INTERACTION BETWEEN TIN/FLUORIDE-CONTAINING SOLUTIONS AND ARTIFICIALLY CREATED DENTAL PELLICLES ON EROSION PREVENTION *IN VITRO*

by

Amnah Abdullah Algarni

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Frank Lippert

Richard L. Gregory

Walter Siqueira

Anderson Hara
Chair of the Research Committee

Andrea Ferreira Zandona
Program Director

N. Blaine Cook
Program Director

Date

DEDICATION

This thesis is dedicated to my deceased parents, who spent their lives supporting me; to my husband, for his love and support; to my sisters, for their prayers and encouragement; to my kids, Mastor and Razan, for their smiles and making my life more meaningful and valuable.

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INTRODUCTION

The prevalence of dental erosion has significantly increased over the last few decades.[1] Dietary acids are considered to be the most important factors causing dental erosion at the population level.[2] Attempts to prevent erosion have focused on either enhancing remineralization or preventing demineralization of the tooth structure. The protective properties of fluoride have been shown in many *in-vitro* and *in-situ* studies.[3-6] The efficacy of stannous solutions in erosion prevention has also been well established in the literature.[3,7-12] There are indications that the presence of both ions is relevant for erosion prevention.[13] The mechanism of the stannous and fluoride ions in erosion prevention is related to the formation of a thin layer on the enamel surface, composed by different precipitates such as $Sn_2(PO_4)OH$, $Sn_3F_3PO_4$, $Ca(SnF_3)_2$, and CaF_2.[14] This layer acts as a physical barrier, inhibiting acid contact with the enamel surface. Due to the low pH of stannous solutions, they may also react with enamel leading to the incorporation of the stannous ion into the enamel structure, which increases the enamel resistance against acid erosion.[13]

Acquired dental pellicle (ADP) is a protein-based selective permeable membrane; it acts as a physical barrier to prevent the acid contact with the tooth surfaces.[15] The protective effect of the ADP against erosion has been thoroughly investigated in both *in-vitro* and *in-situ* studies.1[5-17] The protective level of the pellicle is regulated by its composition, thickness and maturation stage.[15,148] It also shows dose-dependency to acid concentration and duration of exposure.[18] A study conducting protein analysis of *in-vivo* pellicle using proteomic approaches identified 130 proteins

associated with ADP.[21] It has been suggested that some of pellicle's proteins may have a role in erosion prevention.[20] For instance, salivary mucins, particularly MUC5B, have been found to be from the components of ADP basal layer and to be involved in erosion inhibition.[21-23] Moreover, statherin, histatins, and acidic proline-rich proteins (PRPs) compose the basal layer of the ADP and may impact dental erosion by modulating calcium and phosphate concentration within the oral cavity.[20,24,25] Other proteins found to be involved in remineralization are S100 calcium-binding protein, calgranulin, and those of the annexin families.[21]

The protein adsorption to tooth structure during ADP formation is a highly selective process. Therefore, only a small fraction of the proteins from the oral fluids is incorporated into this proteinaceous integument.[42] After protein adsorption, ADP undergoes maturation that may include proteolysis and cross-linking processes.[149] Two main mechanisms have been demonstrated to be involved in ADP maturation, including intrinsic and extrinsic modulation. Intrinsic maturation occurs by pellicle enzymes, while extrinsic occurs by enzymes present in saliva.[150,151] It has been found that enzymes as amylase, lysozyme, carbonic anhydrases, glucosyltransferases, and fructosyltransferase are immobilized in an active conformation in the *in-vivo* ADP and contribute to its modification and in homeostasis.[151] Transglutaminase was shown to be present in enamel pellicles formed *in situ* and exhibits crosslinking action on the primary pellicle precursor proteins such as statherin, cystatins, histatins, and acidic proline rich proteins.[149,150] Moreover, aspartate amino transferase and alanine amino transferase have been detected as integral components of the *in-situ* pellicle, which may indicate their involvement in the intrinsic maturation of the ADP.[152] The

conformational changes and cross-linking process that occur between pellicle proteins after adsorption may explain the tenacity of ADP and its protective effect.

Few studies have investigated the interaction between surface protective agents and pellicle, and the role of their interaction on erosion prevention. In an *in-vitro* study, Hove et al.[26] compared the erosion-inhibiting effect of solutions containing TiF_4, SnF_2, or NaF on pellicle-covered and non-covered enamel. They concluded that pellicle-covered SnF_2- treated enamel specimens reduced tooth loss due to erosion by 45 percent after 2 min and 14 percent after 8 min compared with the control with pellicle (with no SnF_2 treatment).[26] Wiegand et al. investigated the *in-vitro* effect of titanium tetrafluorides and pellicle on erosion prevention on both enamel and dentin, finding that the pellicle enhanced the effect of titanium tetrafluorides, especially in dentin.[29] However, it is not clear how the interaction between tin and the pellicle proteins affects the protective properties of the pellicle. It has been shown that the stannous ion can have a cross-linking action on polyphosphates adsorbed to the enamel surface[28] that possibly relates to its protective action against erosion. We hypothesized that this cross-linking action may also exist with the acquired dental pellicle layer formed on the tooth surfaces and increases protection against erosion.

Until now information is scarce regarding the effects of stannous and fluoride ions on the acquired dental pellicle and subsequently on erosion prevention. Therefore, we proposed the use of an *in-vitro* model to better understand the influence of the ADP on the anti-erosion properties of stannous and fluoride-containing solutions on enamel and dentin surfaces.

OBJECTIVES

The objectives of this *in-vitro* study were: 1) To compare the protective effect of stannous and fluoride-containing solutions on enamel and dentin erosion prevention; 2) To analyze the protein profile of the pellicles treated with stannous and fluoride-containing solutions.

HYPOTHESES

Null Hypotheses

1. There is no difference among stannous solutions, fluoride-containing solutions, and stannous-fluoride-containing solutions on erosion prevention.
2. There is no difference among the protein profiles of pellicles treated with stannous solutions, fluoride-containing solutions, and stannous-fluoride-containing solutions.

Alternative Hypotheses

1. There is at least one significant difference among stannous solutions, fluoride-containing solutions, and stannous-fluoride-containing solutions on erosion prevention.
2. There is at least one significant difference among the protein profiles of pellicles treated with stannous solutions, fluoride-containing solutions, and stannous-fluoride-containing solutions.

REVIEW OF LITERATURE

DEFINITION AND PATHOPHYSIOLOGY OF EROSION

Dental erosion refers to a progressive dental tissue loss due to chemical processes without involvement of bacteria.[31] It occurs due to dissolution of dental tissue by acids when the surrounding environment is unsaturated in relation to the mineral content of tooth tissue.[32] Enamel and dentin are mainly composed of calcium-deficient carbonated hydroxyapatite (HA) crystals (Ca10-x Nax(PO4)6-y (CO3)z(OH)2-uF) at 85 percent and 47 percent by volume, respectively. Acids in saliva (as citric acid and hydrochloric acid) dissociate into hydrogen ions and anions, decreasing the pH of the oral environment and making it unsaturated in relation to the dental surfaces. When hydrogen ions attack tooth structure, they combine with the carbonate and/or phosphate molecules, releasing calcium ions from the apatite crystals. The continuous acid exposure increases mineral loss from tooth structure and can lead to changes in the physical properties of the tooth structure. For example, studies have showed that microhardness decreases significantly after acid exposure, indicating softening of dental tissue.[33] In the persistence of this process, substantial dental surface loss may happen eventually.

ETIOLOGY AND CLASSIFICATION

Erosion is a multifactorial dental condition.[31] The interaction of many factors influences the occurrence of dental erosion including patient-related and diet-related factors.[34] Chemical factors of diet are important for the erosion process.[35] The pH value of soft drinks together with their calcium, phosphate, and fluoride content have a great

influence on the degree of saturation, and consequently, the driving force for demineralization.[35] The pH value of beverages is to be more important when a large volume of acid is present in the oral cavity.[35] However, buffering capacity plays a more important role when beverages remain in the oral cavity for long periods. The higher the buffering capacity of beverages, the longer the time needed for saliva to neutralize the acid, which enhances the process of mineral dissolution.[35] Moreover, the type of acid influences the process of dissolution significantly. For example, an *in-vitro* study found that over the common pH range, citric acid is more erosive than phosphoric acid at any given pH, due to its chelating properties.[36] It has been found that citric acid in orange juices complexes up to 32 percent of calcium in saliva, decreasing the super-saturation of saliva and enhancing the dissolution of tooth minerals.[37] In addition to chemical factors, behavioral factors have a considerable modifying effect on erosion.[88] Frequency and duration of exposure to acidic diet, manner of consumption, and night-time versus day-time exposure all have a strong effect on the erosive potential of dietary acids.[88] Besides, a healthy lifestyle could contribute to dental erosion due to an increase in acidic juices through dietary consumption and frequent tooth brushing.[88]

Erosion can be classified according to the source of acid, which could be either extrinsic or intrinsic. Extrinsic acids are mainly from diet. The erosion potential of diet depends mainly on its pH, buffering capacity, titratable acidity, and drinking or consumption patterns.[38,39] Acid exposure from occupational sources also has been reported, such as from battery and galvanizing factories.[38] In addition, exposure to intrinsic sources of acid may occur mainly due to chronic vomiting, eating disorders, and gastroesophageal reflux disease (GERD).[38]

BIOLOGICAL FACTORS CONTRIBUTING TO EROSION

Saliva, acquired dental pellicle, tooth structure, and positioning in the oral cavity are biological factors of major importance on the development of erosion.[15]

Saliva

Saliva is considered to be the most important biological factor that influences dental erosion.[15] It has a direct effect against erosive challenges by clearing, diluting, and buffering acids.[15] It also renders demineralization by forming acquired dental pellicle ADP over teeth surfaces, and enhances remineralization by providing calcium, phosphate, and fluoride to demineralized dental tissues.[15] The higher salivary flow rate increases the ability of saliva to buffer and neutralize acids, due to the higher content of hydrogen bicarbonate.[15] Therefore, studies have shown that erosion is strongly associated with low salivary flow (hyposalivation).[89]

Role of Acquired Dental Pellicle (ADP)

ADP is a thin bacteria-free protein-based integument forming upon exposure of tooth surfaces to saliva.[21] Adsorption of proteins to form ADP occurs mainly by electrostatic interactions between proteins and tooth surfaces. It is composed of glycoproteins, salivary proteins, non-salivary-derived proteins, carbohydrates, and lipids.[41] The components of ADP come from different sources including exocrine salivary glands, gingival crevicular fluid, oral epithelial cell products, and micro-organism products.[42] The analysis of *in-vivo* pellicle using liquid chromatography-electrospray-ionization tandem mass spectrometry (LC-ESI-MS/MS) led to the identification of 130 different proteins, 14.4 percent of them derived from glandular

secretions, 67.8 percent derived from cells, and 17.8 percent derived from serum through crevicular fluid.[21]

Several studies support the role of ADP in regulation of minerals homeostasis and preventive effect against acid-induced demineralization.[43-46] This preventive effect was found to be related to the prevention of demineralization by reducing acid diffusion rather than preventing mineral loss from dental tissues.[26,45] The acid resistance of ADP depends mainly on its maturation level; therefore, 2-h pellicle dissolves more rapidly from the enamel surface than 6-, 12- and 24-h pellicles.[47] The ultrastructure of ADP has shown to change after acid challenge. It has been observed that the outer global layer is removed after acid exposure, while the basal layer is preserved.[42]

The proteins composing the ADP are important in the progression of dental erosion. Salivary mucins (MUC5B, MUC7) are high-molecular-weight glycoproteins, considered to be one of the main components of the pellicle, and 7 percent to 26 percent of total salivary proteins.[23] Since the affinity of high-molecular-weight mucin (MUC5B) to hydroxyapatite is about six times more than the low-molecular weight one (MUC7), the former adsorbs to the tooth surface and contributes to the formation of the acquired dental pellicle.[90] Moreover, it has been suggested that mucin (MUC5B) presents at the basal layer of the pellicle, strongly related to the protective properties of the pellicle, as it has shown to persist even after 120 min of citric acid exposure.[22] Studies have shown that, at simulated physiological concentrations *in vitro*, mucins adhered to enamel surface inhibiting demineralization due to an erosive challenge.[24,25] It was shown that a pellicle formed by 3 days incubation in mucins from human whole saliva *in vitro* led to 100 percent protection against 1.0-percent citric acid erosion.[25] Other proteins such as

statherin, histatins and acidic proline-rich proteins (PRPs) also compose the basal layer of the ADP and can control dental erosion by modulating calcium and phosphate concentration within the oral cavity.[21,26,27] Histatins, another salivary protein family, are low-molecular-weight salivary proteins secreted by the major and minor salivary glands.[91,92] The three main types of histatins that could be detected as ADP components are histatin 1, histatin 3, and histatin 5.[46] They are multifunctional proteins; in addition to their antifungal effect,[93] it exhibits significant protection against erosive challenges.[46] Phosphoproteins such as histatin and statherin have shown protection against acid demineralization.[46,48] It has been suggested that phosphorylated proteins have more affinity to hydroxyapatite (HA); therefore, phosphorylated residues of histatin 1 and statherin appear to provide greater protection than non-phosphorylated residues.[41,49] Also, albumin, a hydrophilic acidic protein with molecular weight 66.5 kDa, has been suggested to inhibit demineralization.[94]

In addition, proteins may enhance the effect of other anti-erosive agents. For example, in an *in-vitro* study comparing the anti-erosive effect of toothpastes containing 3500 ppm of stannous solution and different proteins including mucin, casein, mucin and casein, or albumin, it was observed that toothpastes with albumin showed the best protection, which suggested a positive interaction between stannous ions and albumin.[50]

Dental Substrate

Enamel is thought to be more prone than dentin to dental erosion due to continuous exposure to oral environment. However, lengthier retention of teeth overtime a lifetime and gingival recession can lead to dentin exposure, making this substrate

vulnerable to acids in the oral cavity and subsequently to dental erosion.[15] Therefore, recent studies have investigated the erosion process and mechanism in both enamel and dentin.

The difference in the composition of enamel and dentin are crucial in order to understand the different behavior of both tissues during erosive challenges. Enamel composed of 85 percent by volume carbonated hydroxyapatide (HA), 12-percent water and 3.0-percent protein and lipid, while dentin contains 47 percent, 20 percent, and 33 percent of carbonated HA, water and protein and lipid respectively.[100] Although both enamel and dentin are composed of HA, dentin contains higher carbonate (5 percent to 6 percent) than in enamel (3 percent), which makes dentin more soluble than enamel.[33] Also, the sizes of HA crystals are much smaller in dentin than in enamel and lead to a larger surface area that makes dentin more susceptible to acid attack.[33]

When exposed to strong acids, enamel demineralization leads to a softening of the surface layer and the subsequent permanent loss of enamel tissue, if the challenge persists.[95] But after an erosive challenge to dentin, demineralization and etching of the inorganic layer occur, leaving the organic layer intact. The organic layer of dentin shows a protective effect against erosive challenges by preventing acid diffusion or mineral release.[96,97] Therefore, the mechanical or chemical removal of this layer leads to a progression of erosive lesion[97] and decreasing remineralization.[98]

Regarding the behavior of eroded enamel and dentin, an *in-situ* study analyzed the surface microhardness recovery of enamel and dentin after exposure to erosive attack using acidic beverages. It was found that enamel and dentin remineralized up to 37.8 percent and 55.4 percent, respectively, after 24 h of exposure to the oral environment.[99]

Nevertheless, when enamel and dentin were treated with fluoride gel after the erosive attack, the remineralization rate of enamel significantly increased to 57.2 percent, yet there was no significant increase in the remineralization rate of dentin.[99]

EROSION PREVENTION STRATEGIES

Erosive Acid Modification

Different strategies have been used to prevent and to repair erosion. First, the acid source responsible for the erosive challenge should be identified and either eliminated, decreased, or modified.[38] Extrinsic acids are mainly from diet, medications, or occupation sources. The erosive potential of diet can be reduced by calcium, phosphate, and fluoride supplementation. The addition of calcium and/or phosphate to erosive drinks makes the environment supersaturated with respect to tooth mineral thus preventing dissolution of dental structure.[55] The calcium-containing low-pH black currant juice significantly reduces the erosive effect of the drink in comparison with a calcium-free one.[101] In another *in-vitro* study compared between calcium-supplemented and non-supplemented commercially available acidic beverages have shown that calcium addition was found to be effective in reducing the erosive potential of those beverages.[102]

Neutralizing Solutions

Both intrinsic acids, as in GERD, and extrinsic acids can be neutralized. Intrinsic acids can be counteracted by using acid suppressive treatments, antacids, psychological therapy, or even surgical intervention if needed.[103] Water, milk, lozenges, antacid chewing tablets, and rinsing with sodium bicarbonate can be used to buffer acids and

increase pH in the oral cavity.[38,103]

Substrate Modification

Erosion prevention by dental substrate modification using lasers either alone or in combination with fluorides also has been thoroughly investigated by many *in-vitro* studies. Nevertheless, most of those studies failed to prove any extra preventive effect of lasers without the use of fluorides. The effect of Nd:YAG laser irradiation on enamel pre-treated with NaF and TiF_4 varnishes and solutions was analyzed *in vitro,* and the laser irradiation was found to have no significant effect on erosion prevention.[51] Another similar but more recent study has showed that Nd:YAG laser irradiation combined or not with fluoride gel/varnish was not more effective than fluoride alone to prevent enamel demineralization.[104] On dentin surfaces also, CO_2 laser irradiation showed no preventive effect when combined with amine fluoride and/or cerium chloride.[105] Moreover, an *in-vitro* study investigated the effect of pulsed CO_2 laser (lambda = 10.6 μm) treatment with or without fluoride gel pretreatment on enamel and dentin erosion. The pulsed CO_2 laser (lambda=10.6 μm) alone showed no preventive effect on enamel or dentin against erosion.[106]

