamel. This could be attributed to the laser parameters used, which were adopted from a clinical trial that treated pits and fissures of third molars and followed the subjects for three years.[158] The authors reported some enamel staining but no complaints from the subjects during the course of the study. Only two of the 22 teeth studied showed signs of dental caries. The brown spots indicate that the laser might have burned the enamel surface. The mechanism by which lasers increase enamel resistance to demineralization is not well understood. It has been suggested that laser irradiation raises the enamel surface's temperature, alters its mineral phase composition, and decreases its permeability and solubility.[93] It has also been shown that

CO_2 lasers can melt enamel, leading to surface damage.[128] To prevent enamel demineralization, the laser must decrease the solubility of enamel and the energy produced must be absorbed and efficiently converted to heat, without damaging the enamel or surrounding tissues.[94] More studies are required to determine safe and appropriate laser parameters for enamel WSLs prevention.

CPP-ACP fluoride varnish is more effective than CO_2 laser in reducing enamel demineralization. In the present study, laser treated enamel showed significant enamel demineralization over time while the MI varnish treated enamel did not. The varnish also produced harder enamel surface than the CO_2 laser irradiation. Previous investigators have reported that fluoride is superior to CO_2 in reducing enamel demineralization. [171-173] Fluoride gels (1.2% NaF) provide higher surface microhardness than the CO_2 laser alone, which was comparable to the untreated control.[171,173] While some studies have shown that fluoride solution (4% TiF4) [172] and gel (1.2% NaF)[174] allow significantly less enamel demineralization (lower amounts of calcium loss) than enamel irradiated with CO_2 lasers. Others have shown that CO_2 laser is superior to fluoride varnish (5% NaF).[175-177] These contradictory results could be due to the different laser parameters used. In addition, the varnish used in the present study could be more effective than varnishes previously used because it contains CPP-ACP, which has a synergistic effect with fluoride.[72,74] MI varnish also has been shown to have a higher fluoride, calcium, and phosphate ions release[77,146] and prevents enamel demineralization more than other varnishes.[145]

CHAPTER III

FOR HOW LONG DOES CPP-ACP FLUORIDE VARNISH PREVENT WSLs FORMATION?

Synopsis

The objective of this longitudinal *in-vitro* study was to evaluate the longevity of the CPP-ACP/fluoride-varnish preventive effect on enamel demineralization. Human molars and premolars were sectioned bucco-lingual and randomly assigned to two groups (N=38/group). Pre-treatment images of the enamel surfaces were obtained under standardized conditions using the FluoreCam. Specimens were covered with an acid-resistant nail polish, leaving a 2x4 mm enamel window exposed. The control group received no treatment and the experimental group received an application of CPP-ACP/fluoride-varnish. To simulate 2, 4, 8, 12 weeks periods, specimens were placed in a tooth-brushing simulator and received 300, 600, 1200, 1800 strokes, respectively (force =280g). Specimens were then thermocycled [(5°C-55°C)/15s dwelling-time] for a total of 150, 300, 600, 1200 cycles. At the end of each simulated-time period, the groups were subjected to 9-days of pH cycling, followed by FluoreCam imaging. Representative samples were sectioned and polished to a thickness of ~100µm for PLM evaluation. Repeated measures-ANOVA was used to determine the effect of time and varnish. The effect of time within each group was further tested using paired t-tests. Between-group comparisons for the effect of varnish at each time-period were conducted using independent t-tests. The results showed that here were statistically significant time ($p<0.001$) and varnish ($p<0.001$) effects on area, intensity, and impact of enamel

demineralization. The control group showed significant and progressive demineralization at 2, 4, 8, and 12 weeks ($p<0.001$). The experimental group revealed no significant demineralization during the first 4 weeks ($P>0.05$). Experimental demineralization increased significantly ($p<0.001$) thereafter, but attaining 2-week control levels after 12 weeks. There were significant between-group differences ($p<0.001$) in enamel demineralization at all time-points. PLM revealed typical WSLs in the control group (lesion depth=190 ± 34μm). The experimental group showed more limited enamel demineralization after 12 weeks (lesion depth=37 ± 9μm). In summary, within the limitations of this *in-vitro* study, CPP-ACP/fluoride-varnish prevents enamel demineralization for at least 4 weeks, and limits demineralization up to 12 weeks.

Introduction

The white opaque spots that appear on enamel surfaces of orthodontic patients are known as white spot lesions (WSLs). WSLs are a clinical sign of enamel demineralization which, if not treated at an early stage, may progress to dental caries or arrest, leaving a permanent white scar.[4] WSLs most commonly occur on the buccal surfaces of the maxillary lateral incisors, followed by canines, premolars, and central incisors, respectively.[10,11,178] They affect esthetics and thus the patients' satisfaction with their smiles, frustrating both the clinician and patient. The prevalence of WSLs has been estimated at approximately 28% for patients treated in university and private dental practice settings.[11,137] They can be seen as early as 4 weeks after the orthodontic appliance placement.[23] Early prevention of the WSLs is one of the goals of modern dentistry.

Fluoride varnish has been successful in reducing the incidence of WSLs in orthodontic patients.[45] The varnish contains a high fluoride concentration (22,600 ppm) and adheres to the tooth surface. Prolonged contact time between the varnish and tooth surface enhances the fluoride uptake of enamel, especially in the outer most layers, and promotes the formation of calcium fluoride (CaF_2) on the tooth's surface.[26,41,179] CaF_2 acts as an intraoral fluoride reservoir, releasing its calcium and fluoride ions when the oral pH falls below 5, and reversing the demineralization process.[26] Fluoride retention and its preventive effect gradually decrease over time.[179]

The longevity of the preventive effect of fluoride varnish has not been clearly defined. According to the ADA recommendations, fluoride varnish should be applied every 3 to 6 months.[180] This recommendation is inappropriate for orthodontic applications because it was based on studies assessing advanced stages of demineralization, which is not applicable to orthodontically associated WSLs. New technologies are now available that can detect lesions at very early stages of development.[148,150-153] Most varnishes have limited preventive effects. One fluoride varnish application at the beginning of the orthodontic treatment did not prevent WSLs after 3 months.[141] Fluoride varnish applications every 6 weeks were 30% more successful in reducing WSLs *in vivo* than a placebo.[140] However, the later study was based on photographic evidence, which requires subjective assessment of clinically visible WSLs.

To prevent WSLs formation, a balance between enamel demineralization and remineralization must be maintained. WSLs development is a dynamic process, in which

enamel demineralization exceeds remineralization.[1,2] Because enamel remineralization requires calcium, phosphate and fluoride ions in the oral environment, manufacturers have tried to improve the efficacy of the fluoride varnish by adding these ions. [63] Recently, fluoride varnish combined with casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) has been developed (MI varnish).[77] CPP-ACP is an amorphous form of calcium phosphate (ACP) stabilized by a phosphopeptide from the milk protein casein (CPP).[145] Topical application of CPP-ACP releases calcium and phosphate ions, which maintains a state of supersaturation with respect to hydroxyapatite enhances remineralization and prevents demineralization. [55,56,68,180] The incorporation of CPP into the salivary pellicle selectively inhibits the adhesion of cariogenic bacteria (S. mutans), by which a non-cariogenic plaque would eventually be produced.[71] Several studies have shown the anticariogenic properties of CPP-ACP [56,68,142-144] and its synergistic effect with fluoride.[72,74] Fluoride varnish containing CCP-ACP has been shown to be more effective in increasing the acid resistance of enamel than other varnishes.[145] Furthermore, it has a greater fluoride, calcium and phosphate cumulative release.[77]

The aim of the present longitudinal in-vitro study was to determine the longevity of CPP-ACP/fluoride varnish preventive effect on WSLs formation.

Materials and Methods

Study design

Enamel demineralization was monitored over 12 weeks (Figure 8). A thermcycling machine and tooth brushing machine were used to simulate 2, 4, 8, and 12-week time-periods. After each period, pH cycling was conducted and enamel

demineralization was evaluated using the FluoreCam system. At the end of the study representative samples were examined using polarized light microscope (PLM).

Samples Size and Power Analysis

Sound human permanent molars and premolars were obtained from the oral surgery department at Texas A&M Collage of Dentistry. The teeth were sectioned into buccal and lingual halves and stored in 0.1% thymol. Assuming an effect size of 0.72, which was based on published estimates,[181] a sample size of n= 38 per group was necessary to achieve a type I error rate of 5% and a power of 99%.[156] Two extra specimens were added to each group to compensate for loss or damage during the study.

FluoreCam imaging

The specimens were randomly assigned to either a control or experimental group. Pre-treatment images of the group's enamel surfaces were recorded under standardized conditions using the FluoreCam. The position of the FluoreCam was standardized as previously described. [154,181] A mold was made for each tooth section using a putty impression material (Exaflex® Putty, GC America, Inc. IL). Each specimen was horizontally inserted into the impression material to the level of the CEJ. The impression material was molded around the tip of the FluoreCam leaving a reference indentation. An image of the enamel surface of each tooth section was captured and the baseline data were recorded, including area (mm^2), light intensity (pixels) and impact of demineralization (pixel. mm^2). FluoreCam imaging was repeated at 2, 4, 8, and 12 weeks.

Treatment Application

The specimens were covered with an acid-resistant nail polish (Revlon®, New York, NY) except for 2X4 mm window of exposed enamel. Photographs were taken to record the location of each window, in case reapplication of the nail polish was necessary. MI Varnish (GC America Inc., Alsip, IL) was applied to the enamel windows of the experimental group (n=40). The control group (n=40) received no treatment. Both groups were kept in 10 ml artificial saliva [1.5 mmol/L Ca, 0.9 mmol/L P, 150 mmol/L KCl, 0.05 µg F/ mL in 0.1 mol/L Tris buffer (pH 7.0)] for 24 hours. The fluoride varnish was then gently scraped from enamel windows of the experimental group using a blade.

Thermcycling and Tooth-brushing

To simulate oral environment thermal conditions, the two groups were thermocycled. [182] The specimens were immersed alternately into two baths of distilled water (5 °C and 55 °C) for 15 seconds (LAUDA-Brinkmann LP, Delran, NJ). [182] The transfer time between the two baths was 5 seconds at room temperature (23 °C). A total of 150, 300, 600, 1200 cycles were performed, representing 2, 4, 8, and 12 weeks, respectively (10 cycles/ day). [182] To simulate the mechanical effect of tooth-brushing, the groups were placed in a tooth-brushing simulator (Proto-Tech Oral Wear products, Portland, OR) with six specimens for one group. [183] The specimens were individually placed in customized rubber molds (Exaflex® Putty, GC America, Inc. IL) mounted in the brushing simulator. Medium bristled toothbrushes (Deluxe Denta-Brite, Eagle, NY) were centered over the exposed enamel windows and oriented to brush in a mesio-distal direction.[183] To simulate a normal manual brushing force, a constant force of 280g and

20 strokes/day were applied. A total of 300, 600, 1200, 1800 strokes were applied for simulating 2, 4, 8, 12 weeks, respectively.[183] A slurry of fluoridated toothpaste (Crest; Procter and Gamble, Cincinnati, Ohio), with a 1:3 paste to water ratio, was constantly circulated during the brushing procedure.[183] New brushes and fresh slurry solution were used for each group. After brushing, the specimens were removed from their molds and the nail polish was reapplied if there was loss during the brushing process.

pH cycling

The two groups were exposed to nine days of pH-cycling [8-day de/remineralization +1-day remineralization]. [157] Each specimen was attached to a Falcon tube cap using an orthodontic wire and soft wax. Two sets of Falcon tubes (VWR, Radnor, PA) were prepared; one set was filled with demineralizing solution (50 mL) and the other with remineralizing solution (25 mL). The specimens were immersed alternately in demineralizing solution for 4 hours and remineralizing solution for 20 hours over an 8-day period.[157] During the cycling procedure, the specimens were rinsed with distilled water to avoid cross-contamination of the solutions. The tubes were kept in an incubator at 37°C and under constant agitation (Excella E24 Incubator Shaker Series, New Brunswick Scientific Co., Inc. Enfield, CT). The pH of the solutions was checked daily and the solutions were replaced every four days. On day 9, the specimens were kept in the remineralizing solution for 24 hours. The demineralizing solution consisted of 0.05 M acetate buffer containing 1.28 mmol/L Ca, 0.74 mmol/L P and 0.03 μg F/mL (pH 5), prepared from Ca $(NO_3)_2$.4 H2O, KH_2PO_4 and NaF, respectively. The proportion of demineralizing solution per area of exposed enamel was 6.25 mL/mm^2. The

remineralizing solution consisted of 1.5 mmol/L Ca, 0.9 mmol/L P, 150 mmol/L KCl, 0.05 µg F/ mL in 0.1 mol/L Tris buffer (pH 7.0). The proportion of remineralizing solution per area of exposed enamel was 12 mL/mm^2. Following pH cycling the nail polish was removed for FluoreCam imaging. The nail polish was then reapplied leaving the same enamel window exposed and the next simulated time period was conducted.

PLM sample preparation

At the end of the study, five specimens from each group were randomly selected and prepared for PLM evaluation. The roots of the teeth were removed with a low speed hand piece. Using an Isomet cutting machine (Isomet, Buehler, Germany), the crowns were serially sectioned perpendicular to the 2x4 mm enamel windows. The thickness of each slice was approximately 300 µm. The slices were ground and polished to a thickness of approximately 100 µm (Ecomet, Buehler Lake Bluff, IL) and soaked in distilled water over night. The specimens were then placed on glass slabs and covered with glass slips; the slab-slip interspace was filled with distilled water. The demineralized enamel lesions were examined under the PLM attached to a camera (Olympus BX51, Olympus Corp, PA). The mean lesion depths of the demineralized enamel were measured in microns using CellSens standard software (Olympus Corp, PA). Three lines were drawn, one at the center and one on each side of the lesion (i.e. enamel window). Each line was perpendicular to the enamel surface and extended to the depth of the lesion. The mean lesion depth was calculated for each group.

Statistical analysis

The data was normally distributed. Independent t-tests were used to evaluate pre-treatment differences. Repeated measure ANOVA, with Greenhouse-Geisser Correction, was used to determine the effects of time and varnish. The effects of time within each group were then tested using paired t-test with Bonferroni corrections. Between-group differences at 2, 4, 8, and 12 weeks were conducted using independent t-tests with Bonferroni corrections.

Results

FluoreCam

At pre-treatment, there were no statistically significant between-group differences for area (P=0.69), intensity (P=0.13) or impact (P=0.33). Post-treatment, there were statistically significant time ($p<0.001$) and varnish ($p<0.001$) effects for area, intensity, and impact. In the control group, area, intensity, and impact of demineralization showed statistically significant increases in every time-period (Table 8, Figures 9-11).

In the experimental group, the area of demineralized enamel decreased significantly after 2 weeks ($P<0.001$). After 4 weeks, there was no significant difference (P=0.94) in area compared to pre-treatment. There was an increase in area (P=0.001) after 8 weeks, which continued till 12 weeks ($P<0.001$) (Figure 9).

The intensity of demineralization in the experimental group showed no statistically significant changes after 2 weeks (P=0.72) or 4 weeks (P=0.61). There was a

significant increase in the intensity of demeralization after 8 ($P<0.001$) and 12 ($P<0.001$) weeks (Figure 10).

The impact of demineralization in the experimental group followed the same pattern as the area of demineralization. Impact decreased significantly after 2 weeks ($P=0.005$). After 4 weeks, there was no significant difference ($P=0.86$) compared to pre-treatment, followed by significant increases at 8 ($P<0.001$) and 12 ($P<0.001$) weeks (Figure 11). There were significant ($p<0.001$) between-group differences in area, intensity, and impact at each of the time periods (2, 4, 8, and 12 weeks) (Figures 9, 10, and 11).

Polarized Light Microscopy (PLM)

The PLM images revealed typical white spot lesions in the untreated control representative samples. The mean lesion depth was 190 ± 34 microns. The experimental group showed more limited areas of enamel demineralization, with a mean lesion depth of 37 ± 9 microns.

Discussion

Prevention of WSLs formation during treatment is one of the biggest challenges facing orthodontics, despite advances in caries prevention. Fluoride therapy has become the gold standard for the prevention and reversal of enamel demineralization.[140,184] Among the available vehicles, fluoride varnish is considered the most effective in the prevention of WSLs.[45,141] CPP-ACP is a new delivering system that allows freely available calcium and phosphate ions to attach the enamel surface and remineralize partially demineralized crystals.[55,56,68,180] The combination of fluoride and CCP-ACP

has a synergistic effect.[72,74] Prior to this present study, the longevity of the preventive effect of CPP-ACP containing fluoride varnish had not been tested.

Untreated control enamel exposed to pH cycling demineralizes over time, but the rate of demineralization decelerates. In the present study, demineralization of the control group increased 127% during the first 4 weeks, 71% during the next 4 weeks, and 62% during the last 4 weeks. This confirms that demineralized enamel is less likely to demineralize than sound enamel.[185-187] It also explains why WSLs development is greatest during the first 6 months of orthodontic treatment, and continues at slower rates thereafter.[139] Demineralization decelerates because acidity (low pH) removes surface impurities, such as magnesium and carbonate from the enamel, which increases surface area and exposes more reactive HA crystals.[21] When the pH neutralizes, the partially demineralized HA crystals uptake calcium, phosphate and fluoride ions and form fluoridated hydroxyapatite crystals, which are less soluble and more resistant to further acid attacks than original HA crystals.[21]

CPP-ACP fluoride varnish initially remineralizes enamel. In the present study, during the first two weeks after fluoride varnish application the enamel remineralized, despite thermal (thermocycling), chemical (pH cycling) and mechanical (brushing) challenges. The mineral gain led to a 12% decrease in the area, a 1.5% decrease in intensity and a 14% decrease in the impact of demineralization. Remineralization probably occurred because human teeth have preexisting areas of enamel demineralization due to cycles of de- and remineralization that occur throughout the day. FluoreCam baseline readings and images showed some minor demineralization areas.

The FluoreCam device was sensitive enough to detect and monitor these areas. Knosel et al, who compared the effect of different varnishes using quantitative light-induced fluorescence (QLF) showed no enamel demineralization or remineralization during the first 2 weeks.[188] However the varnishes they tested did not contain CPP-ACP. CPP-ACP buffers free calcium and phosphate ions, maintaining a state of supersaturation with respect to enamel hydroxyapatite, which facilitates remineralization and prevent demineralization. [55,56,68,180]

Enamel demineralization begins approximately 2 weeks after CPP-ACP fluoride varnish application. After 4 weeks, the enamel mineralization had returned to baseline values, indicating a 100% varnish preventive effect. Knosel and colleagues also showed a 100% preventive effect 2 and 4 weeks after application.[188] Since they did not show remineralization during the first 2 weeks, there must have been increased demineralization over the second week in the present study. Differences between studies can be attributed to two factors. First, the enamel in their study was not subjected to thermal cycling and mechanical forces. Second, their enamel specimens were exposed to shorter cycles of demineralization (30mins/day) than in the present study (4 hours/day). These factors possibly increased the loss of the loosely bonded fluoride (CaF_2) but not the fluoroapatite and thus provided 100% enamel protection.

After 4 weeks, enamel treated with CPP-ACP fluoride varnish undergoes net demineralization. In the present study, during the second and last 4 weeks of the study the preventive effect decreased to 75% and 50%, respectively. Therefore, the net mineral loss (demineralization) must have begun sometime between 4 and 8 weeks. This

suggests that the longevity of the fluoride varnish is possibly 6 weeks, but totally effective for 4 weeks. A previous clinical trial that applied fluoride varnish every 6 weeks reported a 30% increase in WSLs.[140] However, they used a varnish that did not contain CPP-ACP. The effect of the fluoride varnish probably starts to diminish after six weeks because most of the fluoride bonded to the enamel surface is loosely bonded (CaF_2). It gradually de-bonds over time under normal oral conditions.[179] CPP-ACP fluoride varnish may be more effective over a slightly longer period of time than other varnishes.

CPP-ACP containing Fluoride varnish is effective in reducing enamel demineralization over time. The demineralization that occurred 12 weeks after fluoride varnish application was comparable to the demineralization that occurred after 2 weeks in the untreated control teeth. Farhadian et al reported that a one-time fluoride varnish application reduced enamel lesion depths (40%) after 3 months of orthodontic treatment.[141] The varnish that was used in the present study contained CPP-ACP, along with 5% NaF (MI varnish), which is more effective in increasing the acid resistance than other varnishes.[145] It has been reported that MI varnish has the highest cumulative fluoride release among fluoride varnishes (303 µg/ml) [146], in addition to having the greatest calcium and phosphate cumulative release.[77] This suggests that the varnish used in the present study provides a relatively longer preventive effect against enamel demineralization than regular NaF varnishes. More studies are required to compare the long-term effects of CPP-ACP fluoride varnish to other varnishes using new non-destructible sensitive technologies.

Previous recommendations by the ADA may not be applicable to orthodontic WSLs. The ADA recommendation for preventing dental caries is to reapply the fluoride varnish every 3-6 months.[180] Orthodontic treatment with fixed appliances increases the risk of WSLs development, which starts well before 3-6 months. ADA fluoride varnish recommendations are based on studies that used either insensitive or destructive methods. Insensitive techniques, such as photographs, clinical examinations, or scoring systems (MDF score system) might be expected to overestimate the protective effect of the fluoride varnish. Furthermore, destructive methods do not allow lesion development after fluoride treatment application to be monitored. In contrast, optical detective devices such as QLF and FluoreCam are sensitive and undisruptive, providing more sensitive measures for enamel demineralization and remineralization.

FluoreCam may be superior to QLF because it does not require a dark room to operate, it is portable, it analyzes data automatically and it is relatively inexpensive. This technology is based on the autofluorecent phenomenon of enamel exposed to a certain light wavelength. The device excites the surface of a tooth with an intense light and the computer software receives and analyzes the resulting fluorescent image.[150-153] FluoreCam has been shown to be highly reliable in experimental situations *in vitro*, with intra class correlation ranging from 0.87 to 0.95.[189] FluoreCam has also been used to monitor enamel demineralization and remineralization over time.[150-153]

CHAPTER IV

THE EFFECTS OF REPEATED CCP-ACP FLUORIDE VARNISH APPLICATIONS ON PREVENTION OF SMOOTH SURFACE ENAMEL DEMINERALIZATION

Synopsis

The objective of this study was to evaluate the effects of repeated (once, every 4 weeks, or every 6 weeks) CPP-ACP fluoride varnish applications on enamel demineralization. Human teeth were sectioned and randomly allocated to three groups: 4-week, 6-week, and control (N=22/group). Baseline images of the enamel surfaces were obtained using the FluoreCam. All three groups received fluoride varnish applications at the beginning of the experiment; varnish was reapplied every 4 or 6 weeks, for the 4-week, 6-week groups respectively. Following each application, the groups underwent thermo-cycling, tooth brushing and pH cycling. After 12 weeks, the enamel surfaces were reimaged. Within and between-group differences in the area, intensity and impact of demineralization were evaluated. Repeated FluoreCam measurement of 10 specimens showed no statistically significant systematic errors ($p>0.05$); method errors were 0.78 mm^2 and 0.51 pixels for area and intensity, respectively and the ICCs ranged from 0.90 to 0.95. At baseline, there were no significant ($p>0.05$) between-group baseline differences for area, intensity and impact. Statistically significant enamel demineralization ($p<0.001$) occurred over time within each group. There were statistically significant between-group differences in the changes that occurred in area

(P=0.004) and impact (P=0.02), but not intensity (P=0.51). The control had significantly larger areas of demineralization than the 6-week (P=0.04) and 4-week (P=0.001) groups. The change in impact was significantly (P=0.007) greater in the control than 4-week group, but not significantly greater than the 6-week group. There were no statistically significant differences between 4- and 6-week groups in the changes of area, intensity, or impact. Within the limitations of this *in vitro* study, reapplication of the CPP-ACP fluoride varnish reduces enamel demineralization. Because decreases in the preventive effects of the varnish are apparent after 6 weeks, it should be reapplied at least every 4 weeks.

Introduction

White spot lesions (WSLs) surrounding orthodontic brackets are a common clinical problem jeopardizing the esthetic results of the treatment. These lesions can develop as early as 4 weeks following orthodontic appliance placement.[23] Approximately 28% of patients in university and private dental practice settings develop visible WSLs during treatment.[11,137] Prevention of WSLs formation remains a major challenge during fixed orthodontic treatment, despite advances in preventive dentistry.

Fluoride therapy has been the gold standard for the prevention and reversal of enamel demineralization for decades.[140,184] Most fluoride vehicles have limited effects and require repeated applications to be effective. The regular use of home-applied topical fluorides, such as tooth pastes, [23] gels[37], and mouth rinses [37] have proved to be beneficial in reducing the severity of enamel demineralization. The approaches often depend on patient compliance, which is difficult to achieve in most patients.[36] Periodic

professional applications of higher concentrations of fluoride in the form of gels and foams have also been shown to be effective.[38,40,41] However the short fluoride-enamel contact time (4 minute) limits their effects. Among the available vehicles, fluoride varnish is considered the most effective compliance-free approach.[45,140,141,190,191] The key feature of fluoride varnish is its adhesive resin base, which allows a prolonged fluoride-enamel interaction.[192]

Periodic application of fluoride varnish has been shown to be effective in decreasing the risk of dental caries in primary and permanent teeth.[193] According to the ADA recommendations, fluoride varnish should be applied every 3 to 6 months.[180] This recommendation is not applicable to orthodontic WSLs because it was based on studies assessing advanced stages of demineralization. Most varnishes have limited preventive effects on orthodontic WSLs. One fluoride varnish application at the beginning of the orthodontic treatment does not prevent WSLs after 3 months.[141] Reapplication of fluoride varnish every 6 weeks is 30% more successful in reducing orthodontic WSLs than placebo varnish. [140]

The effect of fluoride varnish can be improved by the addition of calcium and phosphate ions. To prevent WSLs formation, enamel remineralization should be equivalent to or supersede demineralization. Enamel remineralization, which requires the bioavailability of calcium and phosphate ions, can be enhanced by the presence of fluoride ions. Phosphopeptides amorphous calcium phosphate complexes (CPP-ACP) have been developed to enhance enamel remineralization. The casein phosphopeptides (CPP) are a group of peptides derived from casein, which is part of the protein naturally

found in milk. Calcium phosphate normally forms a crystalline structure at neutral pH (i.e. insoluble). CPP keeps the calcium and phosphate in an amorphous insoluble state (ACP). The CPP-ACP complex provides a delivery system that allows freely available calcium and phosphate ions to attach to the enamel surface and remineralize the partially demineralized enamel.[55,56,68,180] The combination of fluoride and CCP-ACP has a synergistic effect.[72,74] Recently developed fluoride varnish containing CPP-ACP (MI varnish) has been shown to be more effective for enhancing acid resistance of enamel than other varnishes.[145] Cochrane et al [77] found that MI varnish has higher fluoride, calcium and phosphate ions release than other varnishes. It is associated with the highest release of fluoride over a period of 6 hours, as well as a significantly greater ability to reharden early carious lesions than the other varnishes tested.[146]

To assess and accurately measure changes in enamel WSLs, a variety of detection and quantification analysis techniques are now available. The FluoreCam (DARZA, corporate Headquarters, Noblesville, IN) is an optical, light-based detection and quantification system used to assess early enamel demineralization.[150,194,195] It is based on the same principles as Quantitative Light-induced Fluorescence system (QLF), which has proven to be highly sensitive and reliable.[147-149,196] The FluoreCam has previously been shown to be as reliable as the QLF for detecting enamel demineralization.[154] In contrast to the QLF, FluoreCam is portable and automatically analyzes data. The technology of the FluoreCam device is based on the semi-translucency and auto-fluorescence properties of the enamel. It excites the surface of a tooth with an intense light and the computer software analyzes the resulting fluorescent

image. [150,194,195] Because demineralized enamel emits less intense light than sound enamel, the lesion appears darker. The FluoreCam depicts the lesion based on its size, intensity, and impact. Size represents the area (mm^2) of the altered enamel. Intensity (pixels) represents the intensity of the fluorescent light emitted back from the enamel i.e. amount of enamel demineralization. Impact refers to the overall effect of area and intensity combined.

Prior to the present study, we found that enamel treated with CPP-ACP fluoride varnish was more resistant to demineralization than untreated control (Chapter III). Demineralization after 12 weeks in the treated group was equivalent to demineralization after 2 weeks in the control group. Demineralization started to occur between 4 and 8 weeks. We hypothesized that reapplying CPP-ACP fluoride varnish within this frame of time may prolong its effect and prevent further enamel demineralization. Since the benefits of periodic repeated CPP-ACP fluoride varnish applications have not been tested, the aim of the present study was to determine whether demineralization is related to the frequency of CPP-ACP fluoride varnish application (every 3 or 6 weeks).

Materials and Methods

Sample Size and Power Analysis

Human molars and premolar were collected from the surgery department of Texas A&M University College of Dentistry (IRB ref# 2015 0413-BCD-exp). They were cleaned, sectioned mesiodistally into two halves and stored in 0.1% thymol. Assuming an effect size of 0.72, which was based on published estimates [181], a sample size of 20 per group was necessary to achieve a type I error rate of 5% and a power of

99%.[156] Two extra specimens were added to each group in case of loss or damage during the study.

FluoreCam Baseline Imaging

The specimens were randomly assigned to three groups (control, 6-week, 4-week) using Random.org. Baseline images of the group's enamel surfaces were obtained under standardized conditions using the FluoreCam device.[154,155] The position of the FluoreCam was positioned a fixed distance from a mounting table. Molds were made for each specimen using impression material (Exaflex® Putty, GC America, Inc. IL). The molds were fabricated by horizontally inserting each specimen to the level of the CEJ. The Fluorecam tip was placed on the enamel surface and the material was molded around the tip of the FluoreCam, leaving a reference indentation. An image of the enamel surface of each tooth section was captured and the baseline data were recorded, including area (mm^2), light intensity (pixels) and impact of demineralization (pixel. mm^2). The mold later served as a reference for precisely repositioning the FluoreCam tip during final imaging.