On the other hand, *in-vitro* irradiation of dental enamel with a CO_2 laser at 0.3 J/cm^2 (5 μs, 226 Hz) was found to decrease mineral loss by 97 percent and to harden previously softened enamel.[52] Not only in enamel, irradiation was also found to have a protective effect with no harmful effects in a study of root dentin treated with a diode laser with levels set at 60 J/cm^2. [53]

Anti-erosive Agents

Protein caseinphosphopeptide (CPP) with amorphous calcium phosphate (ACP) has been claimed to promote a supersaturated environment close to dental hard tissue, enhancing remineralization.[107] It has been used either in toothpaste or as an additive to soft drinks.[55] An *in-vitro* study evaluated a CPP-ACP paste for preventing dentin and enamel erosion produced by a soft drink. Using atomic force microscopy and scanning electron microscopy, the researchers found that application of a CPP-ACP paste is effective in preventing dentin and enamel erosion produced by a soft drink.[108] Adding CCP-ACP to cola-type soft drink provides significantly reduced enamel erosion.[109] Moreover, application of tooth mousse reduces erosive wear of dentin and enamel after toothbrush abrasion.[110] An *in-situ* study investigated the remineralization potential of CPP-ACP after exposure to Coca Cola and showed the synergistic effect of fluoride and CPP-ACP.[111]

In addition, since ADP has protective properties against erosive challenges, attempts have been made to strengthen the effect of this naturally occurring organic covering. For instance, the use of casein and mucin have been shown to have considerable acid resistance effect.[43] An *in-situ* study showed the effectiveness of iron-containing mouthrinse in preventing erosion and toothbrush abrasion in human enamel and dentin.[112] This could be due to the formation of ferric phosphate precipitates on tooth surface increasing the resistance against tooth wear.[55] As with iron, sodium hexametaphosphates also have been shown to contribute to anti-erosive protection through the formation of a surface layer.[55] The use of polymers such as xanthan gum and carboxymethyl-cellulose also has been reported.[54] An *in-vitro* study found that a novel

polymers system (carboxymethylcellulose, xanthan gum and copovidone,) significantly reduced surface roughness and enhanced the fluoride uptake by erosive lesions.[113] Another *in-vitro* study investigated the influence of different polymers addition on the erosion potential of orange juice. They have shown that calcium lactate pentahydrate and sodium pyrophosphate tetrabasic polymers significantly reduced the erosive potential of orange juice on enamel and dentin, without interfering with remineralization process.[114]

FLUORIDE AND STANNOUS IONS IN EROSION PREVENTION

The effect of different fluoride formulations on dental erosion has been thoroughly investigated.[55] The anti-erosive effect of high-concentration topical fluorides, including toothpastes, gels, varnishes, and mouth rinses, is based mainly on the formation of precipitates on the tooth surface.[38]

Fluoride-containing toothpastes were more effective in prevention of tooth wear when compared with non-fluoridated ones.[56] Under relatively severe erosive conditions, conventional toothpastes containing 1400 ppm F to 1490 ppm F as sodium fluoride (NaF) exhibited significant protective effect against erosion.[57] In an *in-vitro* study, dentifrices with 5000 ppm F (NaF) and 500 ppm F (NaF) plus 3.0-percent sodium trimetaphosphate (TMP) provided more erosion prevention than a 1100-ppm F (NaF) dentifrice.[58] Moreover, the type of fluoride compound may influence the efficacy of toothpaste against erosion. An *in-vitro* study compared the anti-erosive effect of three toothpastes that contained stannous fluoride (SnF_2), NaF, or sodium monofluorophosphate (SMFP).[3] The authors found that the toothpaste containing stabilized SnF_2 demonstrated a highly significant level of protection compared with all other types of pastes.[3] In an attempt to

simulate clinical situations, an *in-vivo* study was done to compare NaF- and SnF_2-containing toothpastes in erosive demineralization. The toothpaste containing 1000 ppm F as SnF_2, and 1.0-percent stannous pyrophosphate reduced mineral loss significantly compared with toothpaste containing 1500 ppm F as NaF.[59]

Fluoride varnishes and gels also have been suggested to be effective against erosion due to the high concentration of fluoride compounds and the prolonged contact of fluoride with tooth surfaces.[38] Even though its effects are regarded as controversial in the literature, an *in-vitro* study showed an experimental varnish that contains TiF_4 (24500 ppm F) had promising results in prevention of both enamel erosion and abrasion.[60] It showed superior prevention over experimental NaF (24500 ppm F), NaF-Duraphat (22600 ppm F), and placebo varnishes.[60] Another *in-vitro* study compared a fluoride varnish (Duofluorid) and a fluoride gel (Duraphat) against erosion caused by orange juice and Pepsi. The fluoride gel significantly provided better prevention against acidic beverages than varnishes.[61] Nevertheless, in another study, both an NaF varnish and an APF gel were able to inhibit enamel erosion approximately equally.[4] Although NaF varnish contains a higher fluoride concentration, acidic APF gel could create a greater accumulation of CaF_2-like layer on the enamel surface, which would make its effect equal to the varnish.[4]

FLUORIDE AND STANNOUS-CONTAINING MOUTHRINSES

Fluoridated mouthrinses provide considerable protection against tissue loss in comparison with non-fluoridated ones.[62] Mouthrinse that contains 450 ppm fluoride as NaF provides significant protection over less concentrated mouthrinses (112 ppm F, as

NaF).[63] The efficacy of mouthrinses that contain tetrafluorides and stannous ions has been investigated. Their protective effect was more prominent in presence of pellicle in both *in-situ* and *in-vitro* studies.[28,64] It has been found that multiple applications of a 0.5-percent (531.9 ppm F) TiF_4 mouthrinse significantly decreased enamel erosion *in vitro*.[65] Even though all rinses contained 9500 ppm F, a concentration of 3.9-percent SnF_2 showed significant protection for the enamel surfaces, and 1.5-percent TiF_4 provided the best protection against erosive attack. However, 2.1-percent NaF had no significant protective effect in both *in-vitro* and *in-situ* situations.[28,64] However, data from *in-situ* studies revealed controversial results on the beneficial effect of TiF_4 on enamel erosion.[66]

Studies have investigated solutions containing $SnCl_2$ as a source of tin, either alone or combined with fluoride. Different tin/fluoride ratios have been explored in an attempt to determine the proper concentration of both ions. An *in-vitro* study investigated six solutions with different F/Sn ratios as follows: 1500 ppm F was combined with 2800 ppm Sn, 2100 ppm Sn, 1400 ppm Sn, and 700 ppm Sn; while 1000 ppm F was combined with 2100 ppm Sn and 1400 ppm Sn. The pH for all solutions was adjusted to 4.5.[67] The best erosion prevention was obtained by combination of 1500 ppm F with 2800 ppm Sn, and 1000 ppm F with 2100 ppm Sn. Within the solutions with same concentration of tin, the one with higher fluoride concentration was less effective, which indicated that the stannous concentration appeared more important than F, and that the higher the tin concentration, the better the preventive effect.[67] In another *in-vitro* study by the same authors, solutions containing concentrations of tin ranging between 800 ppm and 2800 ppm, and fluoride concentrations of 500 ppm and 250 ppm were investigated. They concluded that an 80-percent reduction in enamel surface loss was achieved by the 2800

ppm Sn/500 ppm F solution, while the lowest reduction (54 percent) was achieved by the 800 ppm Sn/250 ppm F solution.[13] Regarding erosion prevention in dentin, different solutions were compared. An *in-vitro* study found that the AmF- and NaF-containing solutions (250 ppm F) reduced tissue loss by 60 percent, while the stannous solution $SnCl_2$ (815 ppm Sn) reduced erosion by 52 percent.[68] However, solutions containing combinations of fluoride and tin, including AmF/SnF_2 (250 ppm F, 409 ppm Sn) and SnF_2 (250 ppm F, 809 ppm Sn), reduced dentin surface loss by 74 percent and 89 percent, respectively.[68] It has been suggested that SnF_2 is effective even against hydrochloric acid (simulating intrinsic erosion), which is very strong acid with considerably low pH (1.2 and 2.2).[69] In a comparison with 2.0-percent NaF (9,040 ppm F) solution, 0.4-percent SnF_2 (1000 ppm F) solution showed significantly higher inhibition of demineralization.[69]

In addition to the concentrations of tin and fluoride, the pH level influences the efficacy of stannous ions. It has been proved that the fluoride agents at lower pH had better protection against erosion.[6] At the same concentrations, acidic SnF_2 and AmF provided more protection against erosion than NaF.[70] It is been suggested that the anti-erosive effect of metallic ion-containing solutions (as Ti and Sn) are more prominent at low pH,[9] and that they are less stable at higher pH.[14] Therefore, most studies investigated the effect of stannous solutions at pH 4.5.

IN-VITRO EROSION MODELS

The benefits of the *in-vitro* experiments over *in-situ* or *in-vivo* ones are the shorter times needed to conduct the experiment; the use of smaller budgets and fewer staff, and the ability to proceed without study-participant compliance. However, data obtained from

in-vitro models should be interpreted with caution because they do not focus on the biological variations that affect the erosion process in the oral environment.[71] Nevertheless, reasonable and valuable data have been obtained from *in-vitro* studies.[71]

Study Design

To design an erosion model we have to understand and to visualize the erosion process in the oral cavity. When extrinsic acid enters the oral cavity, the acid is mixed with saliva and will contact the teeth surfaces and soften them, which will increase their susceptibility to abrasive wear.[72] When acids are swallowed, some residual acids can remain in the mouth, but eventually be cleared or neutralized by saliva. Therefore, to design an experimental erosion model, many factors must be considered, including the chemical (e.g. acid-tooth interaction, acid concentration and pH), behavioral (e.g. way of drinking beverages), and biological (e.g. salivary flow, ADP) factors.[72] Moreover, the outcome measures of the study have to be determined, either to assess initial surface softening or advanced deep surface loss.

Dental Hard Tissue Substrates

Specimens can be slabs of either enamel or dentin obtained from extracted human teeth, which best represent *in-vivo* situations. Access to human tissue could be difficult; therefore, bovine enamel and dentin (from lower incisors) can and have been used as substitutes for human tissue.[73] An *in-vitro* erosion/abrasion cycling study found that bovine enamel is more susceptible to demineralization than human enamel;[74] while bovine dentin performed similarly to human dentin in the same testing conditions.[75] Even though human hard tissues are preferred for more realistic models, bovine teeth have

been accepted because they can provide adequate relative results.[73] Chemical, morphological, and physical differences among these dental substrates are therefore considered during data interpretation.[76] Hydroxyapatite powder, particles, and discs also have been used as a substitute for enamel.[72] Specimen preparation is determined according to the method of erosion assessment. For instance, in the case of surface microhardness and surface loss profilometry, specimens usually are ground and polished to give more accurate results, while natural surfaces can be used for chemical analyses (calcium, phosphorus and fluoride release).[77]

Cycling Protocol

Very few studies used a single-erosion model, because it does not reproduce the dynamics of erosion development in the mouth. The majority of *in-vitro* erosion studies were conducted using cycling models ranging from three to 720 cycles.[73] Studies that aimed to test initial erosive lesions tend to use fewer cycles, while more frequent cycles are used in studies testing more advanced and aggressive erosive challenges.[73]

When designing an erosion experimental model, lab variables should be properly described and justified to allow comparisons among different studies.[71] Those variables include solution volumes, exposure times, flow rates, temperatures, and stirring methods and rates.[71,72] The determination of these experimental parameters depends mainly on two factors, the simulation of an *in-vivo* situation and the chosen method of erosion assessment.[72] Therefore, in most circumstances, it is preferable to design a model with a short erosion period; however, the model should ensure enough demineralization that can be detected and assessed by the method of choice.[72]

Regarding the testing of preventive treatments, many *in-vitro* studies have used specimens with topical treatment followed by either single or multiple erosive challenges.[72] The frequency of preventive treatment applications also varies according to the objectives of the study.[72] Frequent applications may be incorporated in the erosive model to assess the efficacy of certain preventive products and to determine its appropriate regime.[72,87]

Remineralizing Solution

Some *in-vitro* erosion models incorporate remineralization phase using demin-remin cycling procedures to simulate clinical situations. Artificial mouths and other devices have been developed in attempt to be more realistic and standardized.[71] Different types of remineralizing solutions have been used, including stimulated or unstimulated whole saliva, centrifuged saliva using different centrifugation parameters, and artificial saliva with different formulations.

Type of Acid

The acid type, concentration, and pH to be used as the erosive solution depend on the objectives of the study. When simulating acids from dietary sources, citric acid (pH 2.3-3.8), soft drinks and sodas (pH 2.3–3.2), sport beverages (pH 2.81 to 3.55),[78] acidic candies (pH 2.3–3.1),[79] candy sprays (pH 1.9–2.3), [80] and fruit juices (orange, grapefruit or lemon: pH 3–4)[81] have been used. For intrinsic erosion, hydrochloric acid with pH range 1.2-2.9 has been used to simulate erosion caused by eating disturbances, vomiting, and GERD.[72] The time of acid exposure should ideally reproduce clinical conditions. The average exposure time in the oral cavity has been reported to be approximately 2 min.[82]

The temperature of erosive solution contributes to the degree of erosion[85] proportional to surface loss and inversely proportional to surface nanohardness.[54] Finally, stirring rate can increase the tooth dissolution rate by increasing the speed of the reaction.[86]

METHODS AND MATERIALS

This study was conducted in two phases. In Phase I, the effects of different rinse treatments were tested using an *in-vitro* cycling model. In Phase II, the effects of these rinses on the protein composition of the pellicle were analyzed.

PHASE 1

Study Design

This phase followed a complete randomized design testing the effects of rinse treatment (at 4 levels, $SnCl_2$ /NaF-, NaF-, $SnCl_2$ -containing solutions and deionized water (DIW) as negative control). The experimental units were polished enamel and dentin slabs cut from bovine teeth (n = 10 per group). The response variables were surface loss (in µm) measured at the end of the cycling phase.

Solutions Preparation

Sodium gluconate (FisherSci AC18139; 2.3 g/l solution) and tin chloride ($SnCl_2$, Sigma-Aldrich CAS# 7772-99-3; 800 ppm Sn in the solution equated to 1.277 g $SnCl_2$ /l solution) were added to prepare the $SnCl_2$-containing solutions. Sodium gluconate was allowed to dissolve first, and then tin chloride was added. Two-hundred-fifty (250) ppm fluoride as 0.553 g/l NaF (Sigma-Aldrich CAS# 7681-49-4) was used. To allow for appropriate comparison, all solutions were pH-adjusted to 4.5 using HCl or KOH. Solutions preparations are described in Table I.

Specimen Preparation

Enamel and dentin slabs (4 mm width × 4 length mm × 2 mm thickness) were cut from bovine incisors using a microtome (Isomet, Buehler, Lake Bluff, IL). The specimens were embedded in acrylic resin (Varidur, Buehler) and the resulting blocks (10 mm × 10 mm × 8 mm) containing 1 enamel and 1 dentin specimen were ground flat and polished with water-cooled abrasive discs (500-, 1200-, 2400- and 4000-grit Al_2O_3 papers; MD-Fuga, Struers Inc., Cleveland, OH) and polishing cloth with diamond suspension (1 μm; Struers Inc.) (Figure 1, Figure 2). After the polishing procedures, they were sonicated in detergent solution and selected. Specimens with any cracks or structure defects were discarded. Adhesive unplasticised polyvinyl chloride (UPVC) tapes were placed on the polished surface of each specimen, leaving an area of 4 x 1 mm^2 exposed to subsequent testing (Figure 3). Specimens were then randomly assigned to four experimental groups (n = 10 per group).

Saliva Collection

Human saliva collection is approved according to IRB protocol #0304-58. Stimulated whole saliva was collected 1 h after breakfast from volunteers without active caries, saliva dysfunction, and not on medications. Salivary secretion was stimulated by chewing a gum base for 1 min, and saliva was collected for 2 h directly into 50-ml ice-chilled tubes to collect approximately 1.5 L. After collection, saliva was pooled and immediately centrifuged at 14,000 g for 20 min at 4 °C. The supernatant was separated from the pellet, pooled, and stored in -80°C.

Cycling Procedures

For the cycling, the specimens were fixed on the lids of 12-well plates, allowing them to be individually immersed into the test solutions (Figure 4, Figure 5, Table II). For the first day, all specimens were incubated in the saliva (2 ml/specimen) for 24 h at room temperature under gentle agitation (85 rpm to 90 rpm) (Figures 6, 7). After that, the cycling phase started with a cycle consisting of 5-min immersion in 0.3-percent citric acid (pH 2.6, 4 ml/specimen), followed by 60 min of immersion in clarified saliva (2 ml/specimen). This cycle was repeated 6 times per day for 5 days. The remin solutions were renewed 3 times/day, while the demin solution was renewed after each demineralization episode. After demin the specimens were rinsed in DIW for 10 s, and the excess of water was gently removed with the aid of kimwipes. The exposure to the rinse solutions (4 ml/specimen) was performed in the 30 min after the start of 1st, 3rd and 6th remineralization periods for 2 min. No DIW-rinse was done after exposure to saliva and the treatments. The excess of the rinse solutions was gently removed with kimwipes. All the experimental procedures were conducted at room temperature.

Surface Profilometry

After cycling, the tapes were removed from the specimens and the surface analyzed. An area of 2 mm long (X) × 1 mm wide (Y) was scanned with an optical profilometer (Proscan 2000, Scantron, Venture Way, Tauton, UK) (Figure 8). The scan covered the treated area and the protected reference surfaces on both sides. The step size was set at 0.01 mm and the number of steps at 200 in the X-axis; and at 0.05 mm and 20 mm, respectively, in the Y-axis. The depth of the treated area was calculated based on the

subtraction of the average height of the test area from the average height of the two reference surfaces by using the dedicated software (Proscan Application software v. 2.0.17).