CPP-ACP Fluoride Varnish Application

After initial imaging, the specimens were removed from their molds and covered with an acid-resistant nail polish (Revlon, New York, NY), leaving a 2X4 mm enamel window exposed. The enamel windows of all three groups were covered with a layer of MI Varnish (GC America Inc., Alsip, IL) using the manufacture's applicator (microbrush). They were then soaked in artificial saliva for 24 hours (1.5 mmol/L Ca, 0.9 mmol/L P, 150 mmol/L KCl, 0.05 µg F/ mL in 0.1 mol/L Tris buffer pH 7.0), after

which the fluoride varnish layer was carefully peeled off with a blade. The varnish was reapplied every 4 weeks for the 4-week group (total of 3 applications), and every 6 weeks for the 6-week group (total of 2 applications). Following each reapplication, the specimens were again placed in artificial saliva for 24 hours. The control received no additional treatment (total of 1 application) during the 12 weeks period (Figure 12).

Thermocycling and brushing

To simulate oral conditions, the specimens were subjected to thermal and mechanical challenges immediately after the varnish was removed. The specimens were first placed in a thermocycing machine (LAUDA-Brinkmann LP, Delran, NJ), where each group was immersed alternately into two baths of distilled water (5 °C and 55 °C) for 15 seconds.[182] The transfer time between the two baths was 5 seconds at room temperature (23 °C). The specimens underwent 10-cycles/day; the control and 4-week groups received 300 cycles every 4-week period and the 6-week group received 450 cycles every 6-week period.[182]

The specimens were then placed in a tooth-brushing simulator (Proto-Tech Oral Wear products, Portland, OR). [183] They were embedded in prefabricated molds (Exaflex® Putty, GC America, Inc. IL) and centered under medium bristled toothbrushes (Deluxe Denta-Brite, Eagle, NY), oriented to brush in a mesio-distal direction.[183] To simulate a normal manual brushing, a constant force of 280 g and 20 stroke/day were applied. [183] The control and the 4-week groups received 600 strokes every 4-week period and the 6-week group received 900 strokes every 6-week period. A slurry of fluoridated toothpaste (Crest; Procter and Gamble, Cincinnati, Ohio), with a 1:3 paste to water ratio,

was constantly circulated during the brushing procedure. [183] New brushes and fresh slurry solution were used for each group. After brushing, the specimens were removed from their molds and nail polish was reapplied if there was loss during the brushing process.

pH cycling

The three groups were subjected to 9-day pH-cycling protocol.[157] Each specimen was attached to a Falcon tube cap using an orthodontic wire and soft wax. Two sets of Falcon tubes (VWR, Radnor, PA) were prepared; one set was filled with demineralizing solution (50 mL) and the other with remineralizing solution (25 mL). The specimens of the control and the 4-week groups were immersed alternately in demineralizing solution for 4 hours and in remineralizing solution for 20 hours over an 8-day period.[157] On day 9, the specimens were kept in the remineralizing solution for 24 hours. The 6-week group was subjected to 1.5 times the length of the original pH-cycling protocol.

The demineralizing solution consisted of 0.05 M acetate buffer containing 1.28 mmol/L Ca, 0.74 mmol/L P and 0.03 µg F/mL (pH 5), prepared from Ca $(NO_3)_2.4\ H_2O$, KH_2PO_4 and NaF, respectively. The proportion of demineralizing solution per area of exposed enamel was 6.25 mL/mm^2. [157] The remineralizing solution consisted of 1.5 mmol/L Ca, 0.9 mmol/L P, 150 mmol/L KCl, 0.05 µg F/ mL in 0.1 mol/L Tris buffer (pH 7.0). The proportion of remineralizing solution per area of exposed enamel was 12 mL/mm^2. [157] During the cycling procedure, the specimens were rinsed with distilled water to avoid cross-contamination of the solutions. The tubes were kept in an incubator at 37°C and under constant agitation (Excella E24 Incubator Shaker Series, New

Brunswick Scientific Co., Inc. Enfield, CT). The pH of the solutions was checked daily and the solutions were replaced every four days. After each pH cycling the 4-week and 6- week groups received varnish applications every 4 and 6 weeks, respectively.

FluoreCam Final Imaging

At the end of the study, final FluoreCam imageing was conducted as previously described. The nail polish was removed, the specimens were reinserted into their molds, and a new set of FluoreCam images were captured. To test the reliability and validity of the FluoreCam, 10 randomly selected specimens from each group were reimaged the next day.

Statistical Analysis

The data were normally distributed. Paired t-tests were used to evaluate demineralization within each group (baseline vs final). One-way ANOVAs, followed by LSD post hoc tests, were used to evaluate between-group differences in the changes that occurred *(*Δ*=Final -Baseline*). The reliability of the repeated FluoreCam measurements was determined using intra-class correlations (ICCs) and method random errors. [197] The validity (systematic errors) of the measurements was determined using paired t-tests.

Results

Single measure ICCs were high for both area and intensity (Table 7). The method errors were 0.78 mm^2 and 0.51 pixels for area and intensity, respectively. There were no statistically significant systematic errors for either area or intensity.

At baseline, there were no statistically significant between-group differences in area ($p= 0.79$), intensity ($p= 0.11$), or impact ($p= 0.31$) (Table 9). Statistically significant

enamel demineralization ($p<0.001$) occurred over the 12-week experimental period within each group for each of the measures (Table 9).

There were statistically significant between-group differences in the changes that occurred in area (P=0.004) and impact (P=0.02), but not intensity (P=0.51) (Figure 13). Post-hoc tests showed that the control group had significantly larger areas of demineralization than the 6-week group (P=0.04) and the 4- week group (P=0.001). Changes in the control group impact were not significantly different than changes in 6-week group impact (P=0.30), but they were significantly greater than changes in the 4-week group impact (P=0.007). There were no statistically significant differences between 4- and 6-week groups in the changes of area (P=0.17), intensity (P=0.28), or impact (P=0.09).

Discussion

FluoreCam is a valid and reliable device for evaluating WSLs *in vitro*. FluoreCam provides an indirect method for assessing demineralization, relying upon the relationship between enamel fluorescence intensity and mineralization status of enamel. [150] In the present study, the repeated measurements of the FluoreCam were highly correlated, with ICC ranging from 0.90 to 0.95. The reliability was comparable to our initial study, in which the ICCs ranged from 0.87 to 0.98.[154] The method errors in the present study were all within acceptable limits, 0.78 mm^2 for area and 0.51 pixels for intensity, and similar to method errors previously reported for FluoreCam. [154] Slightly higher random errors (0.79 for area and 0.84 for intensity) were reported for the QLF, when it was used to image enamel demineralization around orthodontic brackets [198]

FluoreCam also provides unbiased repeated measurements, as previously reported for the FluoreCam [154] and QLF.[198]

A onetime application of CPP-ACP fluoride varnish enhances enamel resistance to demineralization. In the present study, although the FluoreCam detected demineralization after a single application of the fluoride varnish over the 12 weeks period, the lesions were difficult to see with naked eye. According to ICCMS (International Caries Classification and management System) [199] such lesions are considered initial, which represents the initial visual changes in enamel after air drying of the enamel. Such lesions are possibly reversible because they are shallow. [199] We previously showed that enamel treated with one application of the CPP-ACP fluoride varnish produces a mean lesion depth of 37 ±9 μm, compared to 190 ±34 μm in untreated control teeth (Chapter III).

Reapplication of CPP-ACP fluoride varnish further protects enamel by limiting the extent of the lesion. In the present study, repeated applications of CPP-ACP fluoride varnish every 4 and 6 weeks reduced the impact of enamel demineralization 24% and 50%, respectively, compared to a single application. Previous studies have demonstrated the benefit of fluoride reapplication in reducing the incidence of WSLs.[140,193] In the present study, repeated application every 4 and 6 weeks led to a significant reduction in area, but not in intensity. Since the intensity (loss of fluorescence) output reflects the depth of the enamel demineralization.[200] This implies that fluoride varnish treatment diminished the size but not depth of the enamel lesions. This can be attributed to the superficial and shallow lesions created following the application of CPP-ACP fluoride

varnish. Large and shallow area of enamel demineralization results in less fluorescent loss than large and deep lesions. As such, changes in the area of the lesion (measured in mm^2) do not necessitate changes in intensity, since the effect size is very small for the intensity (measure in pixels). There may have not been enough statistical power to detect small changings in intensity.

Reapplication of the Fluoride varnish at shorter intervals appears to make a difference. Shorter periodic CPP-ACP fluoride varnish application prolongs the preventive effect. We previously showed that demineralization of enamel after a single application of CPP-ACP varnish begins sometime between 4 and 8 weeks (Chapter III). Although there was no significant between-group difference, enamel treated every 4 weeks showed a tendency toward less demineralization. It appears that a period of 6 weeks may be too long to maintain the efficacy of the varnish. The lack of significance between the 4- and 6-weeks groups was due to small sample sizes and insufficient power. Post hoc power analyses revealed that there was insufficient power to detect between-group differences in area (0.436) and intensity (0.27). Other studies have shown that shorter intervals between reapplications of (5% NaF) fluoride varnish were effective in reversing active enamel caries lesions in the primary dentition.[201,202] Two applications of fluoride varnish over 4 months reversed 81.2% of the active lesions, compared to 37.8% in the control.[202] Weekly applications of fluoride varnish have been shown to be effective in preventing enamel demineralization for one month, but not for 3 months.[201]

Although the reapplication of CPP-ACP fluoride varnish significantly reduces WSLs formation, it does not prevent demineralization over time. In the present study,

enamel demineralization occurred after 12 weeks regardless of the frequency of the varnish application (once, every 4, or 6 weeks). Compared to the control enamel that received no fluoride varnish treatment in the previous study (Chapter III), the present study showed a 61.3% reduction in impact of enamel demineralization for a single application of fluoride varnish, a 70.4% reduction when applied every 6 weeks and an 80.5% reduction when applied every 4 weeks (Figure 14). It has been previously shown that fluoride varnish provides good protection, but it cannot entirely prevent demineralization.[140]

The varnish adheres to the tooth surface, providing prolonged fluoride-tooth contact, which enhances fluoride uptake of enamel and promotes the surface formation of calcium fluoride (CaF_2).[26,41,179] CaF_2 acts as an intraoral fluoride reservoir, which helps reverse demineralization when the oral pH becomes acidic.[26] However the fluoride varnish effect is probably limited because the CaF_2 is loosely bound to enamel and is gradually lost over time. [179] Interestingly, we previously showed that the application of the CCP-ACP prevents enamel demineralization 100% after 4 weeks in vitro (Chapter III).

. On that basis, we expected that reapplication of the varnish at least every 4 weeks would prevent WSLs formation. The demineralization that occurred after the three 4-weekly reapplications may have slightly biased the results. Although care was taken to remove and reapply loose and peeled off nail polish, it is possible that microleakage occurred, leading to additional demineralization over time of small areas of untreated enamel. This suggests that periodic varnish application should be

recommended as an adjunctive therapy for orthodontic patients, but not as the sole preventive measure for WSLs.

This study was not without limitations. The main limitation is that it is an *in vitro* study. The experiment was designed to provide extreme challenges (mechanical and thermal and chemical) that may exceed the oral conditions of most of the orthodontic patients. Patients with poor oral hygiene and at high risk of developing WSLs are less likely to brush their teeth regularly. Therefore, they are probably exposed to less brushing forces than those used in the present study. Mechanical forces from brushing can lead to abrasion of the superficial layer of enamel, which receives and stores most of the fluoride from the fluoride varnish application. Furthermore, we did not include additional topical fluoride sources for daily brushing that patients might be expected to perform every day. Further clinical investigations are required to confirm our findings.

CHAPTER V

CONCLUSION

In summary, with the limitations of these in vitro studies the following can be stated starting with Chapter I:

- CPP-ACP Fluoride varnish has a preventive effect on enamel WSLs formation.
- CO_2 laser apparently has the potential to increase acid resistance however, its application solely for WSLs prevention would not appear to be sensible under the present set of parameters.

Chapter II showed that, although CPP-ACP fluoride varnish does not completely prevent enamel demineralization, the reductions that occur warrant its clinical consideration. It should be considered for routine clinical use to prevent WSLs in orthodontics because:

- CPP-ACP fluoride varnish provides a net remineralization up to 2 weeks after application.
- The preventive effect of the fluoride varnish is 100% for at least 4 weeks.
- The overall demineralization that occurs after 12-weeks from the varnish application is equivalent to 2-weeks of demineralization if the varnish was not applied.

Chapter III showed that fluoride varnish containing CPP-ACP may be a useful aid to protect enamel from WSLs formation. Periodic application of the fluoride varnish

should be advocated as a routine measure to limit WSLs development for orthodontic patients because:

- Reapplication of CCP-ACP fluoride varnish reduces enamel demineralization.
- Because the preventive effects of the FV starts to decrease after 6 weeks, the varnish should be reapplied at least every 4 weeks to ensure that its preventive effect is maintained.

REFERENCES

1. Zero DT. Dental caries process. Dent Clin North Am 1999;434:635-664

2. Featherstone JD. The science and practice of caries prevention. J Am Dent Assoc 2000;1317:887-899

3. Margolis HC, Moreno EC. Physicochemical perspectives on the cariostatic mechanisms of systemic and topical fluorides. J Dent Res 1990;69 Spec No606-613; discussion 634-606

4. Sudjalim TR, Woods MG, Manton DJ. Prevention of white spot lesions in orthodontic practice: a contemporary review. Aust Dent J 2006;514:284-289; quiz 347

5. Shivakumar K, Prasad S, Chandu G. International Caries Detection and Assessment System: A new paradigm in detection of dental caries. J Conserv Dent 2009;121:10-16

6. Silverstone LM. Observations on the dark zone in early enamel caries and artificial caries-like lesions. Caries Res 1967;13:260-274

7. Silverstone LM. The surface zone in caries and in caries-like lesions produced in vitro. Br Dent J 1968;1254:145-157

8. Silverstone LM. Structure of carious enamel, including the early lesion. Oral Sci Rev 1973;3100-160

9. Hicks MJ, Silverstone LM. Acid-etching of caries-like lesions of enamel: a polarized light microscopic study. Caries Res 1984;184:315-326

10. Srivastava K, Tikku T, Khanna R, Sachan K. Risk factors and management of white spot lesions in orthodontics. J Orthod Sci 2013;22:43-49

11. Julien KC, Buschang PH, Campbell PM. Prevalence of white spot lesion formation during orthodontic treatment. Angle Orthod 2013;834:641-647

12. Mattousch TJ, van der Veen MH, Zentner A. Caries lesions after orthodontic treatment followed by quantitative light-induced fluorescence: a 2-year follow-up. Eur J Orthod 2007;293:294-298

13. Ogaard B, Ten Bosch JJ. Regression of white spot enamel lesions. A new optical method for quantitative longitudinal evaluation in vivo. Am J Orthod Dentofacial Orthop 1994;1063:238-242

14. Ekanayake LS, Sheiham A. Reducing rates of progression of dental caries in British schoolchildren. A study using bitewing radiographs. Br Dent J 1987;1638:265-269

15. Gorelick L, Geiger AM, Gwinnett AJ. Incidence of white spot formation after bonding and banding. Am J Orthod 1982;812:93-98

16. Mitchell L. An investigation into the effect of a fluoride releasing adhesive on the prevalence of enamel surface changes associated with directly bonded orthodontic attachments. Br J Orthod 1992;193:207-214

17. Zachrisson BU, Zachrisson S. Caries incidence and oral hygiene during orthodontic treatment. Scand J Dent Res 1971;796:394-401

18. Stratemann MW, Shannon IL. Control of decalcification in orthodontic patients by daily self-administered application of a water-free 0.4 per cent stannous fluoride gel. Am J Orthod 1974;663:273-279

19. Boersma JG, van der Veen MH, Lagerweij MD, Bokhout B, Prahl-Andersen B. Caries prevalence measured with QLF after treatment with fixed orthodontic appliances: influencing factors. Caries Res 2005;391:41-47

20. Kanthathas K, Willmot DR, Benson PE. Differentiation of developmental and post-orthodontic white lesions using image analysis. Eur J Orthod 2005;272:167-172

21. Al-Khateeb S, Exterkate R, Angmar-Mansson B, ten Cate JM. Effect of acid-etching on remineralization of enamel white spot lesions. Acta Odontol Scand 2000;581:31-36

22. Ismail AI, Burt BA, Eklund SA. The cariogenicity of soft drinks in the United States. J Am Dent Assoc 1984;1092:241-245

23. Heymann GC, Grauer D. A contemporary review of white spot lesions in orthodontics. J Esthet Restor Dent 2013;252:85-95

24. Graber TM. Orthodontics: principles and practice. Saunders Philadelphia; 1972.

25. Reynolds EC. Casein phosphopeptide-amorphous calcium phosphate: the scientific evidence. Adv Dent Res 2009;211:25-29

26. ten Cate JM, Featherstone JD. Mechanistic aspects of the interactions between fluoride and dental enamel. Crit Rev Oral Biol Med 1991;23:283-296

27. Levine RS. Fluoride and caries prevention: 1. Scientific rationale. Dent Update 1991;183:105-106, 108-110

28. Takahashi N, Washio J. Metabolomic effects of xylitol and fluoride on plaque biofilm in vivo. J Dent Res 2011;9012:1463-1468

29. Rosin-Grget K, Peros K, Sutej I, Basic K. The cariostatic mechanisms of fluoride. Acta Med Acad 2013;422:179-188

30. Bishara SE, Ostby AW. White spot lesions: formation, prevention, and treatment Seminars in Orthodontics: Elsevier; 2008: p. 174-182.