PHASE 2

Study Design

This phase tested the effect of rinse treatments on the acquired dental pellicle (ADP) protein composition formed by clarified human saliva (CHS) as in Phase I. The same solutions tested in Phase 1 were tested: $SnCl_2$/NaF-, NaF- and $SnCl_2$-containing and DIW (negative control). The experimental units were polished 8 × 8 mm^2 bovine enamel slabs (n = 8 per group). All groups were incubated in clarified saliva for 24 h at room temperature under gentle agitation. Then, all specimens were subjected to a 2-min treatment with the solutions followed by ADP formation for 2 h (Figure 9). Different color-coded forceps were used for each group (Figure 10). The dental pellicle specimens after the three cycles were collected and analyzed for protein profile and composition using proteomic techniques (Table 3).

Specimens Preparation

Thirty-two enamel slabs (8 mm width × 8 length mm × 2 mm thickness) were cut from bovine incisors using a microtome (Isomet, Buehler, Lake Bluff, IL). Specimens were ground flat and polished with water-cooled abrasive discs (500-, 1200-, 2400- and 4000-grit Al2O3 papers; MD-Fuga, Struers Inc., Cleveland, OH) and a polishing cloth with diamond suspension (1 µm; Struers Inc.). After the polishing procedures, they were sonicated in detergent solution and selected. Specimens with any cracks or structure

defects were discarded. All specimens were sonicated again in detergent solution for 3 minutes just before the experiment to remove debris and pellicle (Figures 11, 12).

Harvesting ADP from Specimens

Collection strips of 0.5 cm × 1.0 cm (electrode wick filter paper, Bio-Rad, Hercules, CA) pre-soaked in 3.0-percent citric acid were folded so they could be held from one end with a dental forceps, while the other end was brought into contact with the surface of the specimens (Figure 13 through Figure 17). Each surface of the folded strip was used to collect pellicle from the surface of one specimen, i.e. four collection strips were used per group (1 strip/2 specimens). After collection, the strips were placed into a polypropylene microcentrifuge tube. The collection strips were then kept frozen at -80 °C until used[146] (Figure 18, Figure 19).

ADP Protein Extraction

Procedures for AEP extraction and in-solution digestion were as described by Zimmerman et al. (2013).[146] To extract the AEP proteins from the collection strips, 200 μL of 50 mM ammonium bicarbonate, pH 7.8, was added to each of the polypropylene microcentrifuge tubes containing the 4 collected strips. Each microcentrifuge tube was then sonicated for 1 min, and the recovered solution was then collected and placed into a new microcentrifuge tube for each group. This procedure was repeated for a total of 4 times. The extracted solution was then centrifuged at 14,000 × g for 15 min and the supernatant was extracted. This centrifugal procedure was carried out to prevent the debris from the collection strip that could be released into the solution during the sonication step. The supernatant was dried using a rotary evaporator (Eppendorf,

Parkway, NY) and then resuspended in 100 μL of distilled water. Micro bicinchoninic acid (Micro BCA) assay was carried out to determine the total protein concentration of the extracted solution from each group. All samples were dried using a rotary evaporator and stored at 4 °C until they were needed for further experimentation.[146]

In-Solution Digestion

Dried samples were resuspended in 50 μL of 4 M urea, 10 Mm DTT and 50 mM ammonium bicarbonate at pH 7.8 and incubated for 1 hour at room temperature. Afterwards, 150 μL of 50 mM ammonium bicarbonate was added to the samples, followed by 2.0-percent (w/w) trypsin (Promega, Madison, WI). The sample was then allowed to incubate overnight at 37 °C. Finally, the samples were dried in a rotary evaporator, de-salted by C-18 ZipTip Pipette Tips (Millipore, Billerica, MA) and subjected to mass spectrometry.[146]

ADP Peptidome

The samples were filtered by centrifugal filtration using a 10 kDa molecular weight cut-off (MWCO) membrane (Pall Life Sciences, Ann Arbor, MI). The eluted AEP was centrifuged for 10 min at 14,000 × g using a refrigerated Eppendorf table-top centrifuge (Eppendorf, Parkway, NY). The filtrate containing the proteins/peptides with molecular weights below 10 kDa was collected, dried, and subjected to MS analysis.[146]

Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS)

Mass spectrometric analyses were carried out with a LTQ-Velos (Thermo Scientific, San Jose, CA). This allowed for in-line liquid chromatography with the capillary-fused silica column (column length 10 mm, column ID 75 μm) packed in-house using C-18 resin of 3.0-μm spherical beads and 100-Å pores size (Michrom BioResources, Auburn, CA) linked to the mass spectrometer using an electrospray ionization in a survey scan in the range of m/z values 390-2000 tandem MS/MS. A dynamic exclusion criterion was established as a repeat count of 1 and a repeat duration of 30 s. All samples were dried by rotary evaporator and re-suspended in 15 μL of 97.5-percent H2O/2.4-percent acetonitrile/0.1-percent formic acid and then subjected to reversed-phase LC-ESI-MS/MS. The nano-flow reversed-phase HPLC was developed with linear 65-minute gradient ranging from 5 percent to 55 percent of solvent B (97.5-percent acetonitrile, 0.1-percent formic acid) at a flow rate of 200 nL/min with a maximum pressure of 280 bar. Electrospray voltage and the temperature of the ion transfer capillary were 1.8 kV and 250 °C respectively. Each survey scan (MS) was followed by automated sequential selection of seven peptides for CID with dynamic exclusion of the previously selected ions.[146]

Peptide and Protein Identification

For proteome and peptidome analysis, the obtained MS/MS spectra were searched against human protein databases (Swiss Prot and TrEMBL, Swiss Institute of Bioinformatics, Geneva, Switzerland, http://ca.expasy.org/sprot/) using SEQUEST and Percolator algorithms in Proteome Discoverer 1.3 software (Thermo Scientific, San Jose,

CA). A maximum of two miscleavages were allowed; carbamydomethylation of cysteine; phosphorylation of serine, threonine and tyrosine; and oxidation of methionine were included as dynamic modification. For proteome analysis, a trypsin-specific cleavage site was considered; peptidome analysis was carried out with no specified fragmentation search. Search results were filtered for a false discovery rate of 1.0 percent employing a decoy search strategy utilizing a reverse database. A total of three mass spectrometric runs were carried out in each condition.[146]

STATISTICAL ANALYSIS

Phase 1

Mixed-model ANOVA was used to test the effects of surface (enamel, dentin) and rinse (DIW, NaF, Sn, Sn+NaF) on surface loss. Pair-wise comparisons were made using Tukey's method to control the overall significance level at 5 percent.

Phase 2

An approach was used similar to that adopted in Siqueira et al. (2012)[123] to determine the relative abundance of proteins among experimental groups. Relative abundance of an individual protein from the experimental groups was considered significantly different protein level when the values observed were 0.75 for decreased abundance or 1.25 for increased abundance. Significance level of 5 percent was adopted for all analyses.

RESULTS

PHASE 1

Overall, the solutions and the substrates and their interaction had significant impact on surface loss (Table IV, Table V).

Substrate Comparisons

For the $SnCl_2$ and the $SnCl_2$/NaF groups, dentin had significantly more surface loss than enamel ($p < .0001$) (Table VI).

Solutions Comparisons

On dentin surface: 1) The $SnCl_2$ group had significantly more surface loss than the NaF group ($p < 0.05$) and the $SnCl_2$/NaF group ($p < .0001$), but less than the DIW group (control) ($p < .0001$); 2) The NaF group had significantly more surface loss than the $SnCl_2$/NaF group ($p < .0001$), but less than the DIW group ($p < .0001$); and (3) The $SnCl_2$/NaF group had significantly less surface loss than the DIW group ($p < .0001$) (Table VII; Figure 20).

On enamel surface: 1) The $SnCl_2$ group had significantly more surface loss than the $SnCl_2$/NaF group ($p < .0001$), but had less than the NaF group and the DIW group ($p < .0001$); 2) The NaF group had significantly more surface loss than $SnCl_2$/NaF group ($p < .0001$) but less than the DIW group ($p < .0001$); and 3) The $SnCl_2$/NaF group had significantly less surface loss than the DIW group ($p < .0001$) (Table VII; Figure 21).

PHASE II

After elution of ADP from collection strips and trypsinization, equal amounts of protein peptides were subjected to nanoscale LC-ESI-MS/MS. Four (4) runs per group were carried out. The base-peak chromatogram for reversed-phase chromatography monitored by the mass spectrometer represents the intensity of all peptide ions in the sample in a single scan. SEQUEST search was used to identify the peptide ions following the criteria as described in Methods. The results indicated a high overlap in ADP proteins among the 4 groups (Table VIII through Table XII). A Venn diagram with the number of proteins from each group and their overlaps among the four groups are shown in Figure 30. A total of 72 proteins were present in all four groups. Thirty proteins were exclusively present in the DIW control group. Twenty proteins were exclusively present in the $SnCl_2$/NaF group. Nineteen proteins were exclusively present in the NaF group and other 13 proteins were only present in $SnCl_2$ (Table XIII through Table XVI; Figure 30).

TABLES AND FIGURES

TABLE I

Testing solutions preparation

Solutions	Amounts
$SnCl_2$	800 ppm Sn: 1.277 g $SnCl_2$ /l, 2.3 g Na-gluconate g/l, pH 4.5
NaF	250 ppm F: 0.553 g/l NaF pH 4.5
$SnCl_2$/NaF	800 ppm Sn: 1.277 g $SnCl_2$ /l, 2.3 g Na-gluconate g/l; 250 ppm F: 0.553 g/l NaF, pH 4.5
0.3% citric acid (pH 2.6)	a. Weigh 3.0 g of powdered citric acid anhydrous (C1857, Sigma) and add to a beaker with ~800 ml of DIW b. Adjust the volume to 1 liter, with sterile water c. Determine the pH of the solution using a calibrated pH meter, under agitation. It should be 2.6. d. Record the pH value

TABLE II

Daily cycling procedure for Phase I

Steps	Procedures
Pellicle formation	24h exposure to saliva (2 ml/specimen)
Demin 1	5 min in 0.3% citric acid pH 2.6, (4ml/specimen) DIW rinse, blot dry (Kimwipes)
Remin 1, Treatment 1 (fresh saliva)	30 min in saliva (2 ml/specimen) Blot dry 2 min rinse treatment (4 ml/specimen) Blot dry 30 min in saliva Blot dry
Demin 2	Similar to D1
Remin 2	60 min in saliva Blot dry
Demin 3	Similar to D1
Remin 3, Treatment 2 (fresh saliva)	Similar to R1, T1
Demin 4	Similar to D1
Remin 4	Similar to R2
Demin 5	Similar to D1
Remin 5,	Similar to R2
Demin 6	Similar to D1
Remin6, Treatment3 (fresh saliva)	Similar to R1, T1
Remin	Immersion in saliva overnight

TABLE III

Cycling procedure for Phase II

Steps	Procedures
1	Saliva incubation - 24 h
2	Treatment - 2 min
3	2 h - Saliva incubation

Steps 2 & 3 were repeated 3 times for one day

TABLE IV

ANOVA table

Effect	Num DF	Den DF	F Value	P Value
Group	3	36	211.86	<.0001
Substrate	1	36	71.68	<.0001
Group*Substrate	3	36	39.99	<.0001

TABLE V

Results summary (A: $SnCl_2$, B: NaF, C: $SnCl_2$/ NaF, D: DIW)

Substrate	Group	N	Mean	Standard Deviation	Confidence Limits
Dentin	$SnCl_2$	10	-13.24	1.11	(-14.04, -12.45)
	NaF	10	-10.98	0.87	(-11.60, -10.35)
	$SnCl_2$/ NaF	10	-6.89	0.83	(-7.49, -6.30)
	DIW	10	-17.18	0.83	(-17.78, -16.59)
Enamel	$SnCl_2$	10	-6.14	1.50	(-7.21, -5.06)
	NaF	10	-10.85	1.51	(-11.93, -9.77)
	$SnCl_2$/ NaF	10	-2.05	0.72	(-2.56, -1.53)
	DIW	10	-18.60	3.32	(-20.98, -16.23)

TABLE VI

Substrate comparisons

Comparison	Difference	Standard Error	P value
A: Dentin < Enamel	-7.10	0.63	<.0001
B: Dentin & Enamel n.s.	-0.13	0.63	1.0000
C: Dentin < Enamel	-4.85	0.63	<.0001
D: Dentin & Enamel n.s.	1.42	0.63	0.3491

TABLE VII

Solutions comparisons

Comparison	Difference	Std Error	P value
Dentin: A < B	-2.27	0.70	0.0462
Dentin: A < C	-6.35	0.70	<.0001
Dentin: A > D	3.94	0.70	<.0001
Dentin: B < C	-4.08	0.70	<.0001
Dentin: B > D	6.21	0.70	<.0001
Dentin: C > D	10.29	0.70	<.0001
Enamel: A < C	-4.09	0.70	<.0001
Enamel: A > B	4.71	0.70	<.0001
Enamel: A > D	12.46	0.70	<.0001
Enamel: B < C	-8.80	0.70	<.0001
Enamel: B > D	7.75	0.70	<.0001
Enamel: C > D	16.55	0.70	<.0001

TABLE VIII

Proteins present in all groups. (A: $SnCl_2$, B: NaF, C: $SnCl_2$/NaF, D: DIW)

Accession Number	Protein Name	GPA/ GPD	P Value	GPB/ GPD	P Value	GPC/ GPD	P Value
A7Y9J9	Mucin 5AC, oligomeric mucus/gel-forming	1.20	0.001	2.38	0.278	1.98	0.000
A8K2H9	cDNA FLJ78503, highly similar to Homo sapiens keratin 13 (KRT13), transcript variant 1, mRNA	0.90	0.001	0.92	0.001	1.87	0.000
A8K2U0	Alpha-2-macroglobulin-like protein 1	1.05	0.001	0.96	0.002	1.32	0.000
B1AN48	Small proline-rich protein 3	0.56	0.001	0.99	0.000	1.87	0.010
B4DGT4	cDNA FLJ61241, highly similar to Histone deacetylase 5	0.87	0.001	0.94	0.020	1.38	0.013
B4DPR2	cDNA FLJ50830, highly similar to Serum albumin	0.90	0.001	1.14	0.002	1.77	0.045
B4DZ16	cDNA FLJ58649	0.53	0.001	1.02	0.004	1.42	0.320
B4E1T1	cDNA FLJ54081, highly similar to Keratin, type II cytoskeletal 5	1.13	0.001	1.07	0.031	1.37	0.015
B5ME49	Mucin-16	0.97	0.001	0.93	0.001	1.09	0.020
B7Z5K0	cDNA FLJ52445, highly similar to Homo sapiens membrane-associated ring finger (C3HC4) 7 (MARCH7), mRNA	1.97	0.001	1.98	0.070	2.50	0.001
B7ZMD7	Amylase, alpha 1A (Salivary)	1.19	0.001	0.65	0.000	1.20	0.003
B8ZZJ3	Alstrom syndrome protein 1	0.99	0.001	1.01	0.017	1.34	0.002
C9JA77	Uncharacterized protein	0.69	0.001	0.65	0.000	2.32	0.001
E7EMQ1	Carbonic anhydrase 6	0.90	0.001	1.01	0.038	1.38	0.002
E7EQT2	Mucin-4 beta chain	0.91	0.001	0.91	0.003	1.37	0.003
E7ESM2	Urokinase-type plasminogen activator chain B	0.88	0.001	0.86	0.003	1.34	0.000
E7ETI5	Uncharacterized protein	0.99	0.001	1.18	0.001	1.43	0.002
F4MHJ1	Ubiquitously transcribed tetratricopeptide repeat protein Y-linked transcript variant 97	0.95	0.001	0.98	0.001	1.43	0.005
F6KPG5	Albumin	0.92	0.001	0.84	0.001	1.45	0.012
G3CIG0	MUC19 variant 12	0.97	0.001	0.98	0.001	1.46	0.001
H0Y930	Extracellular matrix protein FRAS1	0.96	0.001	0.69	0.001	1.08	0.001
H6VRF8	Keratin 1	0.81	0.001	0.93	0.001	1.67	0.006
H7BXM7	Uncharacterized protein	1.10	0.001	1.52	0.001	1.61	0.003

(continued)

TABLE VIII (cont.)

H7BYJ0	Uncharacterized protein	0.92	0.001	0.87	0.001	1.60	0.005
O15079	Syntaphilin	0.97	0.001	1.17	0.001	1.48	0.001
P01034	Cystatin-C	1.08	0.002	0.92	0.001	1.37	0.000
P01036	Cystatin-S	0.70	0.002	0.99	0.001	1.51	0.000
P01037	Cystatin-SN	0.98	0.001	0.95	0.001	1.54	0.002
P01833	Polymeric immunoglobulin receptor	1.06	0.000	1.20	0.001	1.40	0.002
P01877	Ig alpha-2 chain C region	0.99	0.010	1.06	0.001	1.38	0.002
P02808	Statherin	0.59	0.030	0.57	0.001	1.08	0.000
P02810	Salivary acidic proline-rich phosphoprotein ½	0.93	0.017	2.25	0.001	2.37	0.023
P02812	Basic salivary proline-rich protein 2	1.16	0.002	0.99	0.001	1.43	0.000
P02814	Submaxillary gland androgen-regulated protein 3B	1.11	0.048	1.00	0.001	1.40	0.001
P04080	Cystatin-B	0.90	0.048	0.55	0.001	1.19	0.006
P06733	Alpha-enolase	0.55	0.002	0.71	0.002	1.42	0.002
P07737	Profilin-1	1.05	0.003	1.25	0.001	1.42	0.001
P09228	Cystatin-SA	0.98	0.019	0.97	0.001	1.35	0.004
P0CG05	Ig lambda-2 chain C regions	0.75	0.051	0.62	0.001	1.10	0.006
P15515	Histatin-1	0.89	0.068	0.78	0.001	1.46	0.002
P15516	Histatin-3	3.20	0.005	1.96	0.001	3.27	0.001
P19961	Alpha-amylase 2B	0.65	0.010	0.96	0.001	1.23	0.003
P28325	Cystatin-D	1.54	0.002	0.91	0.001	1.28	0.001
P35908	Keratin, type II cytoskeletal 2 epidermal	0.79	0.350	0.78	0.001	1.41	0.001
P54652	Heat shock-related 70 kDa protein 2	0.93	0.015	0.78	0.001	2.35	0.001
Q8TAX7	Mucin-7	0.93	0.000	2.02	0.001	1.47	0.002
Q8WVD6	PHTF2 protein	0.75	0.825	0.89	0.001	1.48	0.005
Q8WZ42	Titin	0.93	0.095	0.92	0.001	1.37	0.001
Q9HC84	Mucin-5B	0.79	0.278	0.91	0.001	2.36	0.001
Q9NR09	Baculoviral IAP repeat-containing protein 6	0.95	0.001	0.79	0.001	1.47	0.002
Q9NV58	E3 ubiquitin-protein ligase RNF19A	0.62	0.000	1.95	0.001	1.14	0.001
A0M8Q9	C1 segment protein	0.96	0.234	0.78	0.001	1.58	0.001
A8K5I6	cDNA FLJ78643, highly similar to Homo sapiens cornulin (CRNN), mRNA	0.91	0.020	0.94	0.001	1.44	0.012

(continued)

TABLE VIII (cont.)

B2R853	cDNA, FLJ93744, highly similar to Homo sapiens keratin 6E (KRT6E), mRNA	1.11	0.002	0.97	0.001	1.39	0.012
B3W5Y6	Serpin B3	1.06	0.002	0.95	0.001	1.48	0.004
B4DL17	cDNA FLJ52558, highly similar to Keratin, type I cytoskeletal 13	1.85	0.003	1.37	0.015	1.33	0.001
B4DRR0	cDNA FLJ53910, highly similar to Keratin, type II cytoskeletal 6A	0.89	0.002	1.00	0.003	1.96	0.002
E7EQV7	Uncharacterized protein	0.95	0.002	0.92	0.032	1.49	0.001
G3V1A4	Cofilin 1 (Non-muscle), isoform CRA_a	0.62	0.001	1.01	0.006	1.30	0.011
P01040	Cystatin-A	0.98	0.017	0.86	0.171	1.26	0.023
P01857	Ig gamma-1 chain C region	0.94	0.000	0.90	0.028	1.34	0.010
P04792	Heat shock protein beta-1	1.09	0.038	1.91	0.002	2.61	0.010
P05109	Protein S100-A8	0.74	0.041	0.95	0.002	1.35	0.015
P06702	Protein S100-A9	0.83	0.001	0.88	0.003	1.39	0.003
P07355	Annexin A2	1.15	0.024	1.23	0.001	1.47	0.016
P07737	Profilin-1	2.04	0.003	3.20	0.010	1.02	0.010
P27482	Calmodulin-like protein 3	0.80	0.003	1.01	0.000	1.42	0.001
P31947	14-3-3 protein sigma	0.94	0.002	0.95	0.035	1.45	0.002
P35908	Keratin, type II cytoskeletal 2 epidermal	0.77	0.001	0.88	0.000	1.61	0.000
P47929	Galectin-7	0.84	0.003	0.78	0.001	1.39	0.003
P62805	Histone H4	1.06	0.003	2.96	0.005	2.32	0.000
P68371	Tubulin beta-4B chain	1.25	0.005	0.99	0.005	1.42	0.000

TABLE IX

Proteins present in $SnCl_2$ and NaF groups

Accession Number	Protein Name
A4D1R9	Homeodomain interacting protein kinase 2
B3KVI8	cDNA FLJ16604 fis, clone TESTI4008097, highly similar to Polycomb group protein ASXL1
B3KVV3	cDNA FLJ41584 fis, clone CTONG2020445, highly similar to ATP-binding cassette sub-family A member 12
B4DT53	cDNA FLJ52905, highly similar to Runt-related transcription factor 3
B4E1M1	cDNA FLJ60391, highly similar to Lactoperoxidase (EC 1.11.1.7)
D3DVP6	Macrophage erythroblast attacher, isoform CRA_d
E7EQE3	no name
E7ESP5	no name
E7EWI7	no name
F8WAI1	Immunoglobulin-like and fibronectin type III domain-containing protein 1
H7C0P6	Mitogen-activated protein kinase kinase kinase kinase 4
O43157	Plexin-B1
P49454	Centromere protein F
Q2NKL1	Mineralocorticoid receptor
Q5T1R4	Transcription factor HIVEP3
Q5T3N0	Annexin A1
Q6WKZ4	Rab11 family-interacting protein 1
Q6WRI0	Immunoglobulin superfamily member 10
Q7Z7G8	Vacuolar protein sorting-associated protein 13B
Q9BQK8	Phosphatidate phosphatase LPIN3
Q9UKZ4	Teneurin-1
Q9ULK2	Ataxin-7-like protein 1

TABLE X

Proteins present in $SnCl_2$ and $SnCl_2$ /NaF groups

Accession Number	**Protein Name**
Q9C0A1	Zinc finger homeobox protein 2
A8K008	cDNA FLJ78387
A8K4G7	cDNA FLJ78528, highly similar to Homo sapiens vacuolar protein sorting 4B (yeast) (VPS4B), mRNA
B3KU50	cDNA FLJ39199 fis, clone OCBBF2005189, highly similar to Homo sapiens ankyrin repeat domain 17 (ANKRD17), transcript variant 2, Mrna
B4DII4	cDNA FLJ53493, highly similar to HEF-like protein
B5MCY1	Tudor domain-containing protein 15
C0JYZ1	Dynein, axonemal, heavy chain 11
E9PCX8	Tensin-3
F5H2N0	no name
O60284	Suppression of tumorigenicity 18 protein
P35568	Insulin receptor substrate 1
Q53EU2	GATA binding protein 6 variant
Q9NSI6	Bromodomain and WD repeat-containing protein 1
Q9ULM3	YEATS domain-containing protein 2
Q9UQB3	Catenin delta-2

TABLE XI

Proteins present in $SnCl_2$/NaF and DIW (control) groups.

Accession Number	Protein Name
A4FUT8	JMJD1B protein
A5YKK5	KIAA0232
B3KNA1	cDNA FLJ14021 fis, clone HEMBA1002513, highly similar to Histone deacetylase 6
B3KRV8	cDNA FLJ34970 fis, clone NTONG2005363, highly similar to Castor homolog 1 zinc finger protein
B4DZR3	cDNA FLJ59826, highly similar to Zinc finger protein ZFPM
B7Z8W3	cDNA FLJ53272, highly similar to Homo sapiens LIM domain 7 (LMO7), mRNA
E9PDX3	Kinesin-like protein KIF13A
F6SWM5	C-Jun-amino-terminal kinase-interacting protein 2
F8W7E2	no name
F8WC76	no name
H0Y8W5	Rho GTPase-activating protein 21
H3BLS7	Vacuolar protein sorting-associated protein 13D
Q14517	Protocadherin Fat 1
Q4VXP2	Potassium voltage-gated channel subfamily KQT member 2
Q5CZC0	Fibrous sheath-interacting protein 2
Q6UB99	Ankyrin repeat domain-containing protein 11
Q6UX82	Ly6/PLAUR domain-containing protein 8
Q6ZMI9	cDNA FLJ23911 fis, clone CAE01964, highly similar to Homo sapiens EGF, latrophilin and seven transmembrane domain containing 1 (ELTD1)
Q86YR6	POTE ankyrin domain family member D
Q8N7Z5	Putative ankyrin repeat domain-containing protein 31
Q8TB46	ANKRD50 protein
Q92574	Hamartin
Q9UQ35	Serine/arginine repetitive matrix protein 2
Q9Y4B6	Protein VPRBP
Q9Y6X0	SET-binding protein

TABLE XII

Proteins present in NaF and $SnCl_2$ /NaF groups

Accession Number	Protein Name
A4D1A8	Similar to Piccolo protein (Aczonin)
A7E2D6	NAV2 protein
B4DI39	cDNA FLJ54328, highly similar to Heat shock 70 kDa protein 1
B4DSK7	cDNA FLJ50196, highly similar to Peroxisome proliferator-activated receptor-binding protein
B4DV38	PAP-associated domain-containing protein 5
D3DS86	E3 ubiquitin-protein ligase HECTD1
D3DX93	HCG1745555, isoform CRA_b
E7EPM4	Mucin-17
E9PJL5	Putative uncharacterized protein C12orf63
F5GWX1	no name
F8VW64	RNA-binding protein Nova-1
O75592	Probable E3 ubiquitin-protein ligase MYCBP2
P15822	Zinc finger protein 40
P48169	Gamma-aminobutyric acid receptor subunit alpha-4
P52948	Nuclear pore complex protein Nup98-Nup96
P58397	A disintegrin and metalloproteinase with thrombospondin motifs 12
P78333	Glypican-5
Q05BP9	OLIG2 protein
Q12923	Tyrosine-protein phosphatase non-receptor type 13
Q14204	Cytoplasmic dynein 1 heavy chain 1
Q1RMC5	Claspin homolog (Xenopus laevis)
Q2NKW8	Adenosylhomocysteinase
Q6N030	Putative uncharacterized protein DKFZp686I15212
Q7Z4S6	Kinesin-like protein KIF21A
Q7Z6E9	E3 ubiquitin-protein ligase RBBP6
Q86T35	Putative uncharacterized protein DKFZp451A177
Q8WVD6	PHTF2 protein
Q9H8V3	Protein ECT2
Q9Y2I7	1-phosphatidylinositol 3-phosphate 5-kinase
Q9Y6E7	NAD-dependent protein deacetylase sirtuin-4

TABLE XIII

Proteins exclusively present in $SnCl_2$ group

Accession Number	**Protein Name**
Q96E61	Uncharacterized protein
P06310	Ig kappa chain V-II region RPMI 6410
Q6PIT5	Uncharacterized protein
Q6GMW0	Uncharacterized protein
Q7Z2U7	Uncharacterized protein
Q6GMV8	Uncharacterized protein
P22079	Lactoperoxidase
Q6NS95	Uncharacterized protein
Q8NEJ1	Uncharacterized protein
Q6PIH6	Uncharacterized protein
Q6GMW4	Uncharacterized protein
Q6GMX4	Uncharacterized protein
P02787	Serotransferrin

TABLE XIV

Proteins exclusively present in NaF group

Accession Number	**Protein Name**
Q502W4	Uncharacterized protein
P61626	Lysozyme C
Q6GMV7	Uncharacterized protein
Q9UBC9	Small proline-rich protein 3
Q6PIH4	Uncharacterized protein
P14618	Pyruvate kinase isozymes M1/M2
P12273	Prolactin-inducible protein
Q8TDL5	BPI fold-containing family B member 1
Q71V83	Alpha-A-crystallin
C9JS40	Uncharacterized protein
B3KSF4	Probable ATP-dependent RNA helicase DDX4
O15230	Laminin subunit alpha-5
B3KU03	cDNA FLJ39022 fis, clone NT2RP7003724, weakly similar to Serine/arginine repetitive matrix protein 1
B4DYM8	cDNA FLJ60373, highly similar to Zinc finger CCCH domain-containing protein11A
Q9ULK2	Ataxin-7-like protein 1
F8WEP2	ADP-ribosylation factor-like protein 6-interacting protein 4
E5RJ68	AP-3 complex subunit beta-1
F5H894	Uncharacterized protein
P20930	Filaggrin

TABLE XV

Proteins exclusively present in $SnCl_2$/NaF group

Accession No.	**Protein Name**
Q6GMX8	Uncharacterized protein
P23280	Carbonic anhydrase 6
P07339	Cathepsin D
P29508	Serpin B3
Q9UBX7	Kallikrein-11
P30740	Leukocyte elastase inhibitor
Q6PJF2	Uncharacterized protein
A6NN68	Uncharacterized protein
P02765	Alpha-2-HS-glycoprotein
P13796	Plastin-2
P09228	Cystatin-SA
P02679	Fibrinogen gamma chain
Q13349	Integrin alpha-D
H0YET1	Liprin-beta-2
B7Z9B9	Anoctamin
Q5T6C4	Ataxin-7-like protein 2
D1MPS6	Uncharacterized protein
H0Y7L2	Dedicator of cytokinesis protein 7
Q5T4S7	E3 ubiquitin-protein ligase UBR4
Q9NXG0	Centlein

TABLE XVI

Proteins exclusively present in DIW (control) group

Accession Number	Protein Name
Q6P5S2	UPF0762 protein C6orf58
Q5NV90	V2-17 protein
Q14515	SPARC-like protein 1
P31025	Lipocalin-1
P10599	Thioredoxin
Q9NQ38	Serine protease inhibitor Kazal-type 5
P31947	14-3-3 protein sigma
P04075	Fructose-bisphosphate aldolase A
P00450	Ceruloplasmin
P02749	Beta-2-glycoprotein 1
P20061	Transcobalamin-1
P60174	Triosephosphate isomerase
P54108	Cysteine-rich secretory protein 3
P09211	Glutathione S-transferase P
P22748	Carbonic anhydrase 4
P62937	Peptidyl-prolyl cis-trans isomerase A
P02675	Fibrinogen beta chain
Q6MZM9	Uncharacterized protein C4orf40
P15516	Histatin-3
P01009	Alpha-1-antitrypsin
P06733	Alpha-enolase
P00738	Haptoglobin
P02766	Transthyretin
B4DYR3	cDNA FLJ60976
Q3MIV8	Myosin-11
Q9UPR6	Zinc finger RNA-binding protein 2
H7BXJ7	Uncharacterized protein
H7BYT2	Uncharacterized protein
Q68D65	Putative uncharacterized protein DKFZp686B17277
B4DSN8	cDNA FLJ60863, highly similar to High mobility group protein 2-like 1

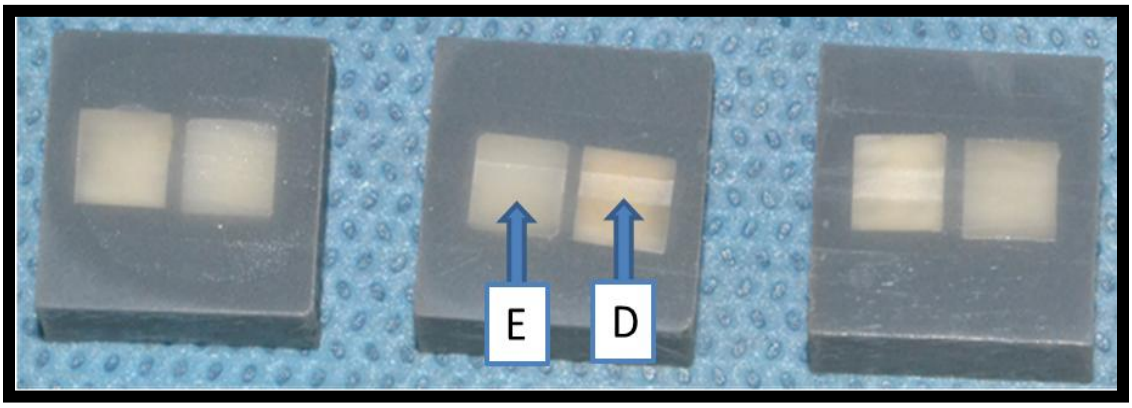

FIGURE 1. Dentin (D) and enamel (E) block (Phase I).

FIGURE 2. Each block is labeled (Phase I).

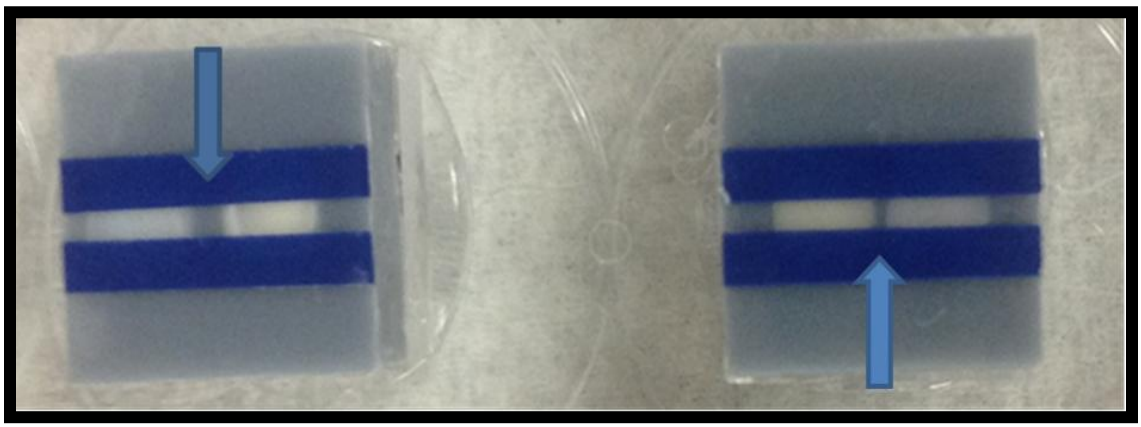

FIGURE 3. Blue tapes (arrows) were placed to determine lesion area (Phase I).

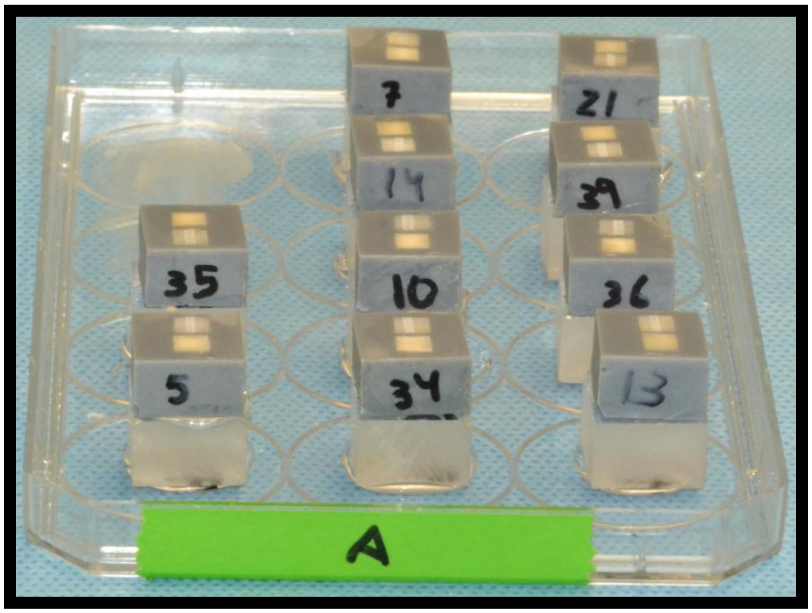

FIGURE 4. Specimens mounted on 12-well plate cover (Phase I).