31. Alexander SA, Ripa LW. Effects of self-applied topical fluoride preparations in orthodontic patients. Angle Orthod 2000;706:424-430

32. Boyd RL, Chun YS. Eighteen-month evaluation of the effects of a 0.4% stannous fluoride gel on gingivitis in orthodontic patients. Am J Orthod Dentofacial Orthop 1994;1051:35-41

33. de Moura MS, de Melo Simplicio AH, Cury JA. In-vivo effects of fluoridated antiplaque dentifrice and bonding material on enamel demineralization adjacent to orthodontic appliances. Am J Orthod Dentofacial Orthop 2006;1303:357-363

34. O'Reilly MM, Featherstone JD. Demineralization and remineralization around orthodontic appliances: an in vivo study. Am J Orthod Dentofacial Orthop 1987;921:33-40

35. Benson PE, Shah AA, Millett DT, Dyer F, Parkin N, Vine RS. Fluorides, orthodontics and demineralization: a systematic review. J Orthod 2005;322:102-114

36. Geiger AM, Gorelick L, Gwinnett AJ, Benson BJ. Reducing white spot lesions in orthodontic populations with fluoride rinsing. Am J Orthod Dentofacial Orthop 1992;1015:403-407

37. Boyd RL. Comparison of three self-applied topical fluoride preparations for control of decalcification. Angle Orthod 1993;631:25-30

38. Johnston DW. Current status of professionally applied topical fluorides. Community Dent Oral Epidemiol 1994;223:159-163

39. Zachrisson BU. Fluoride application procedures in orthodontic practice, current concepts. Angle Orthod 1975;451:72-81

40. Whitford GM, Adair SM, Hanes CM, Perdue EC, Russell CM. Enamel uptake and patient exposure to fluoride: comparison of APF gel and foam. Pediatr Dent 1995;173:199-203

41. Hawkins R, Locker D, Noble J, Kay EJ. Prevention. Part 7: professionally applied topical fluorides for caries prevention. Br Dent J 2003;1956:313-317

42. Jiang H, Hua F, Yao L, Tai B, Du M. Effect of 1.23% acidulated phosphate fluoride foam on white spot lesions in orthodontic patients: a randomized trial. Pediatr Dent 2013;353:275-278

43. Mujeeb A, Mansuri S, Hussain SA, Ramaswamy K. In vitro evaluation of topical fluoride pH and their effect on surface hardness of composite resin-based restorative materials. J Contemp Dent Pract 2014;152:190-194

44. Arends J, Lodding A, Petersson LG. Fluoride uptake in enamel. In vitro comparison of topical agents. Caries Res 1980;146:403-413

45. Benson PE, Parkin N, Dyer F, Millett DT, Furness S, Germain P. Fluorides for the prevention of early tooth decay (demineralised white lesions) during fixed brace treatment. Cochrane Database Syst Rev 2013;12Cd003809

46. Frazier MC, Southard TE, Doster PM. Prevention of enamel demineralization during orthodontic treatment: an in vitro study using pit and fissure sealants. Am J Orthod Dentofacial Orthop 1996;1105:459-465

47. Benham AW, Campbell PM, Buschang PH. Effectiveness of pit and fissure sealants in reducing white spot lesions during orthodontic treatment. A pilot study. Angle Orthod 2009;792:338-345

48. Wenderoth CJ, Weinstein M, Borislow AJ. Effectiveness of a fluoride-releasing sealant in reducing decalcification during orthodontic treatment. Am J Orthod Dentofacial Orthop 1999;1166:629-634

49. Gwinnett AJ, Ceen RF. Plaque distribution on bonded brackets: a scanning microscope study. Am J Orthod 1979;756:667-677

50. Reynolds EC, Johnson IH. Effect of milk on caries incidence and bacterial composition of dental plaque in the rat. Arch Oral Biol 1981;265:445-451

51. Rosen S, Min DB, Harper DS, Harper WJ, Beck EX, Beck FM. Effect of cheese, with and without sucrose, on dental caries and recovery of Streptococcus mutans in rats. J Dent Res 1984;636:894-896

52. Harper DS, Osborn JC, Hefferren JJ, Clayton R. Cariostatic evaluation of cheeses with diverse physical and compositional characteristics. Caries Res 1986;202:123-130

53. Krobicka A, Bowen WH, Pearson S, Young DA. The effects of cheese snacks on caries in desalivated rats. J Dent Res 1987;666:1116-1119

54. Aimutis WR. Bioactive properties of milk proteins with particular focus on anticariogenesis. J Nutr 2004;1344:989s-995s

55. Cochrane NJ, Cai F, Huq NL, Burrow MF, Reynolds EC. New approaches to enhanced remineralization of tooth enamel. J Dent Res 2010;8911:1187-1197

56. Reynolds EC, Cain CJ, Webber FL, Black CL, Riley PF, Johnson IH et al. Anticariogenicity of calcium phosphate complexes of tryptic casein phosphopeptides in the rat. J Dent Res 1995;746:1272-1279

57. Cross KJ, Huq NL, Palamara JE, Perich JW, Reynolds EC. Physicochemical characterization of casein phosphopeptide-amorphous calcium phosphate nanocomplexes. J Biol Chem 2005;28015:15362-15369

58. Azarpazhooh A, Limeback H. Clinical efficacy of casein derivatives: a systematic review of the literature. J Am Dent Assoc 2008;1397:915-924; quiz 994-915

59. Walsh LJ. Contemporary technologies for remineralization therapies: A review. Int Dent SA 2009;434-46

60. Morgan MV, Adams GG, Bailey DL, Tsao CE, Fischman SL, Reynolds EC. The anticariogenic effect of sugar-free gum containing CPP-ACP nanocomplexes on approximal caries determined using digital bitewing radiography. Caries Res 2008;423:171-184

61. Rao SK, Bhat GS, Aradhya S, Devi A, Bhat M. Study of the efficacy of toothpaste containing casein phosphopeptide in the prevention of dental caries: a randomized controlled trial in 12- to 15-year-old high caries risk children in Bangalore, India. Caries Res 2009;436:430-435

62. Ramalingam L, Messer LB, Reynolds EC. Adding casein phosphopeptide-amorphous calcium phosphate to sports drinks to eliminate in vitro erosion. Pediatr Dent 2005;271:61-67

63. Reynolds E. Remineralization of enamel subsurface lesions by casein phosphopeptide-stabilized calcium phosphate solutions. Journal of Dental Research 1997;769:1587-1595

64. Pithon MM, Dos Santos MJ, Andrade CS, Leao Filho JC, Braz AK, de Araujo RE et al. Effectiveness of varnish with CPP-ACP in prevention of caries lesions around orthodontic brackets: an OCT evaluation. Eur J Orthod 2014

65. He WD, Liu YZ, Xu YY, Chen D. [Study on application of CPP-ACP on tooth mineralization during orthodontic treatment with fixed appliance]. Shanghai Kou Qiang Yi Xue 2010;192:140-143(Abstract)

66. Cochrane NJ, Reynolds EC. Calcium phosphopeptides -- mechanisms of action and evidence for clinical efficacy. Adv Dent Res 2012;242:41-47

67. Uysal T, Amasyali M, Koyuturk AE, Ozcan S. Effects of different topical agents on enamel demineralization around orthodontic brackets: an in vivo and in vitro study. Aust Dent J 2010;553:268-274

68. Reynolds EC, Cai F, Shen P, Walker GD. Retention in plaque and remineralization of enamel lesions by various forms of calcium in a mouthrinse or sugar-free chewing gum. J Dent Res 2003;823:206-211

69. Rose RK. Binding characteristics of Streptococcus mutans for calcium and casein phosphopeptide. Caries Res 2000;345:427-431

70. Rose RK. Effects of an anticariogenic casein phosphopeptide on calcium diffusion in streptococcal model dental plaques. Arch Oral Biol 2000;457:569-575

71. Schupbach P, Neeser JR, Golliard M, Rouvet M, Guggenheim B. Incorporation of caseinoglycomacropeptide and caseinophosphopeptide into the salivary pellicle inhibits adherence of mutans streptococci. J Dent Res 1996;7510:1779-1788

72. Cross KJ, Huq NL, Stanton DP, Sum M, Reynolds EC. NMR studies of a novel calcium, phosphate and fluoride delivery vehicle-alpha(S1)-casein(59-79) by stabilized amorphous calcium fluoride phosphate nanocomplexes. Biomaterials 2004;2520:5061-5069

73. Cochrane NJ, Saranathan S, Cai F, Cross KJ, Reynolds EC. Enamel subsurface lesion remineralisation with casein phosphopeptide stabilised solutions of calcium, phosphate and fluoride. Caries Res 2008;422:88-97

74. Reynolds EC, Cai F, Cochrane NJ, Shen P, Walker GD, Morgan MV et al. Fluoride and casein phosphopeptide-amorphous calcium phosphate. J Dent Res 2008;874:344-348

75. Iijima Y, Cai F, Shen P, Walker G, Reynolds C, Reynolds EC. Acid resistance of enamel subsurface lesions remineralized by a sugar-free chewing gum containing casein phosphopeptide-amorphous calcium phosphate. Caries Res 2004;386:551-556

76. Kumar VL, Itthagarun A, King NM. The effect of casein phosphopeptide-amorphous calcium phosphate on remineralization of artificial caries-like lesions: an in vitro study. Aust Dent J 2008;531:34-40

77. Cochrane NJ, Shen P, Yuan Y, Reynolds EC. Ion release from calcium and fluoride containing dental varnishes. Aust Dent J 2014;591:100-105

78. Verma SK, Maheshwari S, Singh RK, Chaudhari PK. Laser in dentistry: An innovative tool in modern dental practice. Natl J Maxillofac Surg 2012;32:124-132

79. Maiman TH. Stimulated optical radiation in ruby 1960

80. Stern RH, Sognnaes RF, Goodman F. Laser effect on in vitro enamel permeability and solubility. J Am Dent Assoc 1966;734:838-843

81. Ballal NV, Kundabal M, Bhat KS. Lasers General Principles - A Review. International Journal of Clinical Dentistry 2011;43:133-146. http://search.ebscohost.com/login.aspx?direct=true&db=ddh&AN=64366182&site= eds-live&group=test

82. Walsh LJ. The current status of laser applications in dentistry. Aust Dent J 2003;483:146-155; quiz 198

83. Guptaa S, Kumarb S. Lasers in Dentistry-An Overview. Trends Biomater. Artif. Organs 2011;253: 119-123.http://medind.nic.in/taa/t11/i3/taat11i3p119.pdf

84. Pohlhaus SR. Dental Laers. Inside Dentistry 2012;812:2 .http://www.dentalaegis.com/id/2012/12/dental-lasers

85. Yamamoto H, Sato K. Prevention of dental caries by acousto-optically Q-switched Nd: YAG laser irradiation. J Dent Res 1980;592:137

86. Hossain MM, Hossain M, Kimura Y, Kinoshita J, Yamada Y, Matsumoto K. Acquired acid resistance of enamel and dentin by CO2 laser irradiation with sodium fluoride solution. J Clin Laser Med Surg 2002;202:77-82

87. Rezaei Y, Bagheri H, Esmaeilzadeh M. Effects of Laser Irradiation on Caries Prevention. Journal of Lasers in Medical Sciences 2012;24:159-164

88. Tavares JG, Eduardo Cde P, Burnett LH, Jr., Boff TR, de Freitas PM. Argon and Nd:YAG lasers for caries prevention in enamel. Photomed Laser Surg 2012;308:433-437

89. Hossain M, Nakamura Y, Kimura Y, Yamada Y, Ito M, Matsumoto K. Caries-preventive effect of Er:YAG laser irradiation with or without water mist. J Clin Laser Med Surg 2000;182:61-65

90. Kim JH, Kwon OW, Kim HI, Kwon YH. Acid resistance of erbium-doped yttrium aluminum garnet laser-treated and phosphoric acid-etched enamels. Angle Orthod 2006;766:1052-1056

91. Ahrari F, Poosti M, Motahari P. Enamel resistance to demineralization following Er:YAG laser etching for bonding orthodontic brackets. Dent Res J (Isfahan) 2012;94:472-477

92. Apel C, Meister J, Schmitt N, Graber HG, Gutknecht N. Calcium solubility of dental enamel following sub-ablative Er:YAG and Er:YSGG laser irradiation in vitro. Lasers Surg Med 2002;305:337-341

93. Zuerlein MJ, Fried D, Featherstone JD. Modeling the modification depth of carbon dioxide laser-treated dental enamel. Lasers Surg Med 1999;254:335-347

94. Rodrigues LK, Nobre dos Santos M, Pereira D, Assaf AV, Pardi V. Carbon dioxide laser in dental caries prevention. J Dent 2004;327:531-540

95. Nelson DG, Shariati M, Glena R, Shields CP, Featherstone JD. Effect of pulsed low energy infrared laser irradiation on artificial caries-like lesion formation. Caries Res 1986;204:289-299

96. Nelson DG, Jongebloed WL, Featherstone JD. Laser irradiation of human dental enamel and dentine. N Z Dent J 1986;82369:74-77

97. Featherstone JD, Barrett-Vespone NA, Fried D, Kantorowitz Z, Seka W. CO2 laser inhibitor of artificial caries-like lesion progression in dental enamel. J Dent Res 1998;776:1397-1403

98. Young DA, Fried D, Featherstone JD. Treating occlusal pit and fissure surfaces by IR laser irradiation BiOS 2000 The International Symposium on Biomedical Optics: International Society for Optics and Photonics; 2000: p. 247-253.