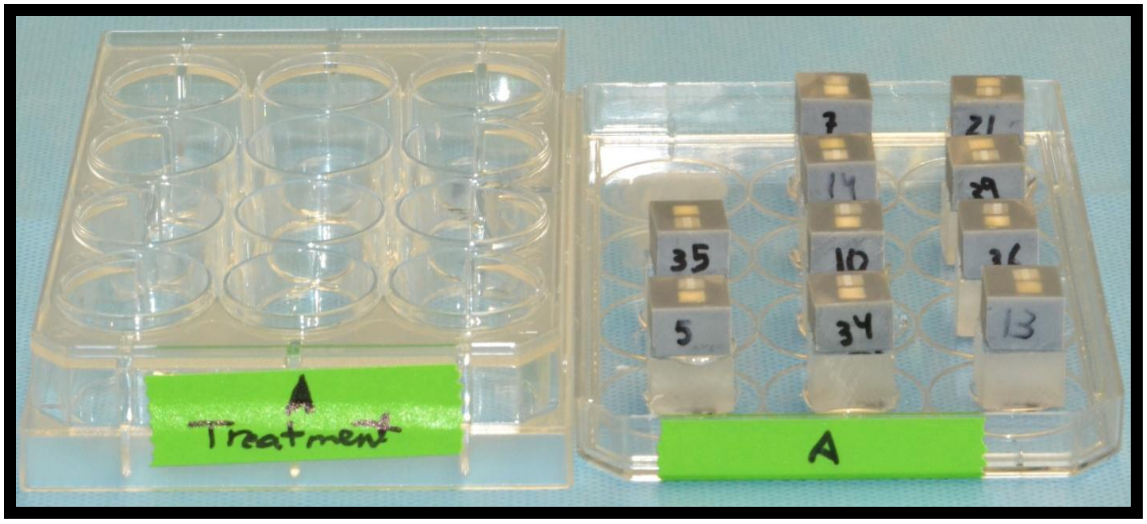

FIGURE 5. Both plates and cover were labeled (Phase I).

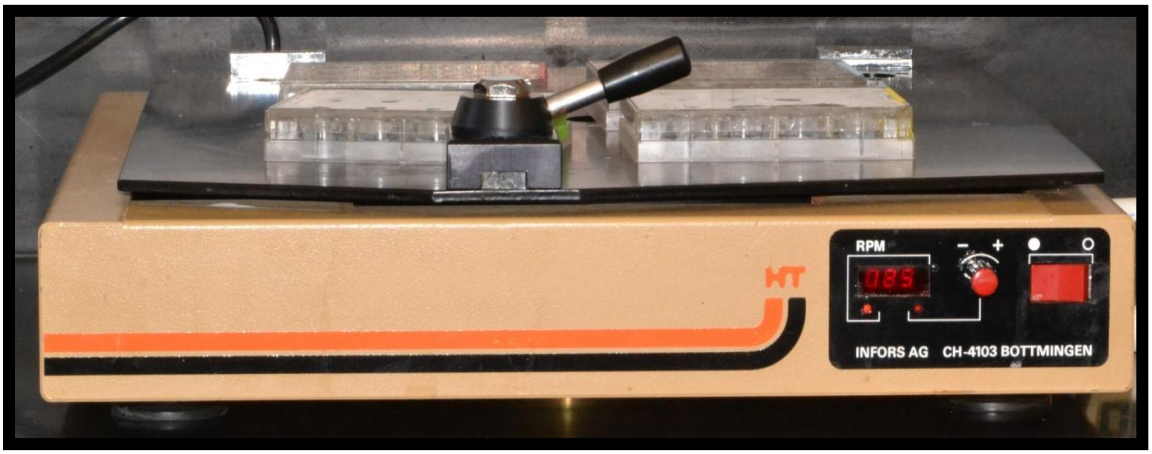

FIGURE 6. Saliva incubation under gentle agitation (both phases).

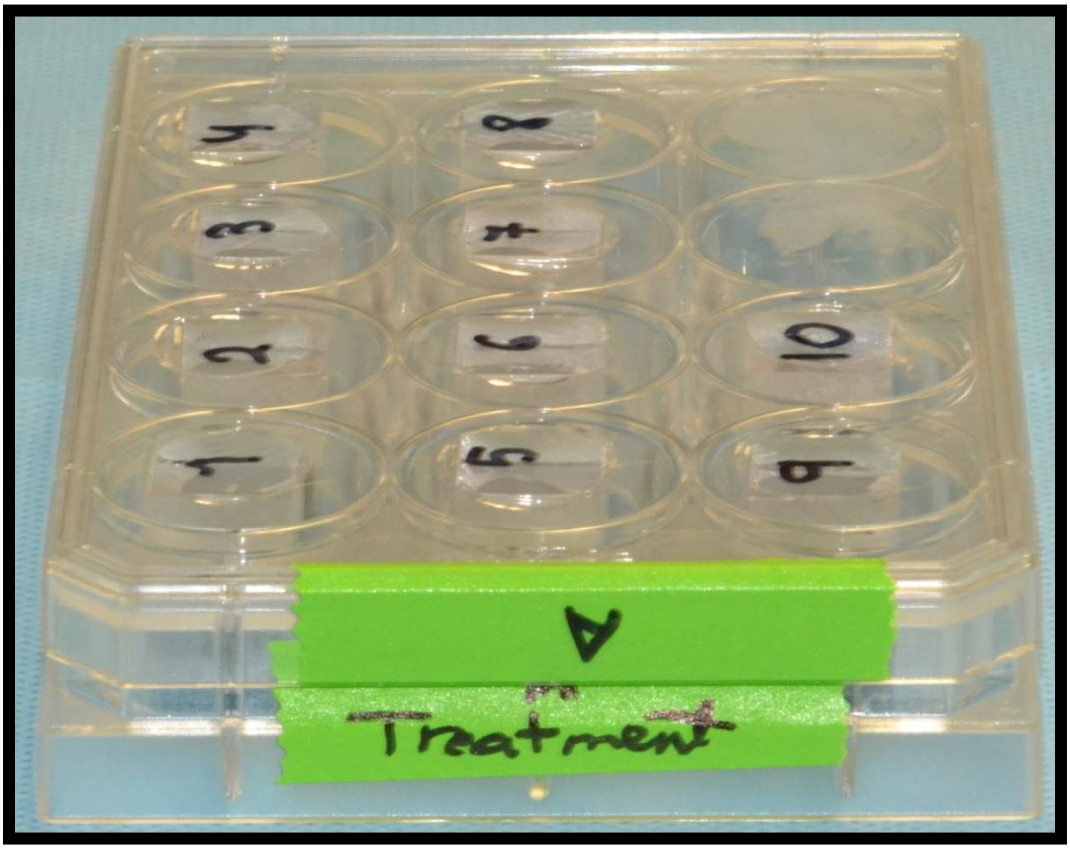

FIGURE 7. Treatment and acid erosion by 12-well plates (Phase I).

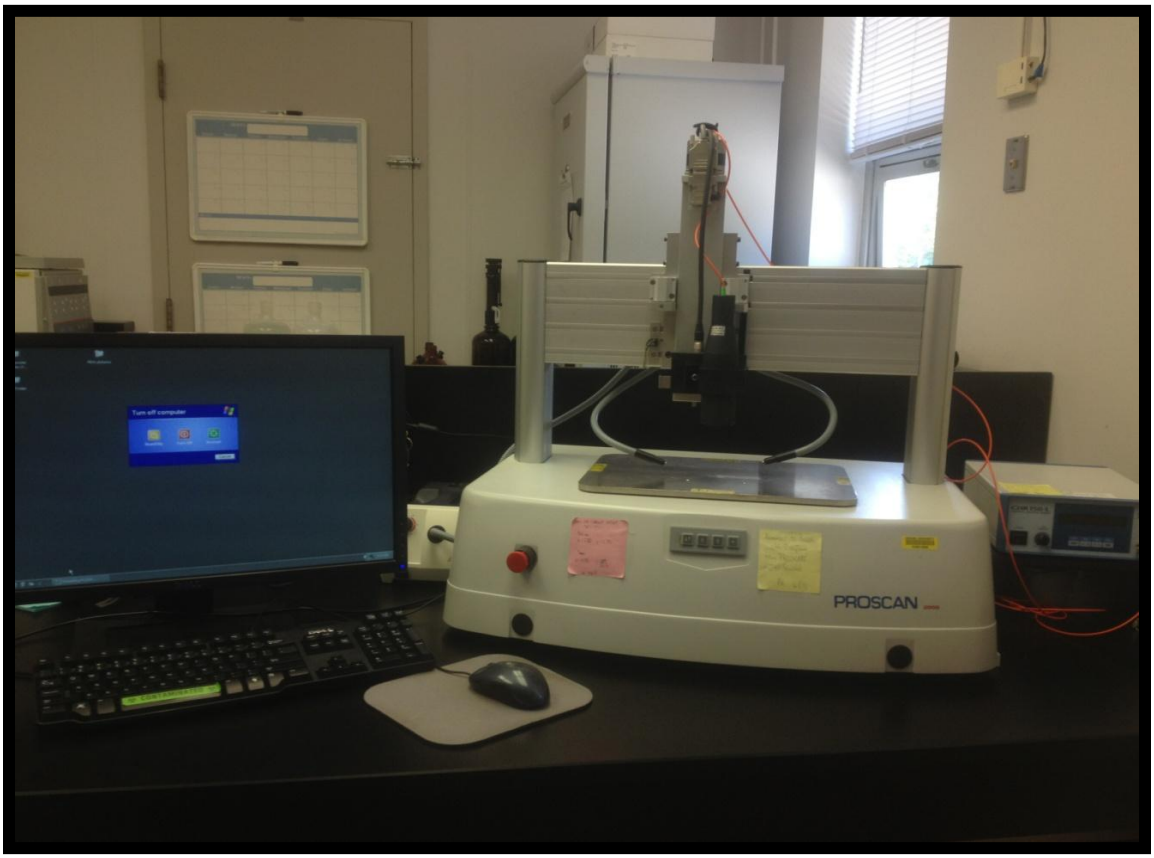

FIGURE 8. Optical profilometer (Proscan 2000) (Phase I).

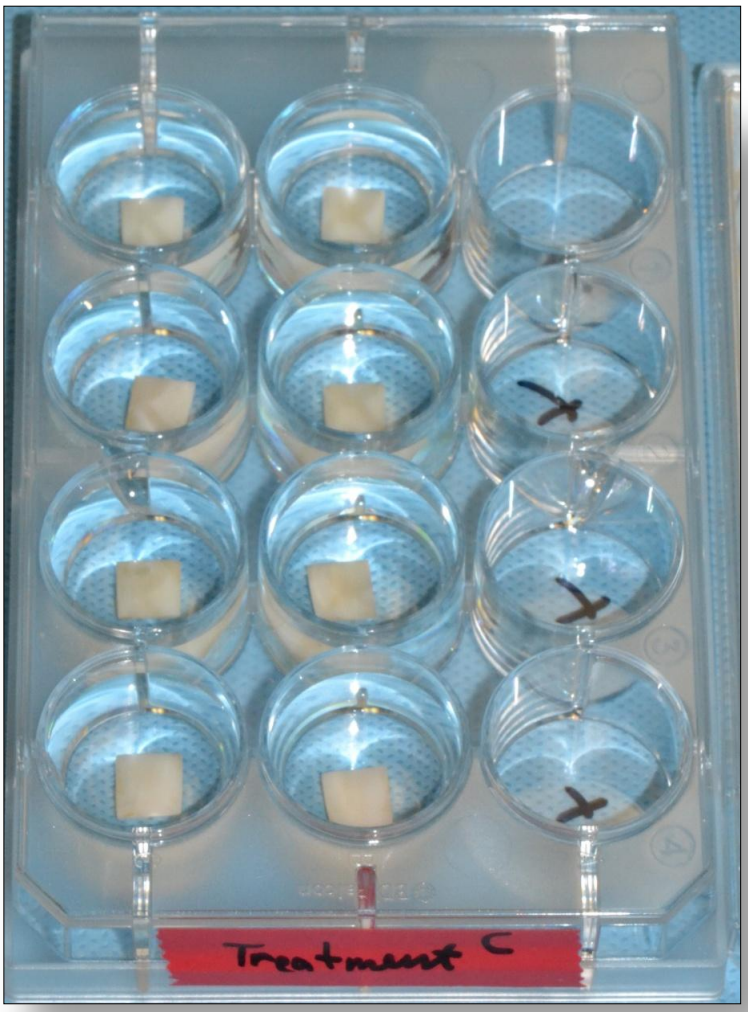

FIGURE 9. Specimens were immersed in solutions and saliva (Phase II).

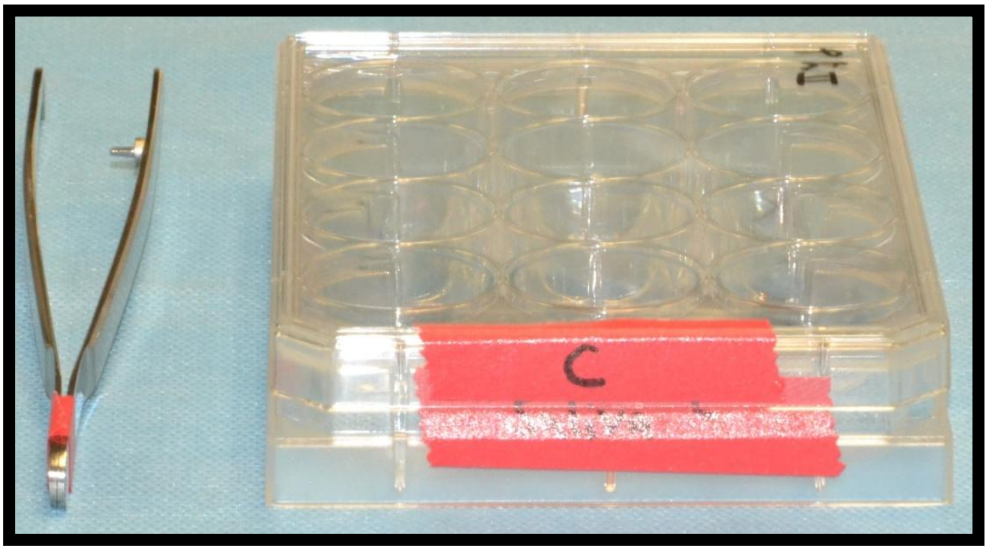

FIGURE 10. Forceps, plates and covers were color coded (Phase II).

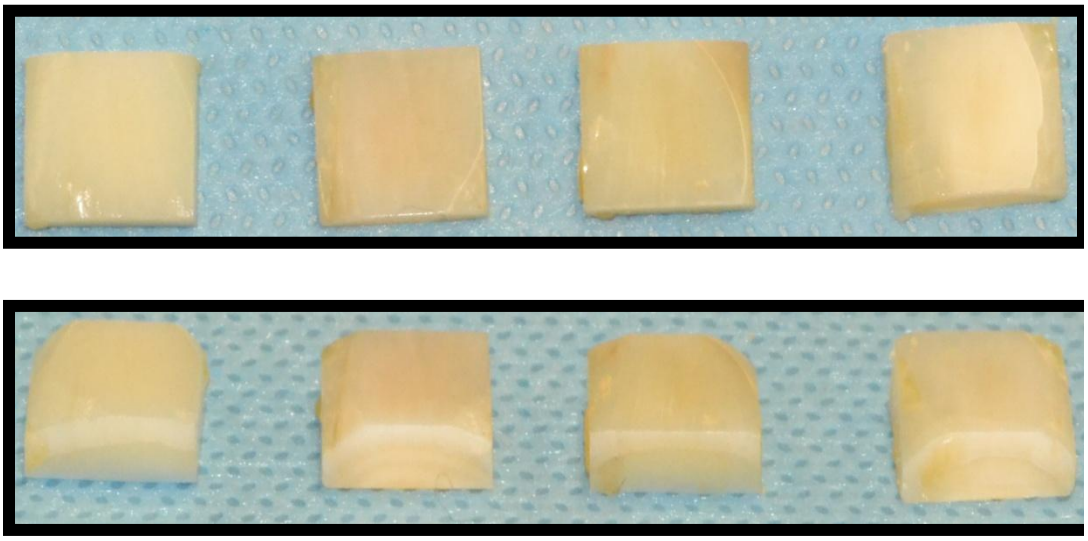

FIGURE 11 (top), FIGURE 12 (bottom). Bovine enamel specimens ($8x8x2mm^3$) (Phase II).

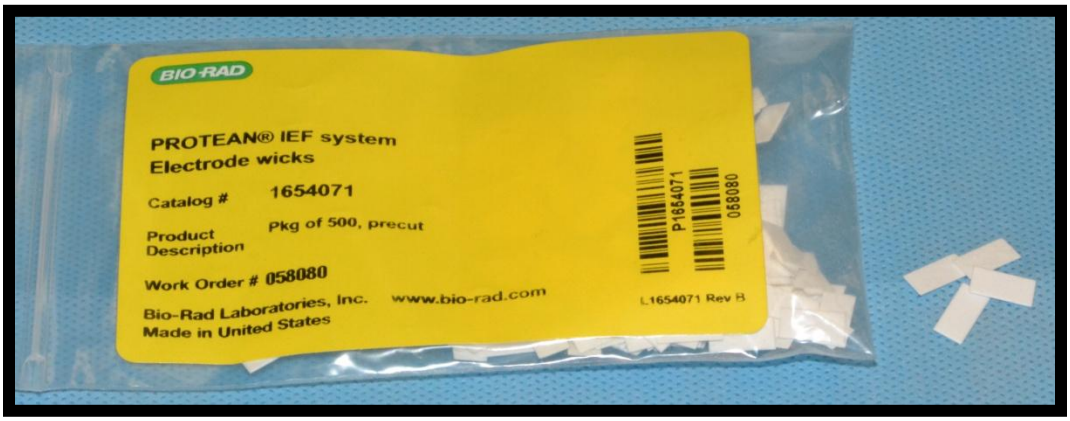

FIGURE 13. Collection strips of 0.5 cm × 1.0 cm (Phase II).