99. Kantorowitz Z, Featherstone JD, Fried D. Caries prevention by CO2 laser treatment: dependency on the number of pulses used. J Am Dent Assoc 1998;1295:585-591

100. Esteves-Oliveira M, Zezell DM, Meister J, Franzen R, Stanzel S, Lampert F et al. CO2 Laser (10.6 microm) parameters for caries prevention in dental enamel. Caries Res 2009;434:261-268

101. Hsu CY, Jordan TH, Dederich DN, Wefel JS. Effects of low-energy CO2 laser irradiation and the organic matrix on inhibition of enamel demineralization. J Dent Res 2000;799:1725-1730

102. Esteves-Oliveira M, Zezell DM, Ana PA, Yekta SS, Lampert F, Eduardo CP. Dentine caries inhibition through CO2 laser (10.6mum) irradiation and fluoride application, in vitro. Arch Oral Biol 2011;566:533-539

103. Esteves-Oliveira M, Pasaporti C, Heussen N, Eduardo CP, Lampert F, Apel C. Prevention of toothbrushing abrasion of acid-softened enamel by CO2 laser irradiation. J Dent 2011;399:604-611

104. Fox JL, Yu D, Otsuka M, Higuchi WI, Wong J, Powell G. Combined effects of laser irradiation and chemical inhibitors on the dissolution of dental enamel. Caries Res 1992;265:333-339

105. Fried D, Ragadio J, Akrivou M, Featherstone JD, Murray MW, Dickenson KM. Dental hard tissue modification and removal using sealed transverse excited atmospheric-pressure lasers operating at lambda=9.6 and 10.6 microm. J Biomed Opt 2001;62:231-238

106. Fried D, Zuerlein MJ, Le CQ, Featherstone JD. Thermal and chemical modification of dentin by 9-11-microm CO2 laser pulses of 5-100-micros duration. Lasers Surg Med 2002;314:275-282

107. Zach L, Cohen G. Pulp response to externally applied heat. Oral Surg Oral Med Oral Pathol 1965;19515-530

108. McCormack SM, Fried D, Featherstone JD, Glena RE, Seka W. Scanning electron microscope observations of CO2 laser effects on dental enamel. J Dent Res 1995;7410:1702-1708

109. Fried D, Seka W, Glena RE, Featherstone JD. Thermal response of hard dental tissues to 9-through 11-μm CO2-laser irradiation. Optical Engineering 1996;357:1976-1984

110. Rechmann P, Fried D, Le CQ, Nelson G, Rapozo-Hilo M, Rechmann BM et al. Caries inhibition in vital teeth using 9.6-mum CO2-laser irradiation. J Biomed Opt 2011;167:071405

111. Liu Y, Hsu CY, Teo CM, Teoh SH. Potential mechanism for the laser-fluoride effect on enamel demineralization. J Dent Res 2013;921:71-75

112. Fowler BO, Kuroda S. Changes in heated and in laser-irradiated human tooth enamel and their probable effects on solubility. Calcif Tissue Int 1986;384:197-208

113. Christoffersen J, Christoffersen MR. Kinetics of dissolution of calcium hydroxyapatite: IV. The effect of some biologically important inhibitors. Journal of Crystal Growth 1981;531:42-54

114. Sato K. Relation between Acid Dissolution and Histological Alteration of Heated Tooth Enamel (with 1 color plate). Caries research 1983;176:490-495

115. Sakae T. X-ray diffraction and thermal studies of crystals from the outer and inner layers of human dental enamel. Archives of oral biology 1988;3310:707-713

116. Ying D, Chuah G, Hsu C-YS. Effect of Er: YAG laser and organic matrix on porosity changes in human enamel. Journal of dentistry 2004;321:41-46

117. Maung N, Wohland T, Hsu C. Enamel diffusion modulated by Er: YAG laser:(Part 1)—FRAP. Journal of dentistry 2007;3510:787-793

118. Borggreven JM, van Dijk JW, Driessens FC. Effect of laser irradiation on the permeability of bovine dental enamel. Arch Oral Biol 1980;2511-12:831-832

119. Nelson DG, Wefel JS, Jongebloed WL, Featherstone JD. Morphology, histology and crystallography of human dental enamel treated with pulsed low-energy infrared laser radiation. Caries Res 1987;215:411-426

120. Mathew A, Reddy NV, Sugumaran DK, Peter J, Shameer M, Dauravu LM. Acquired acid resistance of human enamel treated with laser (Er:YAG laser and Co2 laser) and acidulated phosphate fluoride treatment: An in vitro atomic emission spectrometry analysis. Contemp Clin Dent 2013;42:170-175

121. Hsu CY, Jordan TH, Dederich DN, Wefel JS. Laser-matrix-fluoride effects on enamel demineralization. J Dent Res 2001;809:1797-1801

122. Chin-Ying SH, Xiaoli G, Jisheng P, Wefel JS. Effects of CO2 laser on fluoride uptake in enamel. J Dent 2004;322:161-167

123. Larsen M, Jensen S. Solubility, unit cell dimensions and crystallinity of fluoridated human dental enamel. Archives of oral biology 1989;3412:969-973

124. Steiner-Oliveira C, Rodrigues LK, Soares LE, Martin AA, Zezell DM, Nobre-dos-Santos M. Chemical, morphological and thermal effects of 10.6-microm CO2 laser on the inhibition of enamel demineralization. Dent Mater J 2006;253:455-462

125. Can AM, Darling CL, Ho C, Fried D. Non-destructive assessment of inhibition of demineralization in dental enamel irradiated by a lambda=9.3-microm CO2 laser at ablative irradiation intensities with PS-OCT. Lasers Surg Med 2008;405:342-349

126. Hsu DJ, Darling CL, Lachica MM, Fried D. Nondestructive assessment of the inhibition of enamel demineralization by CO2 laser treatment using polarization sensitive optical coherence tomography. J Biomed Opt 2008;135:054027

127. Lakshmi A, Shobha D, Lakshminarayanan L. Prevention of caries by pulsed CO2 laser pre-treatment of enamel: an in-vitro study. J Indian Soc Pedod Prev Dent 2001;194:152-156

128. Featherstone JD, Barrett-Vespone NA, Fried D, Kantorowitz Z, Lofthouse J, Seka WD. Rational choice of laser conditions for inhibition of caries progression Photonics West'95: International Society for Optics and Photonics; 1995: p. 57-67.

129. de Freitas PM, Rapozo-Hilo M, Eduardo Cde P, Featherstone JD. In vitro evaluation of erbium, chromium:yttrium-scandium-gallium-garnet laser-treated enamel demineralization. Lasers Med Sci 2010;252:165-170

130. Featherstone JD. Caries detection and prevention with laser energy. Dent Clin North Am 2000;444:955-969, ix

131. Liu Y, Hsu CY, Teo CM, Teoh SH. Subablative Er:YAG laser effect on enamel demineralization. Caries Res 2013;471:63-68

132. Goodis HE, Fried D, Gansky S, Rechmann P, Featherstone JD. Pulpal safety of 9.6 microm TEA CO2 laser used for caries prevention. Lasers Surg Med 2004;352:104-110

133. Esteves-Oliveira M, Pasaporti C, Heussen N, Eduardo CP, Lampert F, Apel C. Rehardening of acid-softened enamel and prevention of enamel softening through CO2 laser irradiation. J Dent 2011;396:414-421

134. Schmidlin PR, Dorig I, Lussi A, Roos M, Imfeld T. CO2 laser-irradiation through topically applied fluoride increases acid resistance of demineralised human enamel in vitro. Oral Health Prev Dent 2007;53:201-208

135. Tepper SA, Zehnder M, Pajarola GF, Schmidlin PR. Increased fluoride uptake and acid resistance by CO2 laser-irradiation through topically applied fluoride on human enamel in vitro. J Dent 2004;328:635-641

136. Behnan SM, Arruda AO, Gonzalez-Cabezas C, Sohn W, Peters MC. In-vitro evaluation of various treatments to prevent demineralization next to orthodontic brackets. Am J Orthod Dentofacial Orthop 2010;1386:712.e711-717; discussion 712-713

137. Brown MD, Campbell PM, Schneiderman ED, Buschang PH. A practice-based evaluation of the prevalence and predisposing etiology of white spot lesions. Angle Orthod 2015

138. Opsahl Vital S, Haignere-Rubinstein C, Lasfargues JJ, Chaussain C. Caries risk and orthodontic treatment. Int Orthod 2010;81:28-45

139. Tufekci E, Dixon JS, Gunsolley JC, Lindauer SJ. Prevalence of white spot lesions during orthodontic treatment with fixed appliances. Angle Orthod 2011;812:206-210

140. Stecksen-Blicks C, Renfors G, Oscarson ND, Bergstrand F, Twetman S. Caries-preventive effectiveness of a fluoride varnish: a randomized controlled trial in adolescents with fixed orthodontic appliances. Caries Res 2007;416:455-459

141. Farhadian N, Miresmaeili A, Eslami B, Mehrabi S. Effect of fluoride varnish on enamel demineralization around brackets: an in-vivo study. Am J Orthod Dentofacial Orthop 2008;1334 Suppl:S95-98

142. Poggio C, Lombardini M, Dagna A, Chiesa M, Bianchi S. Protective effect on enamel demineralization of a CPP-ACP paste: an AFM in vitro study. J Dent 2009;3712:949-954

143. Oshiro M, Yamaguchi K, Takamizawa T, Inage H, Watanabe T, Irokawa A et al. Effect of CPP-ACP paste on tooth mineralization: an FE-SEM study. J Oral Sci 2007;492:115-120

144. Pai D, Bhat SS, Taranath A, Sargod S, Pai VM. Use of laser fluorescence and scanning electron microscope to evaluate remineralization of incipient enamel lesions

remineralized by topical application of casein phospho peptide amorphous calcium phosphate (CPP-aCP) containing cream. J Clin Pediatr Dent 2008;323:201-206

145. Tuloglu N, Bayrak S, Tunc ES, Ozer F. Effect of fluoride varnish with added casein phosphopeptide-amorphous calcium phosphate on the acid resistance of the primary enamel. BMC Oral Health 2016;161:103

146. Al Dehailan L, Martinez-Mier EA, Lippert F. The effect of fluoride varnishes on caries lesions: an in vitro investigation. Clin Oral Investig 2016;207:1655-1662

147. Tranaeus S, Shi XQ, Lindgren LE, Trollsas K, Angmar-Mansson B. In vivo repeatability and reproducibility of the quantitative light-induced fluorescence method. Caries Res 2002;361:3-9

148. Gomez J, Tellez M, Pretty IA, Ellwood RP, Ismail AI. Non-cavitated carious lesions detection methods: a systematic review. Community Dent Oral Epidemiol 2013;411:54-66

149. Angmar-Mansson B, Bosch Jt. Quantitative light-induced fluorescence (QLF): a method for assessment of incipient caries lesions. Dentomaxillofacial Radiology 2001;306:298-307

150. Bilgin G, Yanikoglu F, Tagtekin D, Stookey GK, BR S. Remineralization Potential of Different Agents and Assessment by a New Caries Detection Device. Gavin Journal of Dental Sciences 2016;20161:5

151. Korkut B, Korkut D, Yanikoglu F, Tagtekin D. Clinical assessment of demineralization and remineralization surrounding orthodontic brackets with FluoreCam. Asian Pacific Journal of Tropical Biomedicine 2017.http://www.sciencedirect.com/science/article/pii/S2221169116308516

152. Diniz MB, Eckert GJ, Gonzalez-Cabezas C, Cordeiro RC, Ferreira-Zandona AG. Caries Detection around Restorations Using ICDAS and Optical Devices. J Esthet Restor Dent 2016

153. Stookey GK, Isaacs RL, White VA, Nuñez AJ, Collins BD, Williams JS et al. Clinical Monitoring of White Spot Lesions in Children: 12-Month Data (Abstract). Journal Dental Reseach 2011;90A:

154. Abufarwa M, Noureldin A, Campbell PM, Buschang PH. Reliability and validity of FluoreCam for white-spot lesion detection: An in vitro study. J Investig Clin Dent 2017

155. Abufarwa M, Noureldin A, Campbell PM, Buschang PH. Comparative study of two chemical protocols for creating white spot lesions: An in vitro FluoreCam evaluation. J Investig Clin Dent 2017

156. Faul F, Erdfelder E, Lang AG, Buchner A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods 2007;392:175-191

157. Queiroz CS, Hara AT, Paes Leme AF, Cury JA. pH-cycling models to evaluate the effect of low fluoride dentifrice on enamel de- and remineralization. Braz Dent J 2008;191:21-27

158. Kato J, Moriya K, Jayawardena JA, Wijeyeweera RL, Awazu K. Prevention of Dental Caries in Partially Erupted Permanent Teeth with CO2 Laser. Journal of Clinical Laser Mendicine& Surgery 2003;216:6

159. Cuy JL, Mann AB, Livi KJ, Teaford MF, Weihs TP. Nanoindentation mapping of the mechanical properties of human molar tooth enamel. Arch Oral Biol 2002;474:281-291

160. Milly H, Festy F, Andiappan M, Watson TF, Thompson I, Banerjee A. Surface pre-conditioning with bioactive glass air-abrasion can enhance enamel white spot lesion remineralization. Dent Mater 2015;315:522-533

161. Delbem A, Sassaki K, Vieira A, Rodrigues E, Bergamaschi M, Stock S et al. Comparison of methods for evaluating mineral loss: hardness versus synchrotron microcomputed tomography. Caries research 2008;435:359-365

162. Robertson MA, Kau CH, English JD, Lee RP, Powers J, Nguyen JT. MI Paste Plus to prevent demineralization in orthodontic patients: a prospective randomized controlled trial. Am J Orthod Dentofacial Orthop 2011;1405:660-668

163. Llena C, Leyda AM, Forner L. CPP-ACP and CPP-ACFP versus fluoride varnish in remineralisation of early caries lesions. A prospective study. Eur J Paediatr Dent 2015;163:181-186

164. Abdullah Z, John J. Minimally Invasive Treatment of White Spot Lesions--A Systematic Review. Oral Health Prev Dent 2016;143:197-205

165. Featherstone J, Ten Cate J, Shariati M, Arends J. Comparison of artificial caries-like lesions by quantitative microradiography and microhardness profiles. Caries research 1983;175:385-391

166. Kodaka T, Debari K, Yamada M, Kuroiwa M. Correlation between microhardness and mineral content in sound human enamel (short communication). Caries Res 1992;262:139-141

167. Petersson LG, Odelius H, Lodding A, Larsson SJ, Frostell G. Ion probe study of fluorine gradients in outermost layers of human enamel. J Dent Res 1976;556:980-990

168. Chu JS, Fox JL, Higuchi WI, Nash WP. Electron probe micro-analysis for subsurface demineralization and remineralization of dental enamel. J Dent Res 1989;681:26-31

169. de Souza-e-Silva CM, Parisotto TM, Steiner-Oliveira C, Kamiya RU, Rodrigues LK, Nobre-dos-Santos M. Carbon dioxide laser and bonding materials reduce enamel demineralization around orthodontic brackets. Lasers Med Sci 2013;281:111-118

170. Paulos RS, Seino PY, Fukushima KA, Marques MM, de Almeida FCS, Ramalho KM et al. Effect of Nd:YAG and CO2 Laser Irradiation on Prevention of Enamel Demineralization in Orthodontics: In Vitro Study. Photomed Laser Surg 2017;355:282-286

171. Ramos-Oliveira TM, Ramos TM, Esteves-Oliveira M, Apel C, Fischer H, Eduardo Cde P et al. Potential of CO2 lasers (10.6 microm) associated with fluorides in inhibiting human enamel erosion. Braz Oral Res 2014;281-6

172. Mirhashemi AH, Hakimi S, Ahmad Akhoundi MS, Chiniforush N. Prevention of Enamel Adjacent to Bracket Demineralization Following Carbon Dioxide Laser Radiation and Titanium Tetra Fluoride Solution Treatment: An In Vitro Study. J Lasers Med Sci 2016;73:192-196

173. Jordao MC, Forti GM, Navarro RS, Freitas PM, Honorio HM, Rios D. CO2 laser and/or fluoride enamel treatment against in situ/ex vivo erosive challenge. J Appl Oral Sci 2016;243:223-228

174. Steiner-Oliveira C, Nobre-dos-Santos M, Zero DT, Eckert G, Hara AT. Effect of a pulsed CO2 laser and fluoride on the prevention of enamel and dentine erosion. Arch Oral Biol 2010;552:127-133

175. Souza-Gabriel AE, Colucci V, Turssi CP, Serra MC, Corona SA. Microhardness and SEM after CO(2) laser irradiation or fluoride treatment in human and bovine enamel. Microsc Res Tech 2010;7311:1030-1035

176. Souza-Gabriel AE, Turssi CP, Colucci V, Tenuta LM, Serra MC, Corona SA. In situ study of the anticariogenic potential of fluoride varnish combined with CO2 laser on enamel. Arch Oral Biol 2015;606:804-810

177. Rechmann P, Charland DA, Rechmann BM, Le CQ, Featherstone JD. In-vivo occlusal caries prevention by pulsed CO2 -laser and fluoride varnish treatment--a clinical pilot study. Lasers Surg Med 2013;455:302-310

178. Chapman JA, Roberts WE, Eckert GJ, Kula KS, Gonzalez-Cabezas C. Risk factors for incidence and severity of white spot lesions during treatment with fixed orthodontic appliances. Am J Orthod Dentofacial Orthop 2010;1382:188-194

179. Retief DH, Sorvas PG, Bradley EL, Taylor RE, Walker AR. In vitro fluoride uptake, distribution and retention by human enamel after 1- and 24-hour application of various topical fluoride agents. J Dent Res 1980;593:573-582

180. Weyant RJ, Tracy SL, Anselmo TT, Beltrán-Aguilar ED, Donly KJ, Frese WA et al. Topical fluoride for caries prevention. The Journal of the American Dental Association 2013;14411:1279-1291

181. Abufarwa M, Voorhees RD, Varanasi VG, Campbell PM, Buschang PH. White spot lesions: Does etching really matter? J Investig Clin Dent 2017

182. Brown W, Jacobs H, Thompson R. Thermal fatigue in teeth. Journal of dental research 1972;512:461-467

183. Buren JL, Staley RN, Wefel J, Qian F. Inhibition of enamel demineralization by an enamel sealant, Pro Seal: an in-vitro study. Am J Orthod Dentofacial Orthop 2008;1334 Suppl:S88-94

184. Du M, Cheng N, Tai B, Jiang H, Li J, Bian Z. Randomized controlled trial on fluoride varnish application for treatment of white spot lesion after fixed orthodontic treatment. Clin Oral Investig 2012;162:463-468

185. Aasenden R, Brudevold F, McCann HG. The response of intact and experimentally altered human enamel to topical fluoride. Arch Oral Biol 1968;135:543-552

186. DePaola PF, Aasenden R, Brudevold F. The use of topically applied acidulated phosphate-fluoride preceded by mild etching of the enamel: a one-year clinical trial. Arch Oral Biol 1971;1610:1155-1163

187. Belser U, Sporri S, Muhlemann HR. Uptake and retention of fluoride by intact and etched enamel. Helv Odontol Acta 1975;192:69-71

188. Knosel M, Forslund L, Jung K, Ziebolz D. Efficacy of different strategies in protecting enamel against demineralization during fixed orthodontic treatment. J Orofac Orthop 2012;733:194-203

189. Abufarwa M, Noureldin A, Campbell PM, Buschnag PH. Reliability and validity of FluoreCam for White-Spot-Lesion Detection. Journal of Investigative and Clinical Dentistry 2016; Forthcoming

190. Demito CF, Rodrigues GV, Ramos AL, Bowman SJ. Efficacy of a fluoride varnish in preventing white-spot lesions as measured with laser fluorescence. J Clin Orthod 2011;451:25-29; quiz 40

191. Vivaldi-Rodrigues G, Demito CF, Bowman SJ, Ramos AL. The effectiveness of a fluoride varnish in preventing the development of white spot lesions. World J Orthod 2006;72:138-144

192. Majithia U, Venkataraghavan K, Choudhary P, Trivedi K, Shah S, Virda M. Comparative evaluation of application of different fluoride varnishes on artificial early enamel lesion: An in vitro study. Indian J Dent Res 2016;275:521-527

193. Lenzi TL, Montagner AF, Soares FZ, de Oliveira Rocha R. Are topical fluorides effective for treating incipient carious lesions?: A systematic review and meta-analysis. J Am Dent Assoc 2016;1472:84-91.e81

194. Korkut B, Korkut D, Yanikoglu F, Tagtekin D. Clinical assessment of demineralization and remineralization surrounding orthodontic brackets with FluoreCam. Asian Pacific Journal of Tropical Biomedicine 2017;74:373-377

195. Diniz MB, Eckert GJ, González-Cabezas C, Cordeiro RdCL, Ferreira-Zandona AG. Caries Detection around Restorations Using ICDAS and Optical Devices. Journal of Esthetic and Restorative Dentistry 2016;282:110-121

196. Tranaeus S, Shi X-Q, Trollsas K, Lindgren L-E, Angmar-Mansson B. In-vivo quantification of natural incipient caries lesions using the quantitative light-induced fluoroscence method: a reproducibility study BiOS 2000 The International Symposium on Biomedical Optics. Sweden: International Society for Optics and Photonics; 2000: p. 235-241.

197. Galvão MCdS, Sato JR, Coelho EC. Dahlberg formula: a novel approach for its evaluation. Dental Press Journal of Orthodontics 2012;171:115-124

198. Benson PE, Pender N, Higham SM. Quantifying enamel demineralization from teeth with orthodontic brackets—a comparison of two methods. Part 1: repeatability and agreement. The European Journal of Orthodontics 2003;252:149-158

199. Ismail AI, Pitts NB, Tellez M, Banerjee A, Deery C, Douglas G et al. The International Caries Classification and Management System (ICCMS) An Example of a Caries Management Pathway. BMC Oral Health 2015;15 Suppl 1S9

200. Wu J, Donly ZR, Donly KJ, Hackmyer S. Demineralization Depth Using QLF and a Novel Image Processing Software. Int J Dent 2010;2010958264

201. de Amorim RG, Leal SC, Bezerra AC, de Amorim FP, de Toledo OA. Association of chlorhexidine and fluoride for plaque control and white spot lesion remineralization in primary dentition. Int J Paediatr Dent 2008;186:446-451

202. Autio-Gold JT, Courts F. Assessing the effect of fluoride varnish on early enamel carious lesions in the primary dentition. J Am Dent Assoc 2001;1329:1247-1253; quiz 1317-1248

203. Ogaard B, Duschner H, Ruben J, Arends J. Microradiography and confocal laser scanning microscopy applied to enamel lesions formed in vivo with and without fluoride varnish treatment. Eur J Oral Sci 1996;1044 (Pt 1):378-383

204. Todd MA, Staley RN, Kanellis MJ, Donly KJ, Wefel JS. Effect of a fluoride varnish on demineralization adjacent to orthodontic brackets. Am J Orthod Dentofacial Orthop 1999;1162:159-167

APPENDIX A

FIGURES

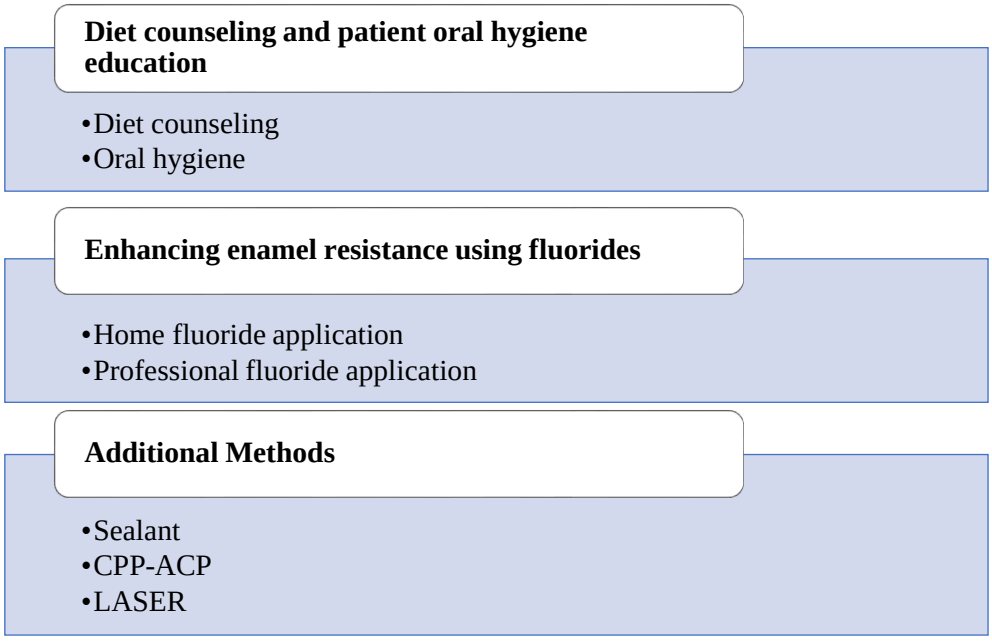

Figure 1 Summary of the preventive measures that are recommended for the prevention of WSLs formation.

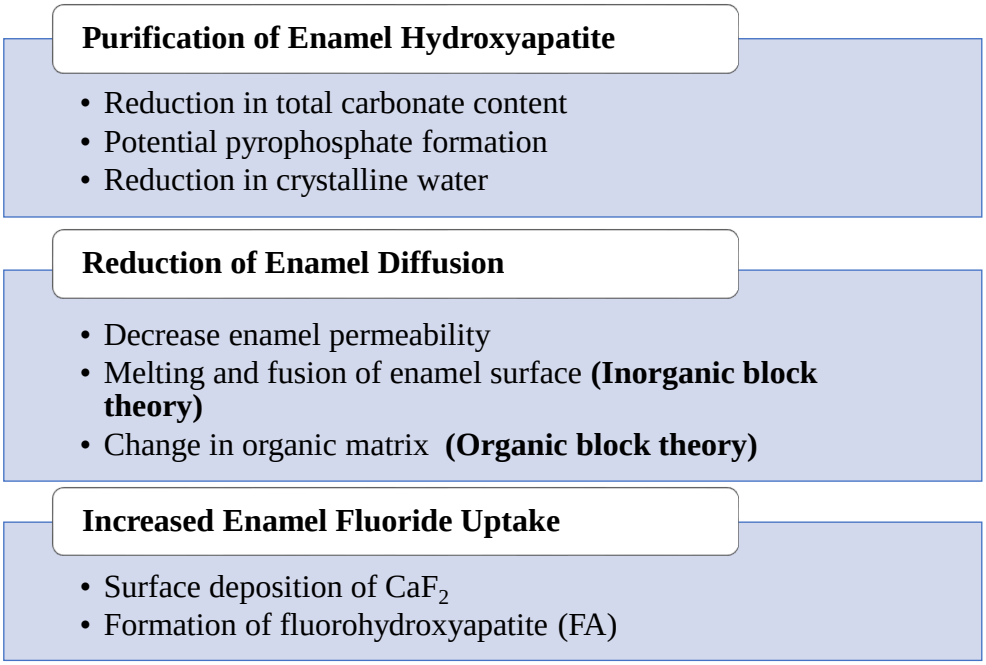

Figure 2 Summary of suggested mechanisms of the laser preventive effect on enamel demineralization.

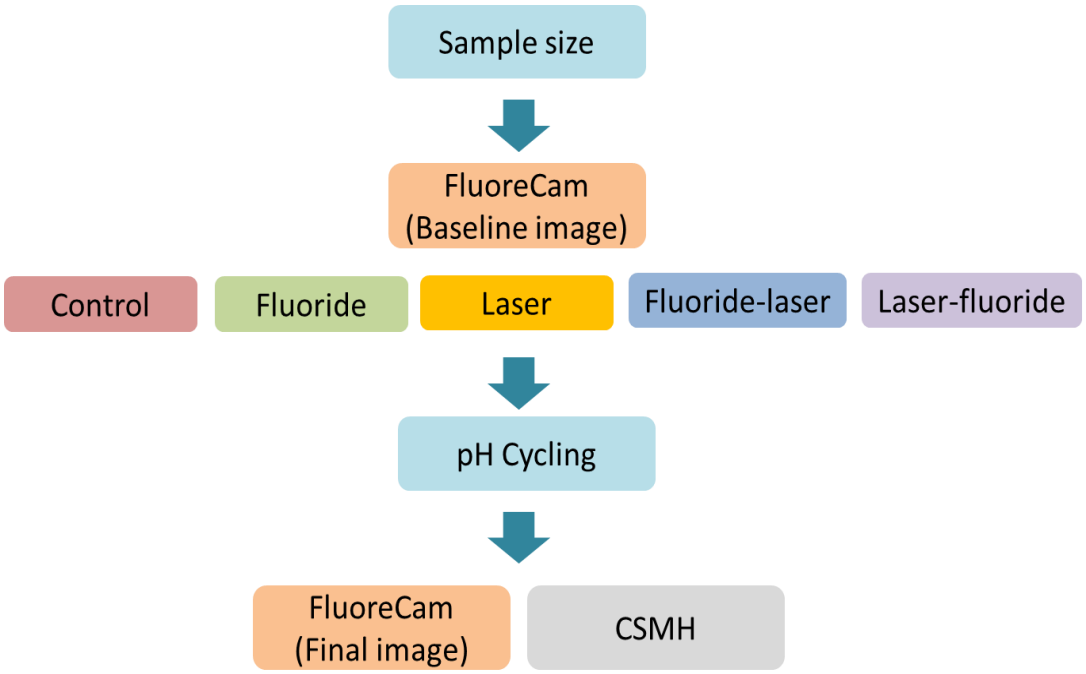

Figure 3 Follow chart illustrating the design of the experiment.