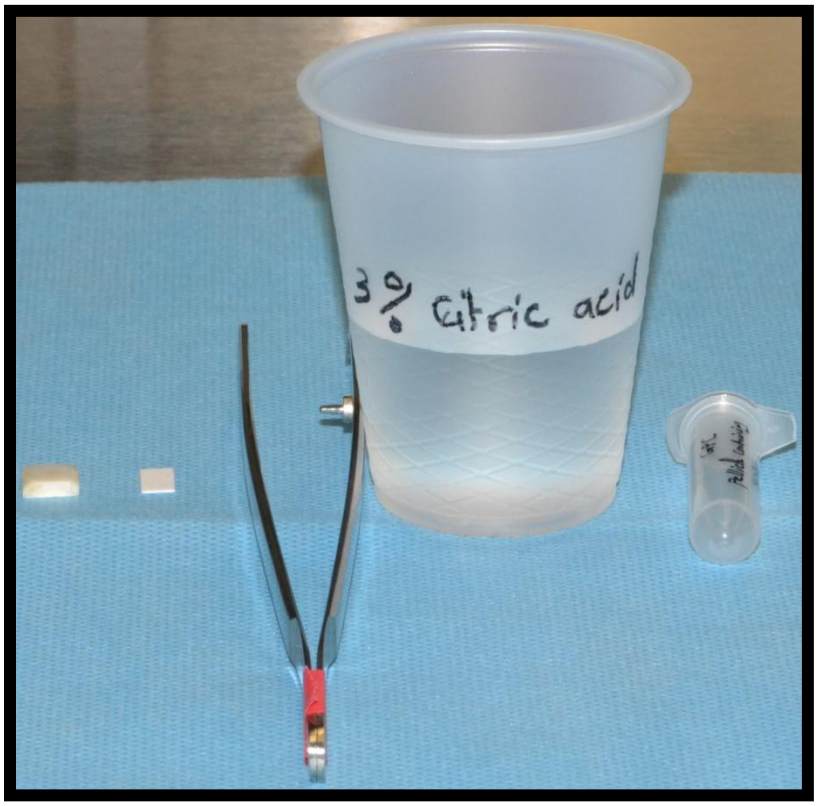

FIGURE 14. Materials needed for pellicle collection; specimens, strips, citric acid, forceps and microcentrifuge tubes (Phase II).

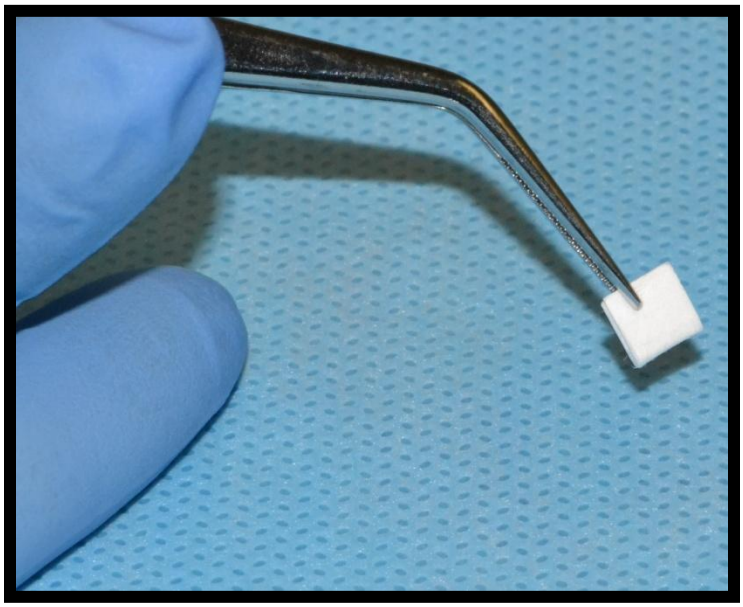

FIGURE 15. Folded strip (Phase II).

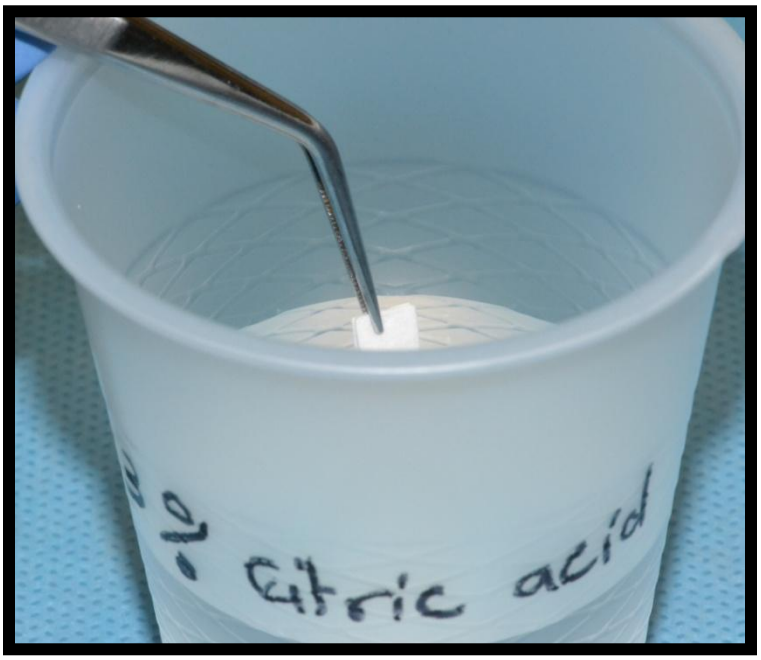

FIGURE 16. Strip being soaked in 3% citric acid (Phase II).

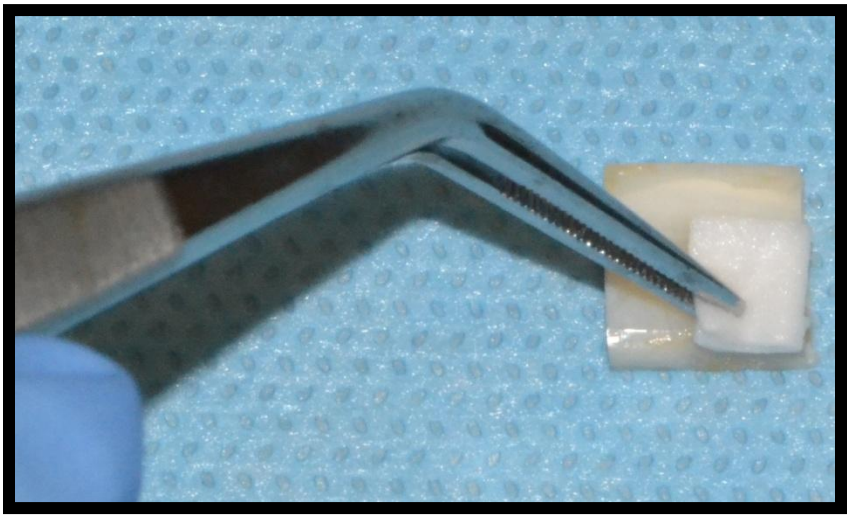

FIGURE 17. Pellicle collection from the surface of the specimens (Phase II).

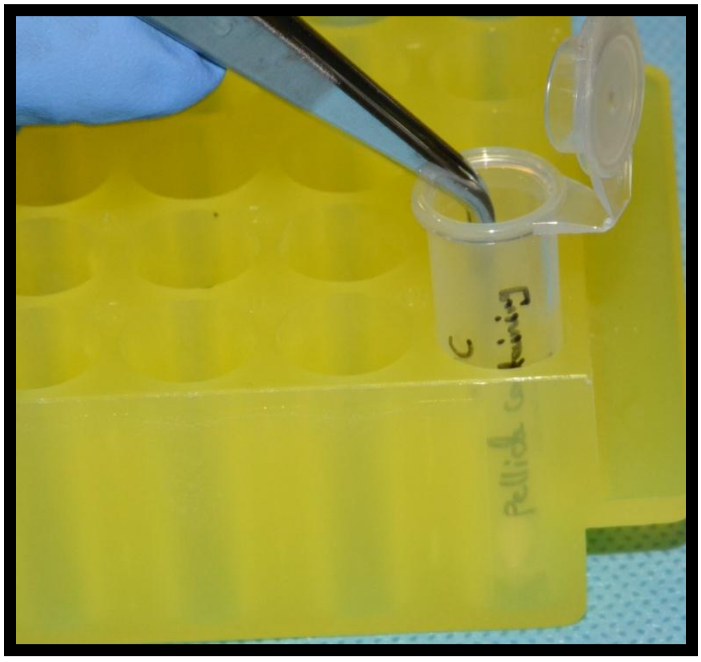

FIGURE 18. Strips being placed in microcentrifuge tubes after collection (Phase II).

FIGURE 19. 4 strips/group (Phase II).

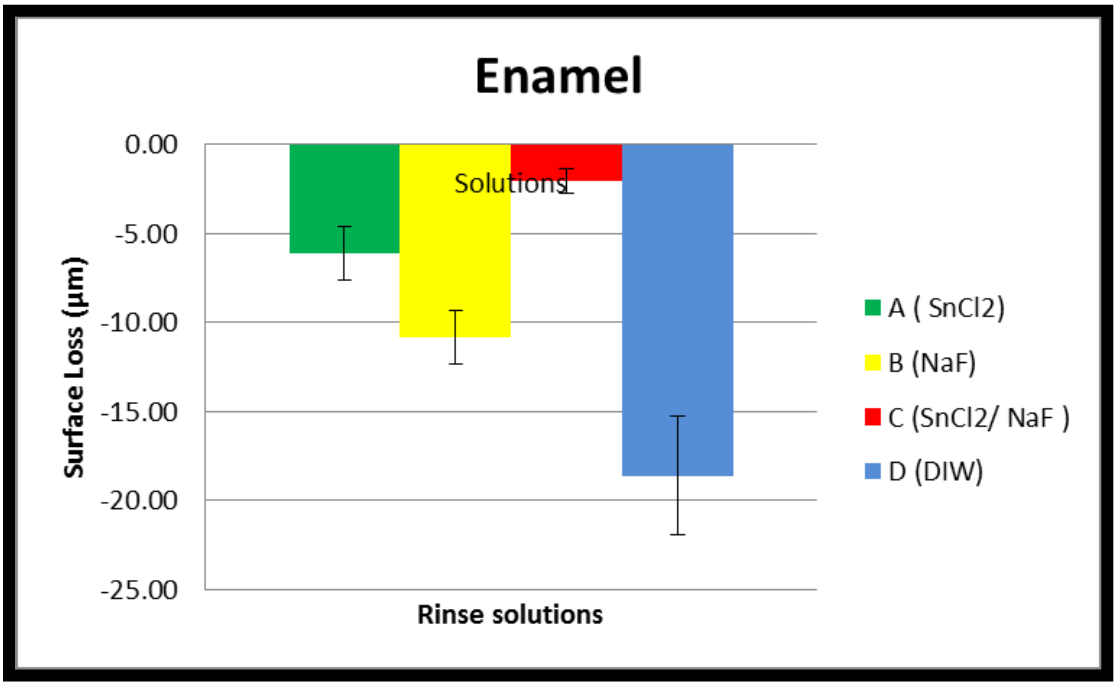

FIGURE 20. Bar graph displays the surface loss in enamel for each group (Phase I) .

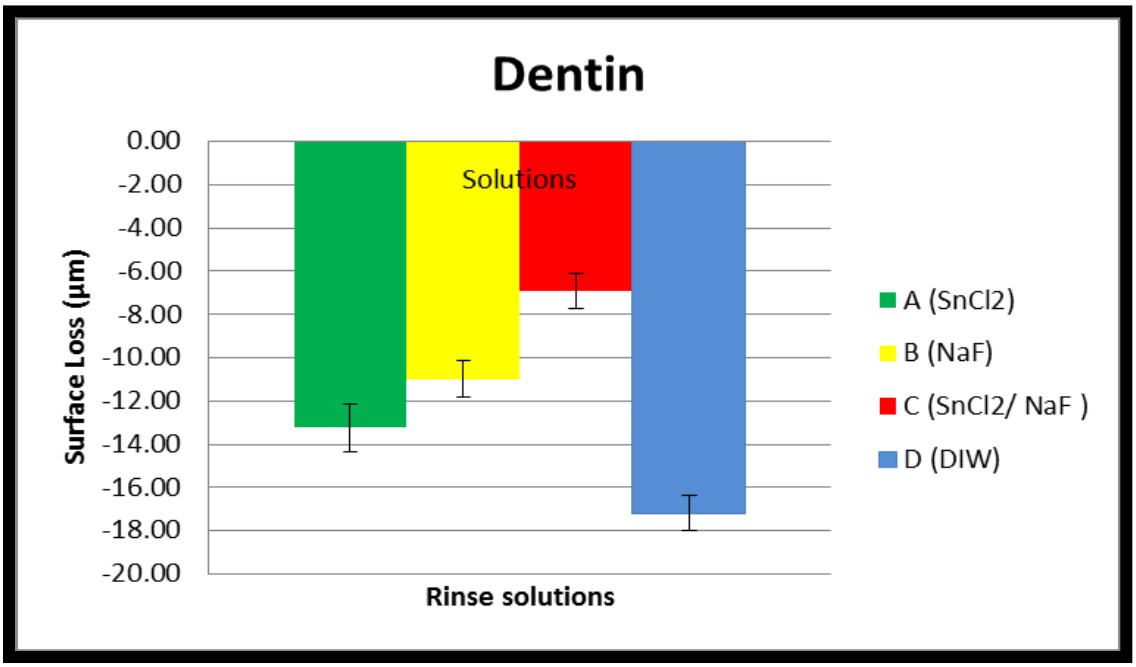

FIGURE 21. Bar graph displays the surface loss in dentin for each group (Phase I).

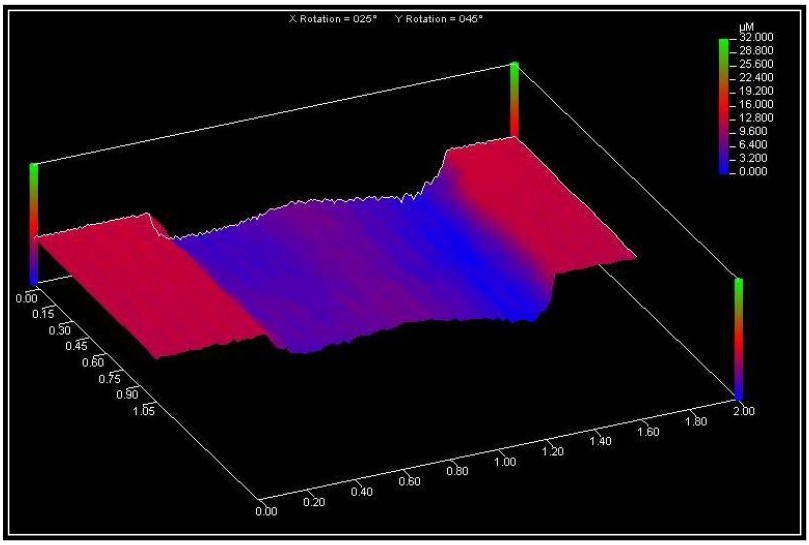

FIGURE 22. SnCl2 group. Example of profilometric analysis image (Phase I): enamel.

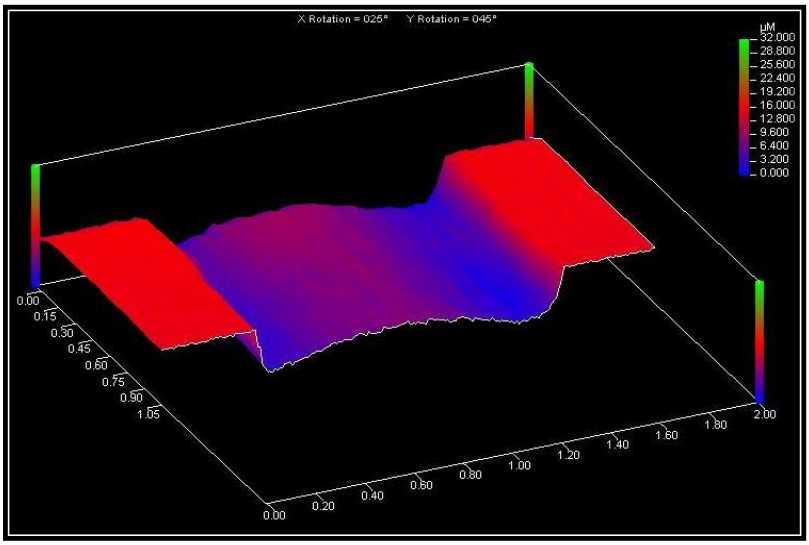

FIGURE 23. NaF group. Example of profilometric analysis image (Phase I): enamel.

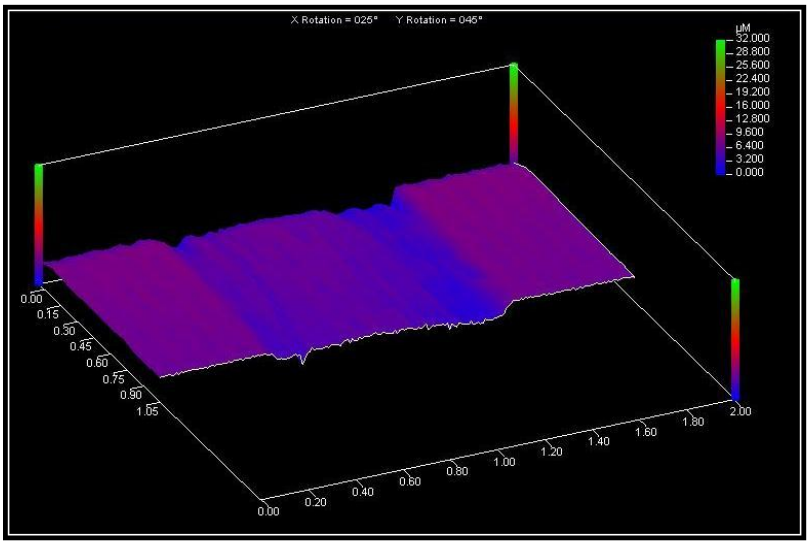

FIGURE 24. SnCl2/NaF group. Example of profilometric analysis image (Phase I): enamel.

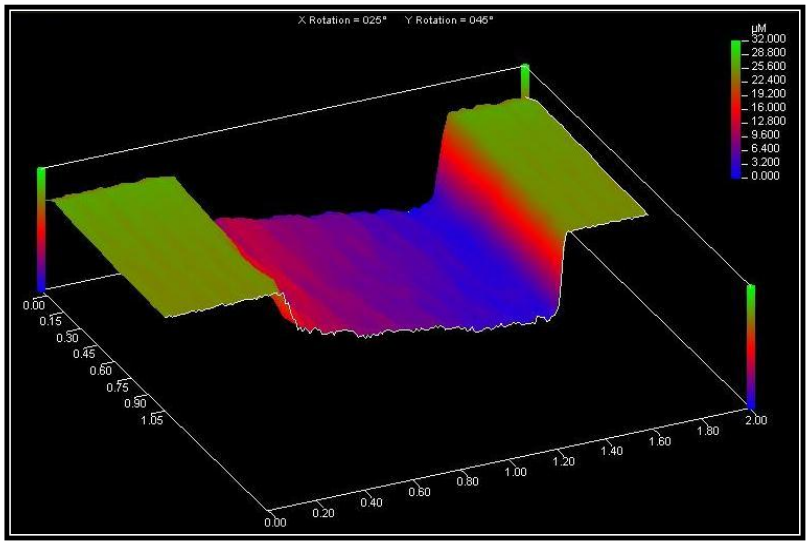

FIGURE 25. DIW (control) group. Example of profilometric analysis image (Phase I): enamel.

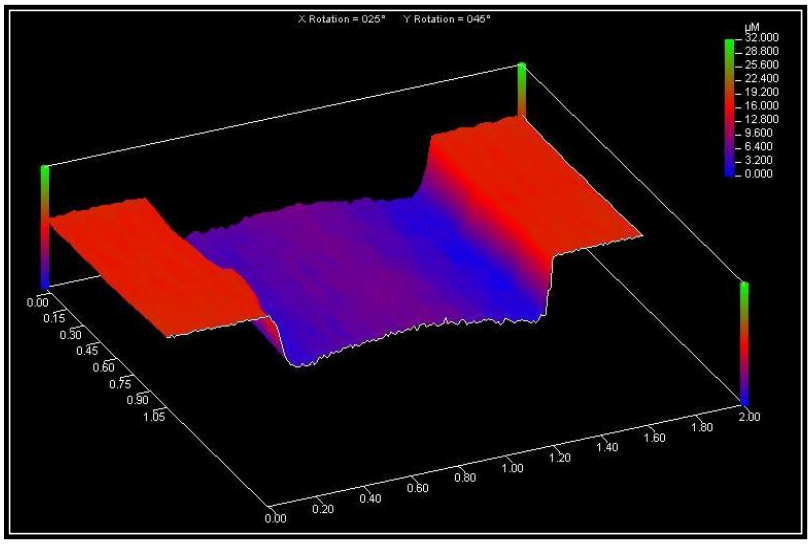

FIGURE 26. SnCl2 group. Example of profilometric analysis image (Phase I): dentin.

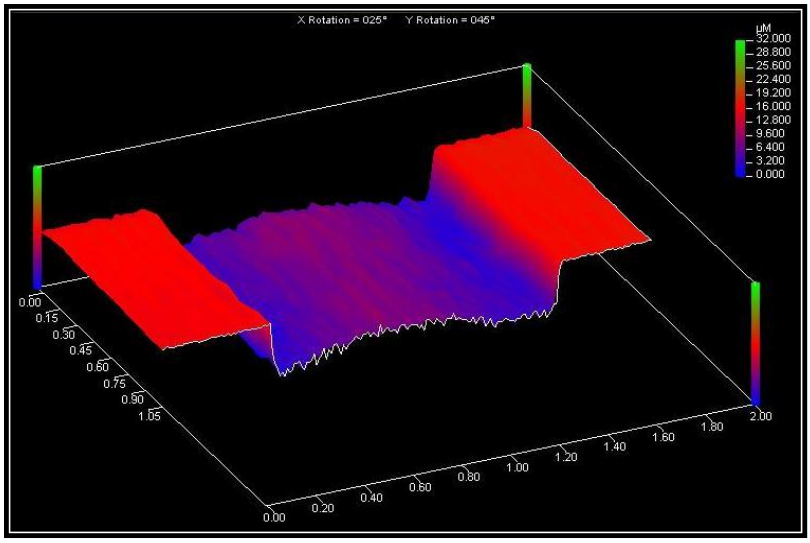

FIGURE 27. NaF group. Example of profilometric analysis image (Phase I): dentin.

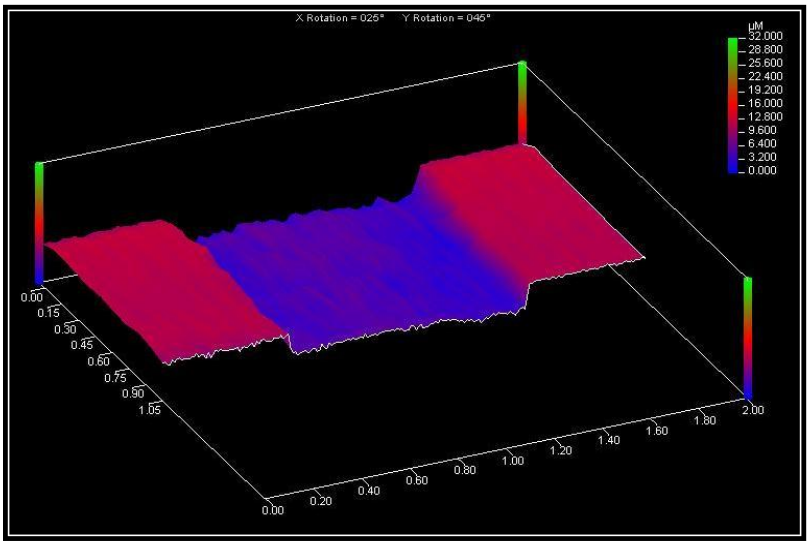

FIGURE 28. SnCl2/NaF group. Example of profilometric analysis image (Phase I): dentin.

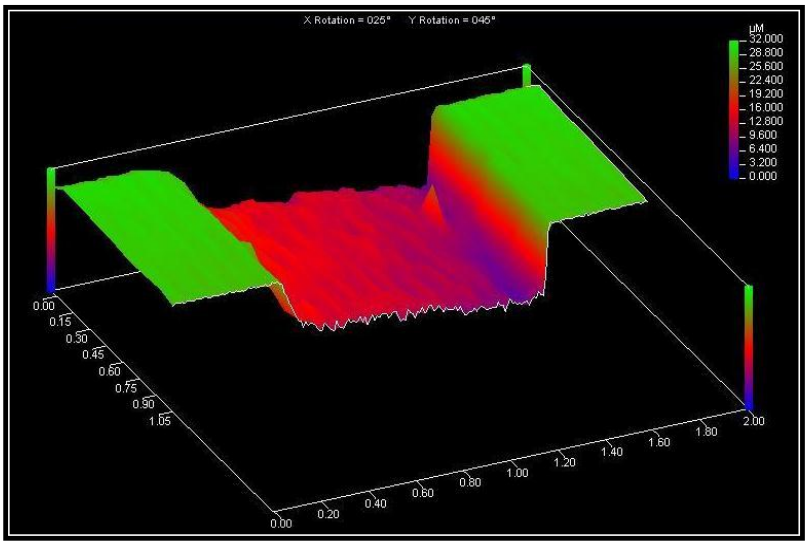

FIGURE 29. DIW (control) group. Example of profilometric analysis image (Phase I): dentin.

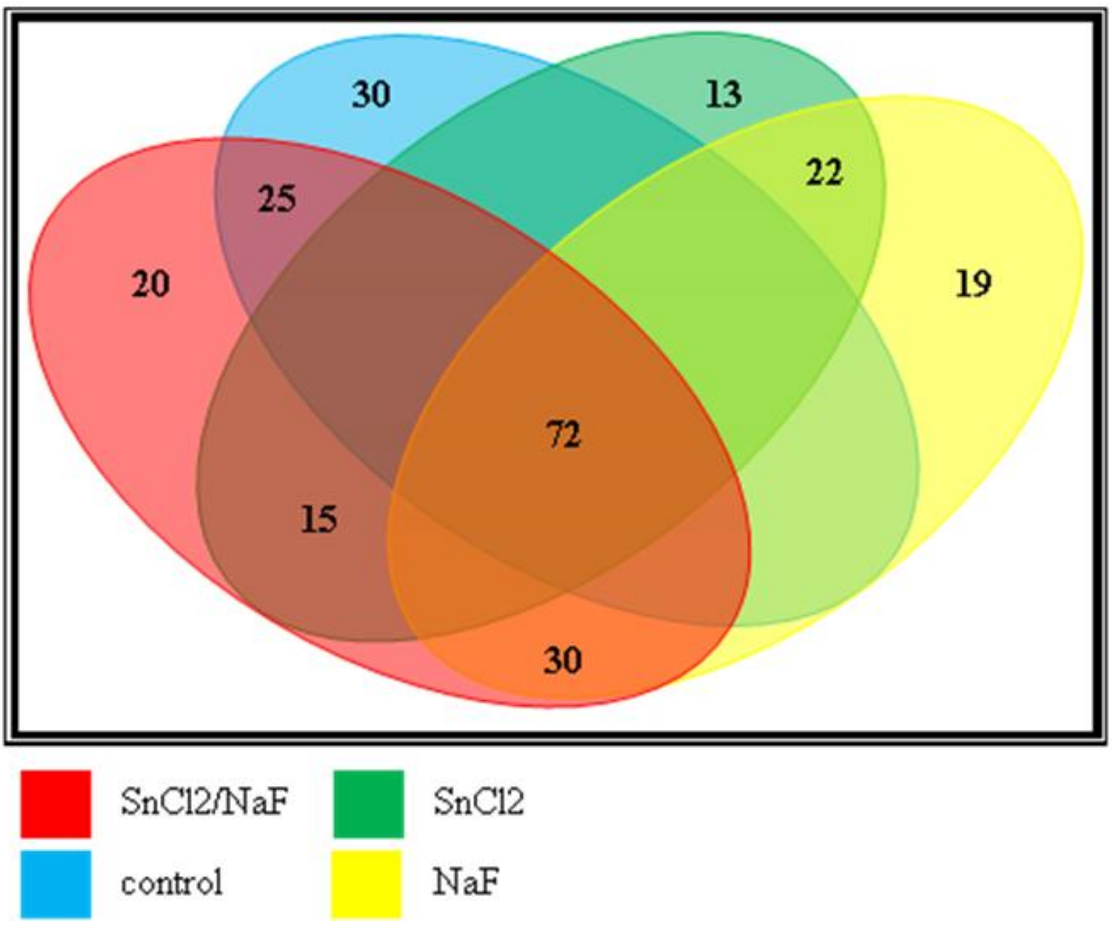

FIGURE 30. Venn diagram of ADP proteins identified in each group and across groups (Phase II).

DISCUSSION

PHASE I

Model and Evaluation Methods

Phase I was designed to investigate dental surface loss resulting from the interaction between pellicle proteins and stannous and fluoride-containing rinse solutions during cycles of erosive challenges. Initially, the samples were incubated in clarified human saliva for 24 h to allow a mature acquired dental pellicle (ADP) to form before starting the cycling procedure. This was done to enhance the interactive effects of stannous and fluoride ions on the ADP. The cycling model used in the present study was based on previous protocols and involved episodes of erosion (acid exposure) six times a day, for 5 min each[8,13,14]and a rinse solution application three times a day, for 2 min each.[9,11-14,64,115] Specimens were kept in clarified human saliva while not in the demin or rinse solutions to simulate remineralization and ADP re-formation. The choice of clarified human saliva over artificial formulations was done, so that a more realistic pellicle could have been formed. In an attempt to mimic clinical conditions, rinse treatment was performed after the first, the third, and the sixth erosive challenges representing exposure to the test rinses after the main meals (breakfast, lunch, and dinner, respectively). Citric acid was used to simulate dental exposure to most of the acidic beverages available, corroborating previously reported studies on the area.[6,8,9,11-14,115]

Several laboratory techniques are available for the evaluation of dental erosion on tooth surfaces, including surface profilometry, microradiography, chemical analysis, micro-indentation, and scanning electron microscopy (SEM).[77,121,122] In this study,

profilometry was used because it allows for the quantification of surface loss in a sensitive and reproducible way. The detection limit of this method has been reported to be approximately <0.3 µm and <0.5 µm for enamel and dentin, respectively.[106] Moreover, profilometry is the most commonly applied quantitative method to determine both dentin and enamel.[77] Non-contact profilometry was used because it has good flexibility for analyzing deep erosive lesions and avoids interference with the soft partially eroded dental surfaces, as commonly observed on the contact technique.[77]

$SnCl_2$-rinse Effect on Enamel and Dentin

The $SnCl_2$ solution was able to reduce enamel surface loss by 67 percent, compared with the DIW control. This result is similar to previous findings showing that a commercially available stannous mouthrinse (Meridol, GABA International), as well as a $SnCl_2$ (815 ppm, pH 2.6) test rinse could reduce the loss of enamel by 90 percent[118] and 65 percent[9] respectively. The $SnCl_2$ is capable of depositing appreciable amounts of tin on enamel, forming a stable protective layer.[117] It has been shown by SEM that this tin layer could be detected even after a 2-min exposure to 1.0-percent citric acid (pH 2.3).[9] Although the tin-containing testing solutions mentioned above had some differences compared to the one used in the present study, and despite that their specific mechanisms of action are unclear, it is evident that they can offer protection to the tooth surfaces against erosion. $SnCl_2$ also showed superior erosion protection when compared with the NaF rinse, which is in contrast with a previous study[9] where similar protection was observed. Differences among the test solutions and the erosion models could explain the contrast. Although the rinse solutions used in this study had similar concentrations to the

previous study, the pH values were different. In this study, the pH values for all solutions were adjusted to 4.5, while in the previous study $SnCl_2$ and NaF rinses presented pH of 2.3 and 3.5, respectively. Therefore, it can be speculated that the lower pH value of the NaF-rinse may have contributed to its better protection.[9,147] The erosive challenge was also different regarding acid concentration, pH, and total exposure time. The present study used 0.3-percent citric acid (pH 2.6), 5 min exposure for six times a day, for a total of 5 days; while the previously mentioned study used 1.0-percent citric acid with pH 2.3, 2 min exposure for six times a day, for a total of 10 days. Finally, clarified human saliva was used in this study, in contrast with the artificial saliva used in the previous study, which may have had an important role due to the interaction of the ADP and test rinses.

In dentin, $SnCl_2$ solution significantly reduced surface loss when compared with control (23 percent). The observed protection may be related to the same mechanisms as explained for enamel and is consistent with a previous study.[68] The relatively less prominent effect of tin-containing solutions in dentin compared with enamel might be due to its lower content of minerals, reducing the surface area for stannous action, which is known to be highly reactive with hydroxyapatite.[12] Another potential explanation may be due to the absence or formation of a less protective acquired pellicle on dentin than on enamel.

NaF-rinse Effect on Enamel and Dentin

The 250-ppm NaF rinse provided 42-percent reduction of enamel surface loss compared with the control. The mechanism of action of fluoride in erosion prevention is reported to be due to the formation of a CaF_2- or CaF_2-like layer on the enamel surface

after topical application. This would provide a physical mineral barrier for the erosive acids to dissolve before reaching the underlying enamel.[98] Nevertheless, the effect of a CaF_2-like layer is more obvious at higher concentrations[98] and could explain the less prominent anti-erosive effect of NaF solution in this study compared with the effect of the $SnCl_2$-rinse. This observation contrasts with an *in-vitro* study testing a more concentrated NaF solution (500 ppm F, pH 4.5),[12] where no significant better protection was found in comparison with the untreated control.[12] A possible explanation for the contrasting results is that a more aggressive erosive challenge was used in that study, consisting of 2.5 mmol/L HCl (pH 2.6), for 25 min in consecutive manner with no remineralization.[12]

The protocols of application and testing conditions are important factors determining the relative erosive protection provided by the NaF-rinses. An *in-situ* study compared erosion prevention of a single application of NaF 500 ppm F pH 4.5 and AmF/NaF/$SnCl_2$ solutions against erosive attack using 1.0-percent citric acid with pH 2.3 for 5 min 6 times/day for 7 days.[8] Minimum surface loss reduction was observed for the NaF solution (19 percent) in comparison with the control.[8] In that study, an aggressive erosion model was used, including higher concentration of citric acid with low pH accompanied with a single application of the treatment solution per day. It was suggested that this led to a dissolution of CaF_2 precipitates making the preventive effect of NaF less prominent.[8] An *in-vitro* study that tested the effect of NaF (500 ppm F, pH 4.5) and AmF/NaF/$SnCl_2$ solutions in erosion progression prevention used 0.65-percent citric acid pH 3.6 as an erosive solution and clarified human saliva for remineralization.[115] The NaF solution significantly slowed down the erosion progression when compared with the

control, yet AmF/NaF/SnCl2 solution provided better protection, which is in agreement with the present findings.[115]

In this study, NaF solution reduced surface loss on dentin by 36 percent compared with the control, which is significantly more than $SnCl_2$ but less than the combination of both. The protective effect of NaF is most probably due to retention of fluoride in peritubular, intertubular, and intratubular dentin after topical application because of the porosity and the water content of dentin, providing a fluoride reservoir.[98,119,120] Although the same treatment ranking order has been observed in another *in-vitro* study (NaF was better than $SnCl_2$ but less than the combination), the NaF effect was more obvious at 59 percent and 34 percent with and without the organic matrix, respectively.[68] As mentioned before, supersaturated artificial saliva used by that study could enhance remineralization in the presence of fluoride, which was not the case in the current study. In an *in-situ* study, NaF-rinse (500 ppm F, pH 4.5) provided 23-percent protection against erosion,[8] which may indicate that lower efficacy should be expected in clinically relevant conditions.