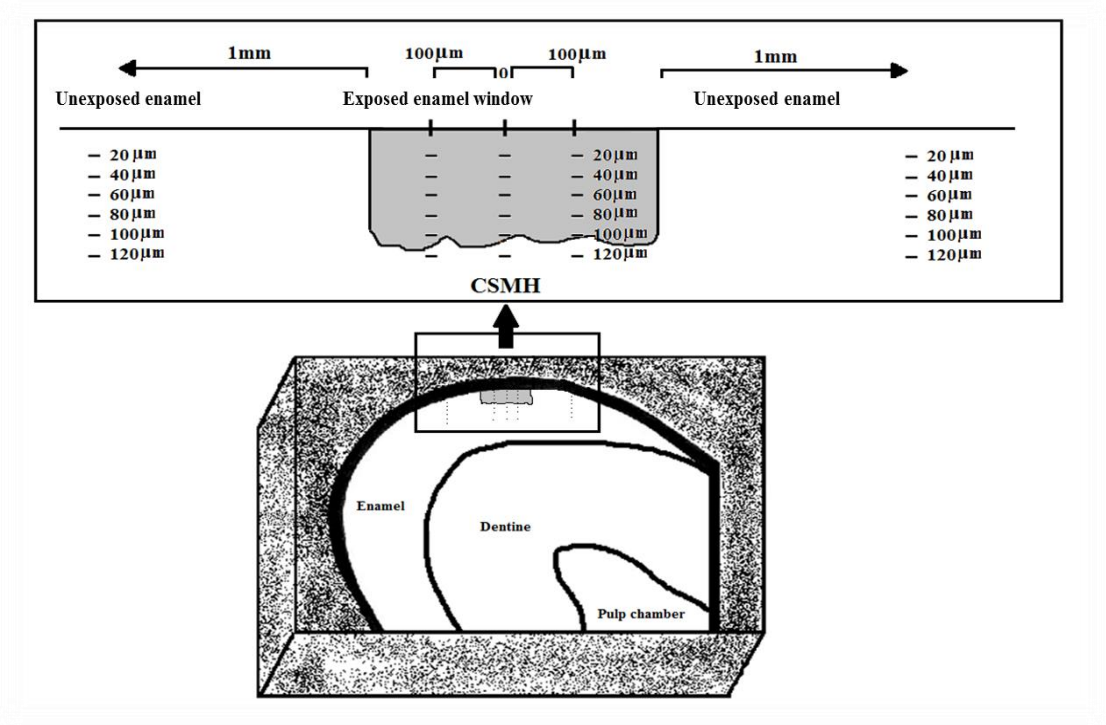

Figure 4 Illustration of a cross-section crown-part of a tooth section and the locations and depths of the CSMH indentations in relation to the exposed enamel window and the enamel surface.

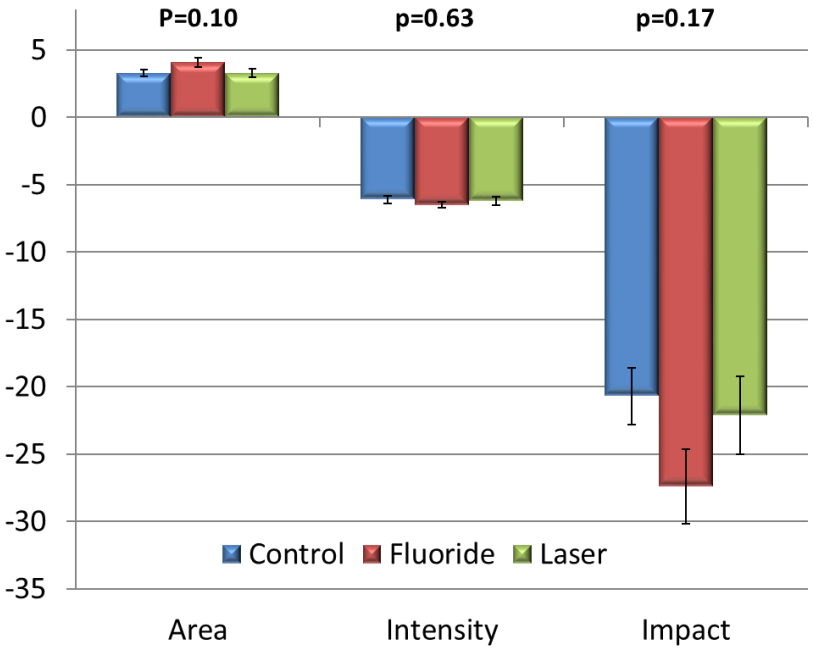

Figure 5 FluoreCam baseline data including area, intensity and impact of enamel demineralization of the three groups and their probabilities.

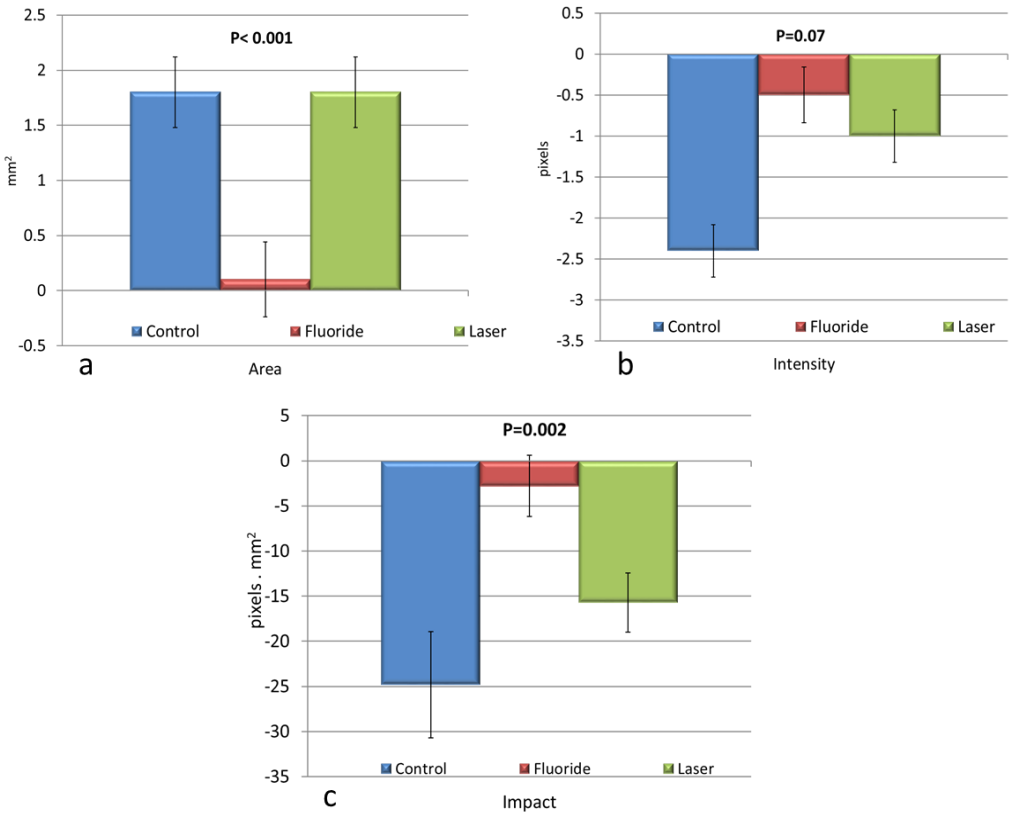

Figure 6 Differences in the FluoreCam data for (a) area, (b) intensity, and (c) impact of enamel demineralization that occurred (Δ=Final –baseline).

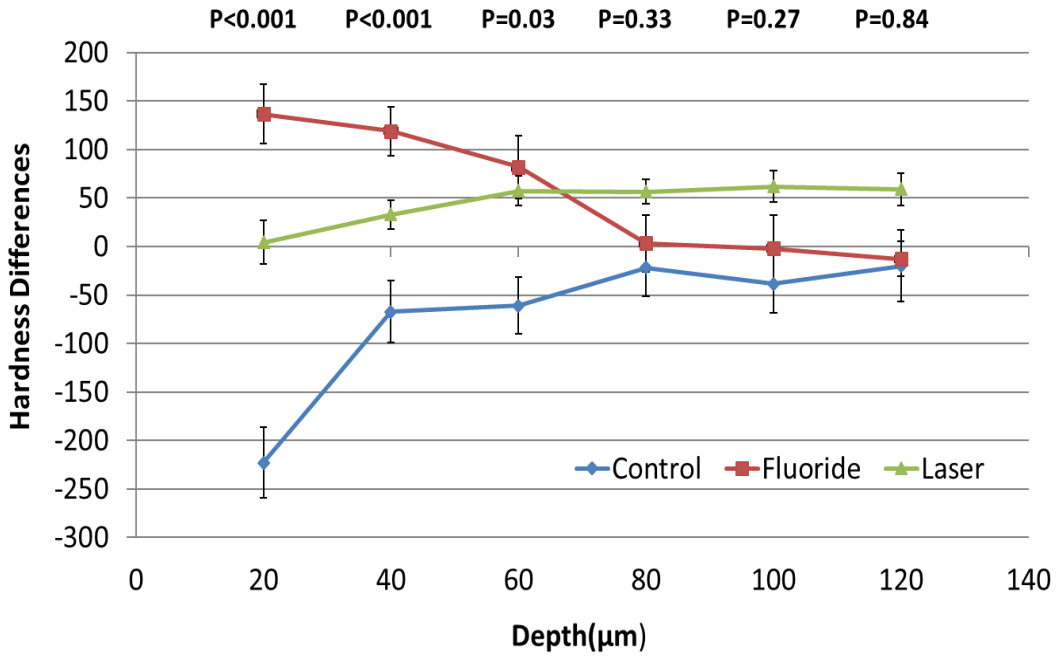

Figure 7 Differences in enamel cross-sectional microhardness (Δ = exposed – unexposed enamel) at 20, 40, 60, 80, 100, and 120 µm from the enamel surface for the control, fluoride, and laser groups.

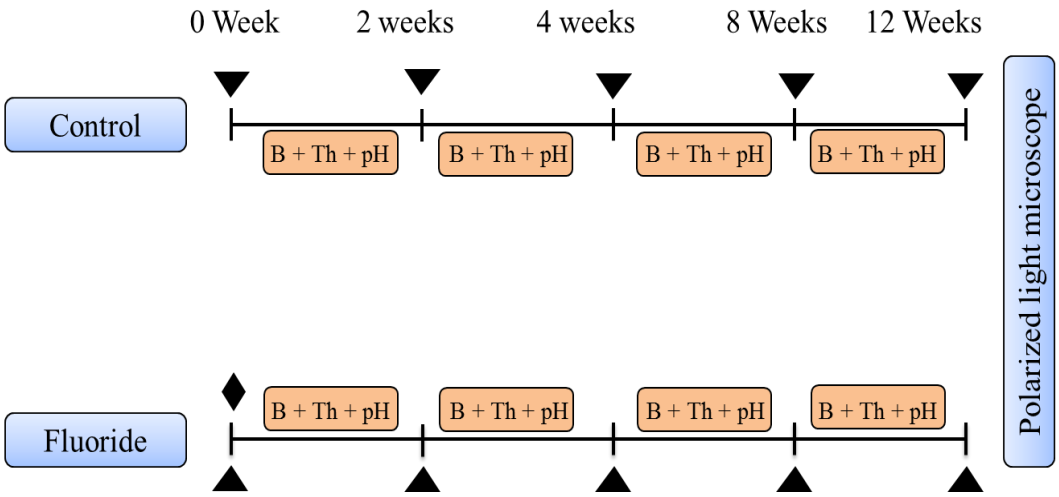

Figure 8 Illustration of the study design.

▲: FluoreCam imaging, ♦ Fluoride varnish applied to the experimental group, B+Th+pH: Brushing, Thermocycling and pH cycling, PLM: Polarized light microscope.

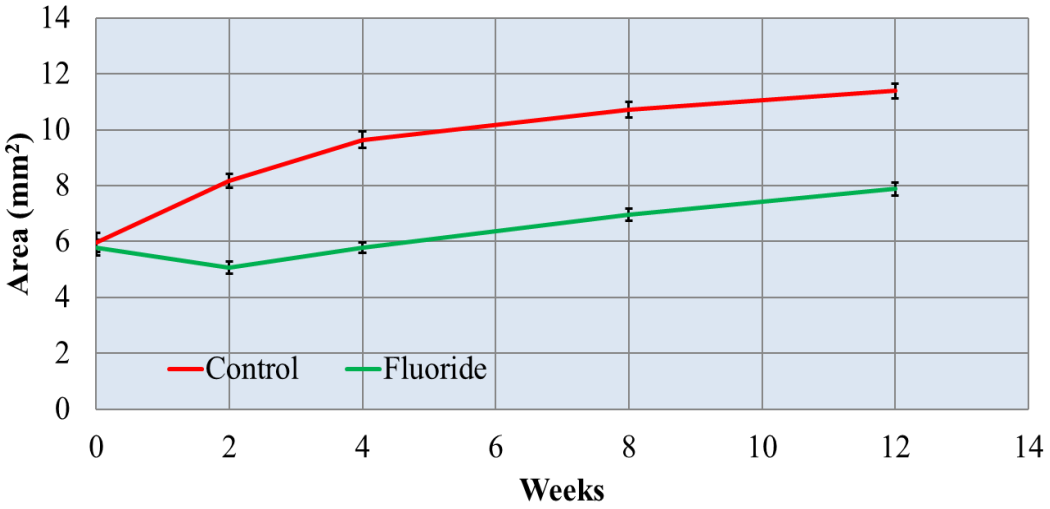

Figure 9 Changes in area of enamel demineralization over time in the control and varnish groups, along with probabilities from independent t-test.
Probability <0.001

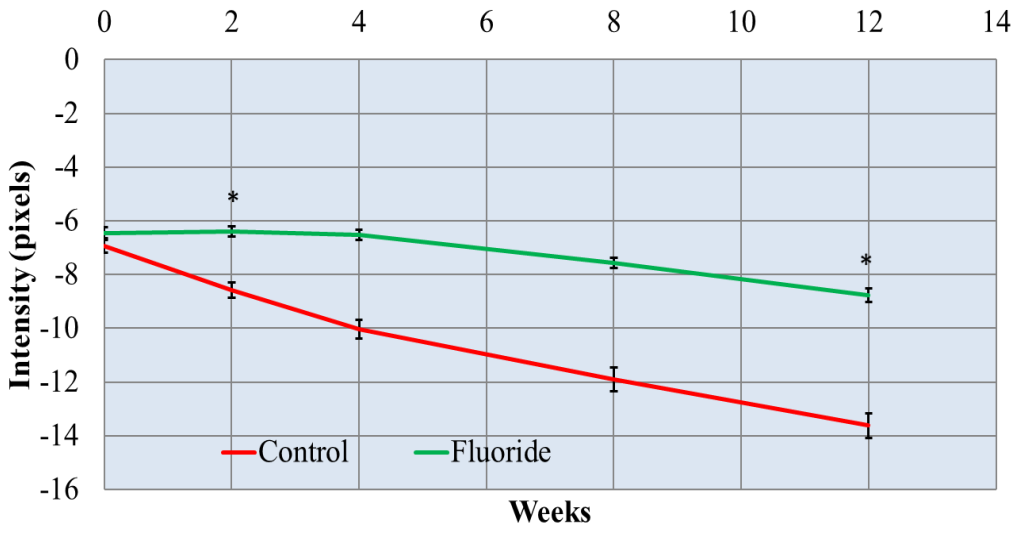

Figure 10 Changes in the intensity of enamel demineralization over time in the control and varnish groups, along with probabilities from independent t-test. Probability <0.001