NaF/$SnCl_2$-rinse Effect on Enamel and Dentin

$SnCl_2$/NaF provided an 89-percent surface loss reduction in enamel. This observation indicates that the combination of tin and fluoride ions in the same rinse was significantly more effective in surface loss reduction than that provided by each ion alone. The effectiveness of stannous fluoride solution as an anti-erosive agent has been shown by different studies.[8,9,11,12,14,115] It has been suggested that the erosion-protective potential of tin-containing fluoride solutions in enamel is related to the incorporation of

tin in the surface layer of enamel, increasing its resistance against acids attacks.[14] In addition, a layer of precipitates containing Sn_2OHPO_4, $Sn_3F_3PO_4$, $Ca(SnF_3)_2$, and CaF_2 found to be formed on the enamel surface after application.[14,116] This layer acts as a protective barrier enhancing the acid resistance of enamel[14,116] and is considered to be more stable and resistant to acid attack than the one formed by fluoride alone (CaF_2).[9,12] From this comparison, the combination of both ions has a synergistic action on both enamel and dentin.

Sixty percent surface loss reduction was shown by a $SnCl_2$/NaF solution on dentin. A similar mechanism of action on enamel is assumed to be applicable for dentin as well. However, it can be speculated that the different protective effect of $SnCl_2$/NaF solution in enamel and dentin might be due to the lower content of minerals in dentin, as well as less prominent pellicle formation on dentin than enamel surface.

Dentin vs. Enamel

The comparison of enamel and dentin surface loss showed that when they were treated with NaF-rinse or DIW, no significant differences could be found. This observation can be explained taking two factors into account, the organic matrix of dentin, and also the strength of the acid used to simulate erosion. The organic matrix can act as a physical barrier preventing not only the further diffusion of acid into sound dentin, but also the tooth mineral release from the lesion to the surrounding environment. The matrix also has been shown to provide buffer effect against acids.[119] In this study, the organic matrix was not removed, which may be the reason why similar surface loss was observed for the NaF and DIW groups. Keeping the organic matrix has been described as

less representative of clinical situations, where continuous removal and degradation of the matrix occurs once in contact with the oral environment. The organic matrix of dentin can be removed either chemically using enzymes (collagenase),[68] or mechanically by brushing.[50] However, this degradation has not been fully investigated and understood clinically and is a point of disagreement among researchers, which is why we opted for not modeling this phenomenon in the present study.

Some of our previous unpublished work using the same cycling model with a less aggressive acid (0.3-percent citric acid, pH 3.8) showed an overall higher relative surface loss for dentin, whereas when a more aggressive acid (1.0-percent citric acid, pH 2.3) was used, an overall higher relative surface loss was observed for enamel. This observation is in agreement with previous studies[8,68] and highlights the importance of the acids and the dentin organic matrix on the comparison of results between enamel and dentin.[119]

Role of Acquired Dental Pellicle (ADP)

Only inferences can be made on the possible role of ADP in the results of this study, as groups with no ADP were not included in the study. Difficulties establishing a protocol for saliva dialysis did not allow us to obtain clarified human saliva without the presence of the proteins (to eliminate the presence of ADP), while keeping the same mineral content (for similar remineralization potential). Therefore, it can be only speculated in Phase I that the better erosive prevention observed with $SnCl_2$ and $SnCl_2$/NaF on enamel than on dentin may be associated to the stannous ion's ability to interact with the pellicle. An *in-vitro* study suggested that the stannous ion has cross-linking action on pellicle proteins that enhance its protective effect. This finding also

supports data from the present study.[30] To further explore these findings, protein analyses of ADP treated with test solutions were done in Phase II of this study.

PHASE II

Methods, Pellicle Analysis and Model Justification

Different approaches have been used to acquire the data important to study and to understand proteomics, including gel electrophoresis and chromatography techniques.[123] In the past, 2D-PAGE (two-dimensional gel electrophoresis) and/or 2DIGE (two-dimensional differential gel electrophoresis) were the most common techniques for quantitative proteomic analyses.[123] Nevertheless, the limited visualization of proteins in the gel, low sensitivity, and reproducibility decreased the accuracy of these methods.[128] To overcome these limitations, the use of liquid chromatography accompanied with mass spectrometry has been proposed.[123] The label-free LC-ESL MS/MS methodology has been used as described by Siqueira et al. 2012 to explore the relative quantitation of *in-vitro*-acquired dental pellicle (ADP) formed on HA surfaces treated with fluoride - containing solutions.[123] This approach seems particularly appropriate for the exploratory analysis of pellicle protein profiles between experimental and control groups, as it identifies common proteins among groups comparing their relative abundance.[123] Proteins exclusively present in each group were also identified and described.[123] Therefore, the influence of treatments on the pellicle protein composition can be done at common and exclusive protein levels.

In the present study, a total of 72 common proteins were identified, which is higher than what was found in a previously related study.[123] Therefore, the difference in

testing conditions between the two studies has to be considered. In the previous study, HA was pretreated with high-concentration fluoride solutions (4,950 ppm, 9,040 ppm, 22,600 ppm F) and incubated in clarified human saliva for 2 h before protein analyses. All procedures were done at 37 C°[123]. In Phase II of the current study, enamel slabs were used as substrate and were incubated in clarified human saliva for 24 h before being submitted to three cycles of 2 min treatment-2 h saliva incubation at room temperature. It has been shown that fluoride application considerably reduced proteins adsorption on enamel surface, and with higher fluoride concentrations, lower proteins adsorption has been observed.[123,141] In addition to the difference in fluoride concentration, the maturation stage of the pellicle contributes to the amount and the nature of proteins adsorbed on enamel.[148] For example, due to the competition with other proteins, such as acidic proline rich proteins, mucins participate slightly in pellicle formation in the beginning.[148] However, the involvement of mucins increases with time,[148] which was the case in our study, in which 24-h saliva incubation was done before treatment. To enable observation of the best rinse effect and protein expression on the analyses performed, the laboratorial procedures of Phase I and Phase II had to be done differently. This should be kept in mind when relating the results of Phase II to those from Phase I. Our main goal from the cycling procedure of Phase II was to create greater amounts of pellicle treated with tested solutions to be able to detect as large a protein number as possible.

Rinse Effect on Pellicle-Protein Composition

The proposed null hypothesis was rejected because there were significant differences among the protein profiles of pellicles treated with the different rinses in this

study. Focusing on the proteins found in all groups, it was possible to identify some previously reported to be associated with enamel demineralization and remineralization. In general, $SnCl_2$ /NaF-treated group showed more protein abundance than NaF- and $SnCl_2$-treated groups when compared with the control. This observation suggests that the combination of tin and fluoride ions may enhance protein retention and enhance the protective effect of ADP against dental erosion. Salivary mucins including MUC5B (MG1), MUC7 (MG2), MUC4, MUC 16, MUC19, and MUC5AC were identified as common proteins between all groups. Most of them (MUC5AC, MUC4, MUC19, MUC7, MUC5B) showed significantly higher abundance in $SnCl_2$/NaF-treated group than other test groups compared with the control. Generally, salivary mucins are considered to be the major constituents of ADP.[136] Further, mucins, particularly MUC5B and MUC7, are characterized with heterotypic complexing property, which gives them the ability to interact with other salivary molecules to provide multiple biological protective functions.[137] Their interactions with other proteins also concentrate proteins (as phosphoproteins) on the surface of the teeth enhancing the preventive effect of ADP against erosion and even abrasion.[43,137] Moreover, the large mucin MUC5B was suggested to be an important component of the salivary pellicle that protects the oral surfaces against sodium dodecyl sulfate (SDS), which is a typical surfactant present in oral care products.[138] In the $SnCl_2$-treated group, there was no statistical difference in the abundance of all mucins in comparison with the control, while in the NaF-treated group, only MUC7 showed greater abundance than the control group.

Histatin 1 and histatin 3 were identified in all groups. Pellicle profile for $SnCl_2$/NaF-treated group showed significantly higher abundance of both histatins (1 and

3), while other test groups showed higher abundance of histatin 3 but not histatin 1. Regarding differences between histatin1 and histatin 3, the former contains 1 mole of phosphate/mole of protein; therefore, it is considered to be phosphoprotein, while the latter lacks phosphate.[133] Although both types have been shown effective in antifungal and demineralization prevention, it is likely that because histatin 1 is a phosphoprotein, its main function may be that of a precursor of the ADP and inhibitor of hydroxylapatite crystal growth, while the less phosphorylated histatin 3 may act mainly as an antifungal and an antimicrobial agent.[46,134] An *in-vitro* study found that histatin 1 enhanced significantly the overall rate and the extent of remineralization for enamel with pre-formed artificial subsurface lesions, and that its effect was greater than that of statherin.[135] This finding supports the superior anti-erosive effect of stannous fluoride solutions in Phase I of this study.

$SnCl_2$ /NaF-treated group showed a higher abundance of three types of proline rich proteins (PRPs) that have been detected in all groups including acidic proline rich phosphoproteins 1 and 2, small proline rich protein, and basic proline rich protein. PRPs are from the major salivary proteins that form the pellicle because of its high affinity to HA.[139,141] The acidic proline rich proteins strongly bind to the calcium from HA maintaining the concentration of ionic calcium in saliva,[139] which may suggest its role in the anti-erosive effect of ADP. They also can inhibit precipitation of minerals on teeth surfaces.[139] The basic proline rich protein is a non-phosphorylated protein with a significant amount of proline. However, the function of basic proline rich protein is not well established in the oral cavity.[123,140] In the NaF-treated group, acidic PRP was significantly higher than the control. The $SnCl_2$-treated group showed lower, but not

significantly lower abundance than the control. Interestingly, $SnCl_2$/NaF and NaF-treated groups showed similar values of acidic PRP abundance, which may indicate a synergistic effect of F with this particular protein, except in higher concentration (22,600 ppm F) where acidic PRP was absent.[123] This possible interaction deserves further investigation.

Cystatin C, cystatin S, cystatin SN, cystatin SA, cystatin B, cystatin D and cystatin A were detected as common proteins for all groups. All detected cystatins, except cystatin B, showed a higher abundance in the $SnCl_2$ /NaF-treated group, whereas only cystatin D was higher in the $SnCl_2$-treated group compared with control. In comparison to statherin, the affinity of cystatin to hydroxyapatite surfaces has been considered lower; however, their influence on the growth kinetics of hydroxyapatite has been shown to be greater.[143] Therefore, they are considered to be from the major components of the ADP.[141] Comparing the function of salivary cystatins, SA and SN are involved in the prevention of periodontal tissue destruction by acting as protease inhibitors.[142,144] While cystatin S had no protease inhibitor effect, it binds more to calcium than SA or SN, which suggests its main role to be in maintaining the mineral balance of the tooth.[142]

Statherin was significantly less abundant in the $SnCl_2$- and NaF-treated groups, while a combination of both showed no significantly different abundance compared with the control. Regarding its interaction with fluoride, this observation is consistent with a previous study in which statherin decreased as fluoride concentration increased.[124] Statherin has a high affinity to hydroxyapatite and binds to enamel surface by both electrostatic and hydrogen bonding interactions through its acidic N-terminal that contains two phosphoserines.[124,125] It acts as an efficient inhibitor of mineral precipitation

on teeth surfaces and crystal growth inhibitor.[125] The strong binding of statherin peptides to HA may also contribute to the mechanism by which statherin inhibits enamel demineralization.[129,130] Moreover, statherin has been shown to be a potential preventive/therapeutic agent in the treatment of enamel erosion and dental caries.[130]

Protein S100-A8 and 9 showed higher abundance in $SnCl_2$/NaF-treated group over control. Those proteins have the ability to bind to calcium and zinc; therefore, it has been suggested that they may bind to the CaF_2 precipitates on the tooth surface and act as a reservoir for calcium.[124,145] Also, other proteins such as amylase, carbonic anhydrase 6, and albumin were higher in $SnCl_2$/NaF-treated groups than the control, and those proteins were found to be components of *in-vivo* formed pellicle.[21]

Overall, the $SnCl_2$/NaF-treated group showed higher protein abundance than other test groups in comparison with the control. This observation may suggest the combination of tin and fluoride enhances retention of pellicle proteins and possible interaction between those proteins, which may contribute with the superior anti-erosive effect of $SnCl_2$/NaF-treated group over other tested groups observed in Phase I. Interestingly, the $SnCl_2$-treated group showed overall lower values of protein abundance, which may indicate that the stannous ion may decrease the affinity of proteins to hydroxyapatite. Data from this study suggest that the mechanisms of action of tin and fluoride as anti-erosive agents may depend not only on formation of precipitates on the tooth surface, but also on the interactions between pellicle and tin and fluoride on the tooth surface. These results suggest that further investigation on these interactions are warranted, aiming to develop more effective preventive and therapeutic measures against dental erosion.

SUMMARY AND CONCLUSIONS

The objectives of this study were to compare the anti-erosive properties of fluoride-containing and stannous solutions on enamel and dentin, and to characterize the protein profile of the acquired dental pellicle (ADP) treated with the test solutions. The experiments were conducted on two phases. In Phase I; the anti-erosive effect of $SnCl_2$/NaF, $SnCl_2$, NaF solutions were compared with the DIW (control) using profilometry. While in Phase II the protein profiles of ADP treated with the test solutions were investigated using liquid chromatography electrospray ionization tandem mass spectrometry (LCESI-MS/MS).

Within the limitations of this study, the following conclusions can be drawn:

1. $SnCl_2$/NaF combination had the best anti-erosive effect among the tested solutions.
2. $SnCl_2$ had significantly superior preventive effect over NaF on enamel, but not on dentin.
3. Tested solutions significantly changed the protein profile of ADP.
4. $SnCl_2$/NaF had a significantly greater abundance of proteins compared with the control (DIW).

Based on the results of the present study, it can be concluded that the combination of fluoride and tin significantly prevents surface loss due to erosive acids and modifies acquired pellicle protein composition.

Clinical significance: Based on the results of this study, $SnCl_2$ and NaF solutions provide significant protection against dental erosion, which is modulated by their interaction with the proteins of the acquired dental pellicle.

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ABSTRACT

INTERACTION BETWEEN TIN/FLUORIDE-CONTAINING SOLUTIONS AND ARTIFICIALLY CREATED DENTAL PELLICLES ON EROSION PREVENTION *IN VITRO*

by

Amnah Abdullah Algarni

Indiana University School of Dentistry
Indianapolis, Indiana

BACKGROUND: Fluoride and stannous ions have been reported to be relevant for dental erosion prevention. However, their interaction with the acquired dental pellicle (ADP), a clinically relevant erosion protective factor, is not well known and needs to be investigated. OBJECTIVES: To investigate the anti-erosive properties of fluoride-containing solutions and stannous solutions on enamel and dentin surfaces with a previously formed ADP. To characterize the protein profile of the ADP treated with the test solutions. METHODS: Phase I tested four solutions: $SnCl_2$/NaF, NaF, $SnCl_2$ and deionized water (DIW) (as negative control). Forty bovine enamel and dentin specimens

($4\times4\times2$ mm^3) were prepared and randomly distributed into 4 groups (n = 10). The specimens were incubated in clarified human saliva (CHS) for 24 h for pellicle formation and then they were subjected to a cycling procedure that included a 5-min erosive challenge (0.3-percent citric acid, pH 2.6); a 2-min treatment with the solution (between 1st, 3rd and 6th cycles); a 2-h immersion in CHS, and overnight immersion in CHS. Cycles were repeated 6x/day for 5 days. The outcome measure was surface loss (SL) using profilometry. Phase II: Thirty-two (32) bovine enamel specimens ($8\times8\times2$ mm^3) (n = 8) were similarly prepared and incubated in saliva for 24 h and then treated with the solutions for 2 min followed by CHS immersion for 2 h. This cycle was repeated 3x for one day. The pellicles formed and treated with the test rinse solutions were collected, digested, and analyzed for specific protein content using liquid chromatography electrospray ionization tandem mass spectrometry (LCESI-MS/MS). RESULTS: Phase I: for enamel, $SnCl_2$/NaF, $SnCl_2$, NaF solutions provided 89 percent, 67 percent, and 42 percent SL reduction respectively compared with the control, while in dentin they provided 60 percent, 23 percent, and 36 percent, respectively, all significant at $p < 0.05$. Phase II: Seventy-two (72) common proteins were identified in all groups, 30 exclusive to DIW, 20 to $SnCl_2$/NaF, 19 to NaF, and 13 to $SnCl_2$. $SnCl_2$/NaF increased the abundance of pellicle proteins than each one alone. CONCLUSION: $SnCl_2$/NaF showed the best anti-erosive effect on both enamel and dentin. The findings suggest that the composition of acquired pellicle changes with different solutions, which may be related to their anti-erosive effect.

CURRICULUM VITAE

Amnah Abdullah A. Algarni

September 1983	Born in Jeddah, Saudi Arabia
June 2001	High School Diploma 34th Secondary School. Jeddah, KSA
July 2001 to July 2007	Bachelor of Dental Medicine & Surgery (BDS) Faculty of Dentistry, King AbdulAziz University (KAU) Jeddah, KSA
July 2008 to present	Demonstrator (Teaching Assistant) Operative Dentistry Division, Department of Restorative Dentistry, College of Dentistry, Taibah University Al Madinah Al Monawara, Saudi Arabia.
August 2010 to July 2013	Operative/Preventive Dentistry Graduate Program, Indiana University School of Dentistry, Indianapolis, Indiana.
August 2013	Accepted by doctoral program in Dental Science Indiana University School of Dentistry Indianapolis, IN

Professional Organizations

The Academy of Operative Dentistry
Saudi Dental Society
American Dental Education Association (ADEA)