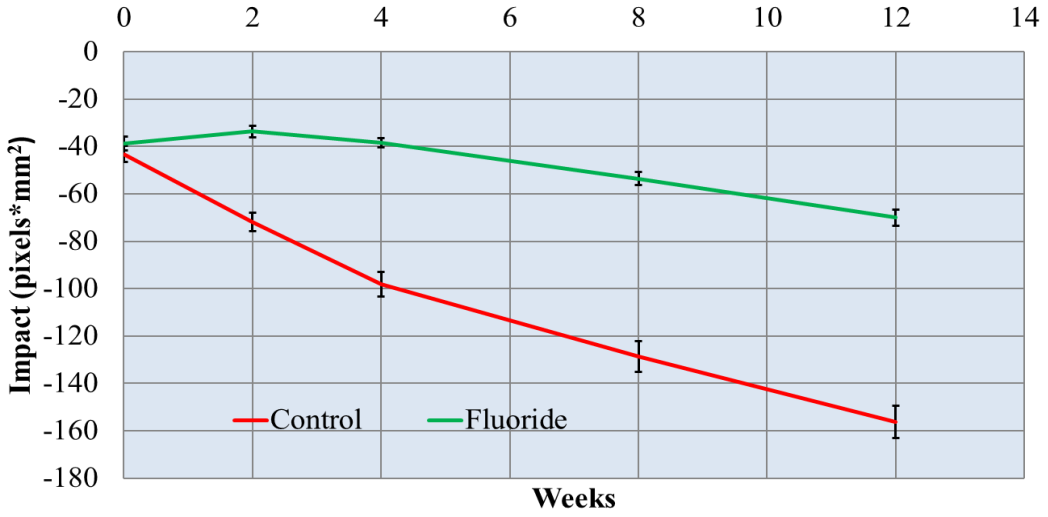

Figure 11 Changes in the impact of enamel demineralization over time in the control and varnish groups, along with probabilities from independent t-test. Probability <0.001

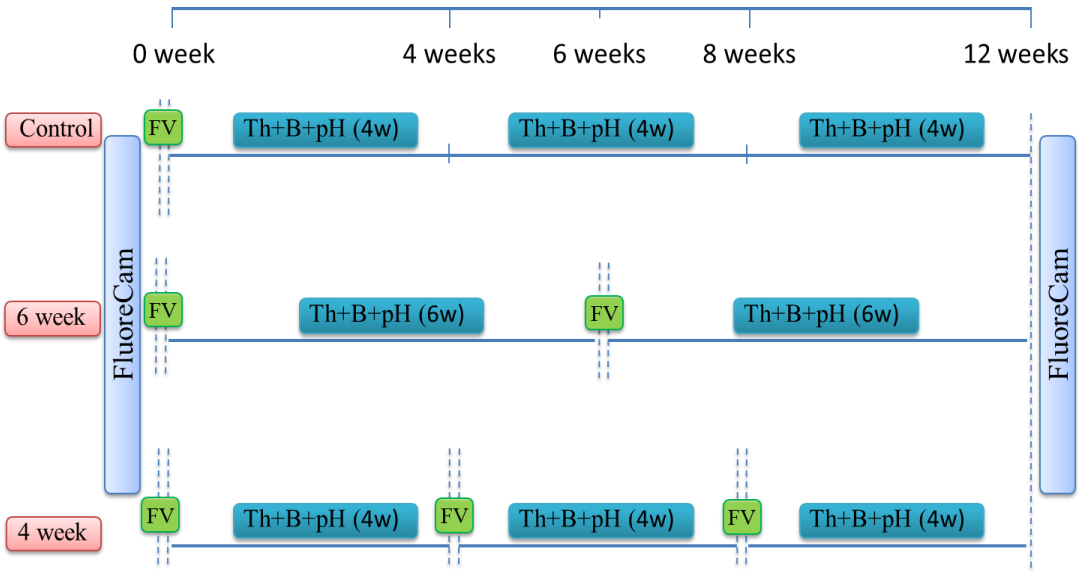

Figure 12 Illustration of the study design.

FV: Fluoride varnish application, double dotted lines: 24 hours in artificial saliva, Th+B+pH: Thermocycling (Th) Brushing (B), and pH cycling (pH).

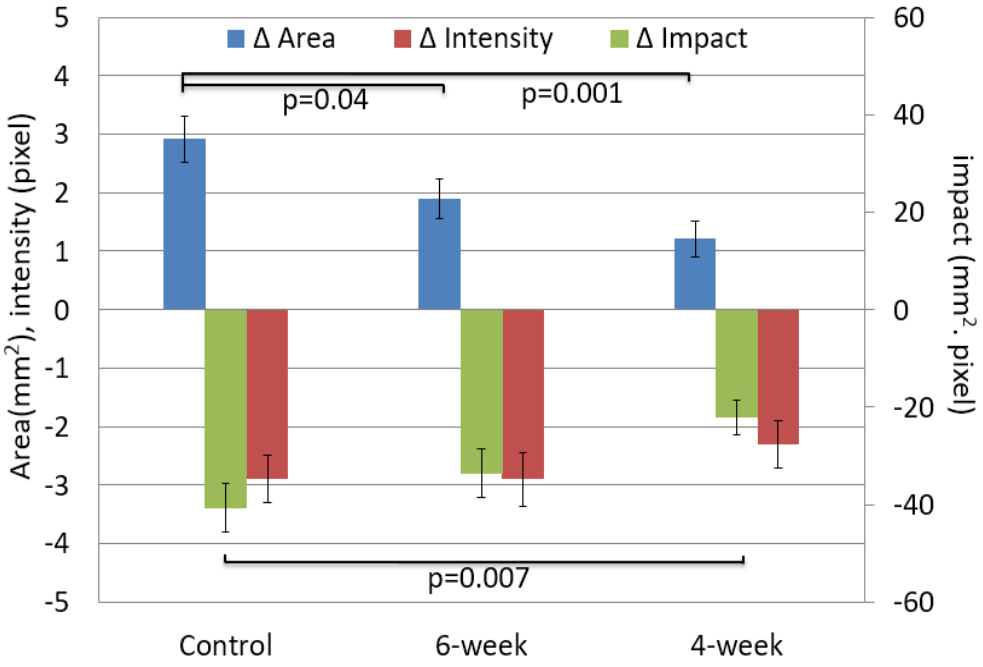

Figure 13 Changes (Δ =Final - baseline) in the control group (1 application), 6-week group (2 applications), and 4-week group (3 applications).

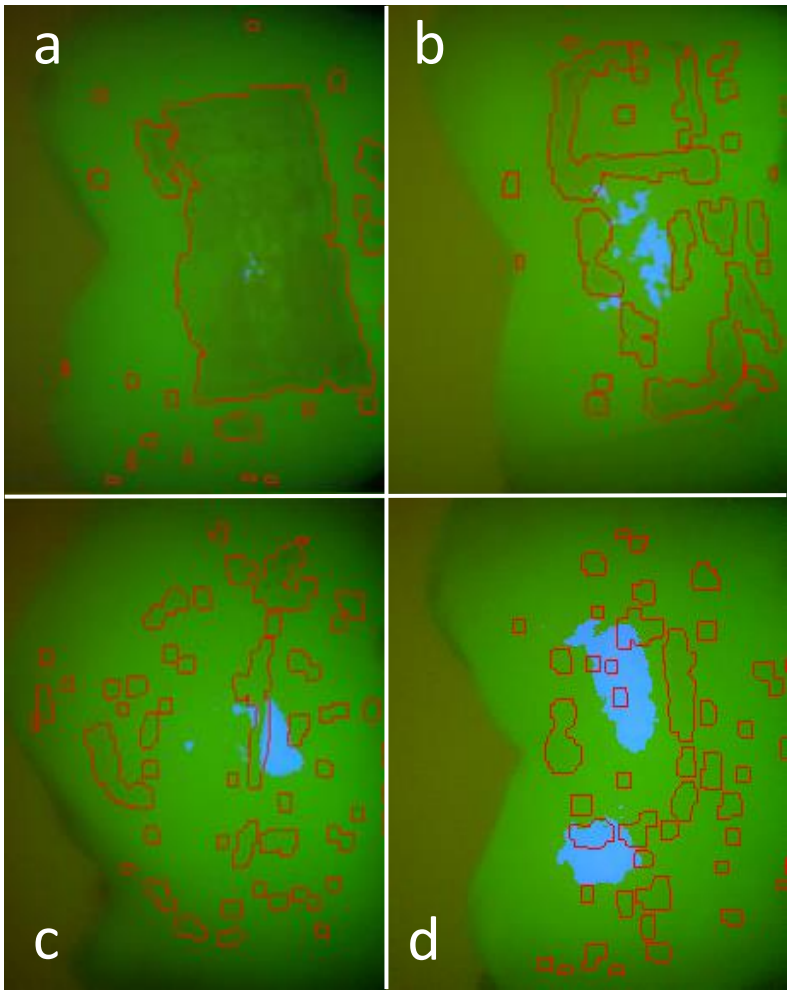

Figure 14 FluoreCam images of (a) Untreated control (from previous study), (b) one application control, (c) two applications (every 6 weeks), (d) three applications (every 4 weeks).

APPENDIX B

TABLES

Table 1 Effectiveness of fluoride varnish review.

Study	Fluoride %	Application	Method of assessment	Demineralization Reduction (%)
Farhadian[141]	12.6% CaF_2 and 6% NaF	Once and assessed after 85 to 95 days	Polarized light microscopy	40%
Rodrigues[191]	2.2% F	Every 3 months and assessed after 1 year	Intraoral photographs	44%
Ogaard [203]	2.2% F	Once and assessed after 1 month	Confocal laser scanning microscopy Transverse microradiography	48%
Todd [204]	2.2% F	Once and assessed after 37 days	Sonic digitizer	50%
Demito[190]	2.26% F	Every 3 months and assessed after 3 and 6 months	Laser fluorescence	70%

Table 2 Dental lasers and their use.

Laser	Wavelength (nm)	Absorption	Tissue application	Examples
Argon	488 515	Poorly absorbed by tooth structure.	Soft	Treatment of acute inflammatory periodontal disease and highly vascularized lesions.
Diode	635 670 810 830 980	Highly absorbed by pigmented tissue. Poorly absorbed by tooth structure and water.	Soft	Cutting gingiva and mucosa, hemostasis, soft tissue surgeries in close proximity to dental structure.
Nd-YAG	1064	Poorly absorbed by tooth structure	Soft -hard	Gingival contouring, non-surgical sulcular debridement, treatment of oral ulcers and hemostasis.
Erbium	2780 2940	Highly absorbed by HA* and water.	Soft -hard	Caries removal, tooth preparation, root debridement, enamel etching and minor soft tissue surgery
CO_2	9600 10600	Highly absorbed by HA and water.	Soft -hard	Rapid soft tissue removal (biopsies), hemostasis, treating mucosal lesions, bone cutting.

*HA: hydroxyapatite.

Table 3 Studies using different CO_2 laser wavelengths and percentage of caries reduction.

Author	Year	Wave length	Beam	Inhibition %
Nelson et al.	1986	9.3μm	Pulsed	50%
Nelson et al.	1986	9.3μm	Pulsed	50%
Kantorowitz et al.	1998	10.6μm	Pulsed	87%
Featherstone et al.	1998	9.6μm	Pulsed	70%
Young et al.	2000	9.6μm	Pulsed	50%
Oliveria et al.	2009	10.6μm	Pulsed	81%
Hsu et al.	2000	10.6μm	Pulsed	98%

Table 4 Parameters used by Featherstone and Hsu groups.

Parameters	Featherstone et al.	Hsu et al
Duration pulse	100 μs	5 ms
Energy density	12 J/cm^2	3.4 J/cm^2
Repetition rate	10 Hz	20 Hz
Number of pulses	25 (overlapping)	-

Table 5 Differences and probabilities of the FluoreCam Δ *area*, Δ *intensity* and Δ *impact* of the Fluoride, laser, and control groups.

Groups	**Δ Area**		**Δ Intensity**		**Δ Impact**	
	Mean	P-value	Mean	P-value	Mean	P-value
Fluoride vs Control	-1.7	0.002*	1.87	0.08	21.98	0.002*
Fluoride vs Laser	-1.91	0.001*	0.58	0.99	14.17	0.07
Laser vs Control	0.20	0.99	1.28	0.36	7.81	0.63

* Significant probability < 0.05

Table 6 Differences and probabilities of the microhardness differences (Δ *hardness= exposed – unexposed*) of the fluoride and laser groups in contrast with control and the fluoride and laser groups in contrast to each other.

Depth	Fluoride vs Control		Laser vs Control		Fluoride vs Laser	
	Difference	p- value	Difference	p- value	Difference	p- value
20 µm	268.6	<0.001**	160.8	<0.001**	107.8	0.001*
40 µm	132.7	<0.001**	85	0.001**	47.7	0.06
60 µm	76.4	0.007*	39.3	0.17	37.1	0.16
80 µm	48.7	0.15	30.5	0.39	18.3	0.63
100 µm	51.4	0.11	23.3	0.50	28.2	0.42
120 µm	18.4	0.57	14.1	0.69	4.4	0.91

* Significant probability < 0.05
** Significant probability < 0.001

Table 7 FluoreCam reliability [Interclass correlation coefficient (ICC) and method errors] and validity (systematic error) for area and intensity.

Output	ICC	Method Errors	Systematic error	
			Mean	P-value
Area (mm^2)	0.90	0.78	0.06	0.60
Intensity (pixel)	0.95	0.51	0.16	0.12

Table 8 Mean changes in area, intensity and impact of enamel demineralization over time for the control and varnish group, along with probabilities from paired t test.

Output	Group	0-2		2-4		4-8		8-12	
		Mean	P-value	Mean	P-value	Mean	P-value	Mean	P-value
Area	Control	2.22	<0.001	1.46	<0.001	1.08	<0.001	0.68	<0.001
	Varnish	-0.70	<0.001	0.72	<0.001	1.17	<0.001	0.92	<0.001
Intensity	Control	-1.63	<0.001	-1.45	<0.001	-1.87	<0.001	-1.73	<0.001
	Varnish	0.05	0.72	-0.13	0.20	-1.03	<0.001	-1.20	<0.001
Impact	Control	-28.6	<0.001	-26.37	<0.001	-30.54	<0.001	-27.6	<0.001
	Varnish	5.22	0.005	-4.82	0.003	-15.09	<0.001	-16.52	<0.001

Table 9 Baseline and final FluoreCam measures (mean ± SDs) of the three groups, along with their within-group probability.

Group	Area (mm^2) Baseline	Final	P value	Intensity (pixel) Baseline	Final	P-value	Impact (mm^2. pixel) Baseline	Final	P-value
Control	4.8 ± 1.5	7.8 ±1.6	<0.001	-6.1 ±0.8	-9.3 ±2.2	<0.001	-30 ±11.4	-74 ±27	<0.001
6-Week	5.0 ± 1.6	6.3 ±1.3	<0.001	-6.8 ±1.3	-9.8 ±1.5	<0.001	-34.6 ±16.6	-68 ±27	<0.001
4-week	5.2 ± 1.9	6.9 ±1.5	<0.001	-6.8 ±1.6	-9.2 ±2.3	<0.001	-37.3 ±19.5	-59 ±19.5	<0.001