FIRST-HAND NICOTINE/TOBACCO EXPOSURE ON *STREPTOCOCCUS MUTANS* BIOFILM AND EFFECTS OF SODIUM CHLORIDE, POTASSIUM CHLORIDE AND POTASSIUM IODIDE SALTS

by

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DEDICATION

I would like to thank and show gratitude to Allah for his many blessings, graces, and virtues. You gave me the strength and the patience to continue moving throughout this difficult life; you facilitated all the tough times that faced me, and you always have been my guide. Thank you so much. And I also ask you to bless me and have mercy on me in both lives, and to grant my parents, my family, friends, beloved ones, and me a place in paradise.

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INTRODUCTION

Dental caries is one of the most prevalent chronic oral diseases. Sixty percent to 90 percent of school children and nearly 100 percent of adults around the world have caries.[1] In the USA in 2007, around 91 percent of individuals older than age 20 had some level of caries experience, and 27 percent had untreated carious lesions.[2] About 38 percent of children aged two through eight years in the US had some caries experience, and 14 percent were recognized with untreated dental caries.[3] In addition, Dye et al. stated that one in five children aged five to 11 years and one in seven adolescents aged 11 to 19 years have at least one untreated dental caries lesion.[4] Dental caries can be defined as a multifactorial disease resulting from an ecological shift of the microbial species inside the mouth, leading to mineral loss from the tooth structure.[5] The concept behind dental caries has been expanded to involve many aspects such as the caustic bacteria, nutrition, host and other factors related to economy and education.[6-9] In addition, the concept has been explained as a dynamic process of demineralization and remineralization cycles that affects the quantity of minerals inside the tooth structure. If demineralization dominates, loss of minerals from the enamel may occur and teeth may become cavitated. But if remineralization dominates, the lesion could be arrested and teeth could gain the minerals back.[10,11] This concept leads many dental practitioners to understand the difference between cavitated and non-cavitated lesions as non-cavitated lesions could be remineralized without any need for aggressive intervention, i.e. tooth drilling and filling.[12] For a long time, scoring non-cavitated lesions was eliminated from caries indexes because these lesions are difficult to diagnose.[13] However, this concept

was refuted by many studies stating that dental practitioners can be trained to recognize such lesions.[14, 15] As a result, non-cavitated lesions have been added in many caries detection systems. The fact that these lesions could be reversed before cavitation raises many questions regarding how the enamel could be remineralized. So, dental practitioners should be able to recognize non-cavitated lesions as these lesions could be remineralized without surgical intervention.[16]

Remineralization Strategies

Many strategies have been developed to arrest or reverse the process of demineralization. Fluoride is considered the most widely used anti-caries agent as fluoride dentifrices have contributed to decreasing the amount of caries incidence in many countries.[17,18] In 2003 Marinho et al. conducted a systematic review of 70 clinical trials that compared fluoride dentifrice with a placebo. It was found that fluoride is capable of decreasing the DMFS score by 24 percent among permanent teeth.[19] Moreover, fluoride mouthwash has a significant effect in reducing caries incidence.[20, 21] In addition, many studies discussed the ability of casein phosphopeptide (CPP) amorphous calcium-phosphate (ACP) complexes to inhibit demineralization and increase the remineralization process. These compounds increase the amount of calcium and phosphate in the dental plaque. So, they restore the same minerals that were lost during the demineralization process.[22-25] Recently, silver diamine fluoride (SDF) provided fascinating results regarding its ability to arrest both enamel and dentin caries.[22] In 2002 Chu et al. conducted a clinical trial study among 376 school children using SDF. It was found that annual application of SDF is more effective than 5.0-percent sodium fluoride varnish, and it was found that SDF is effective no matter if the caries was removed or

not.[27]

Another aim to decrease the incidence of caries has been focused on reducing the load of *Streptococcus mutans* and dental plaque. Many agents have been used for these purposes such as chlorhexidine, essential oils, triclosan, cetylpyridinium chloride, sanquinarin, sodium dodecyl sulphate, tin, zinc, and copper ions. With the exception of chlorhexidine, the effectiveness of these agents is still questionable.[26] Many salts such as sodium chloride (NaCl), potassium chloride (KCl) and potassium iodide (KI) are available in everyday life and are easy to obtain. The antimicrobial activities of these salts have been demonstrated in the literature. It was found that the salt tolerance test, that contains TSB and 6.5-percent NaCl, inhibits the growth of *Streptococcus pyogenes* and *Streptococcus bovis*.[28] Also, the combination of NaCl and KCl demonstrated some kind of inhibition against many bacterial species including *Staphylococcus aureus*.[29] KI has been used to treat many fungal and respiratory tract infections.[30] Moreover, some studies investigated the effect of these salts on *S. mutans*, but no study has investigated the effect of these salts specifically on *S. mutans* biofilm formation or nicotine-induced *S. mutans* biofilm formation.

Objectives/Specific Aim

Hypothesis

The presence of a specific concentration of NaCl, KCl and KI inhibit first hand nicotine-induced *S. mutans* biofilm formation (nicotine; 0-32 mg/ml).

The null hypothesis is that the presence of a specific concentration of NaCl, KCl and KI salts increase or have no effect on *S. mutans* biofilm formation with first hand nicotine exposure.

REVIEW OF LITERATURE

Three main pathological factors contribute to dental caries: fermentable carbohydrate, presence of cariogenic bacteria and salivary dysfunction.[33]

FERMENTABLE CARBOHYDRATE

Sugar has been recognized for a long time as one of the most consumed dietary compounds in humanity. Most evidence shows that there is a significant relationship between sugar and dental caries, and dietary habits could affect the progression of such lesions. In a study that was conducted in Australia, named the Hopewood House study, children in this house were fed food with no sugar contents. This study conducted between 1947-1952 found that those children had less caries experience in comparison to children in other schools.[34] In the Vipeholm report, scientists conducted an experiment in mentally challenged subjects between 1945 and 1952. They investigated the effect of adding sugar to the subjects' food, the frequency of sugar intake and sugar consistency. They found that adding sugar resulted in little increase of caries experience, but consuming sugar as a snack significantly increased the cariogenicity of sugar.[35] The risk of sugar intake frequency was stated also in other studies.[36, 37] In 1976, Scheinin et al. investigated the cariogenicity of three sweeteners: sucrose, fructose and xylitol. They found that sucrose was associated with the highest caries rate. On the other hand, fructose and xylitol exhibited 32 percent and 85 percent lower caries rates than sucrose, respectively.[38] This study and other studies [39, 40] showed that sucrose is the most cariogenic sugar. It was found that in order for dental caries to occur, sugar has to be available to be utilized by the cariogenic bacteria. Increasing the feeding time or intake

frequency of carbohydrates increases the acidity of the oral cavity with the carbohydrates incorporated in the biofilm leading to acid production and a significant demineralization process.[36, 41] Even though the risk of carbohydrates has decreased more than the pre-fluoride era, people should still be aware of the importance of dietary content in relation to dental caries.[42] Furthermore, The United States Department of Agriculture's Food Guide Pyramid indicates that foods should have little sugar content.[43]

CARIOGENIC BACTERIA

Many researchers have reported that dental caries is mainly associated with *S. mutans* and the *Lactobacillus* species. It was found that the number of *S. mutans* is 70 times higher in caries-infected subjects when compared to caries-free subjects.[44] There are two important features for the cariogenic bacteria: the ability to produce acid in order to dissolve the enamel (acidogenic) and the ability to survive in a high acidity environment (aciduric). Over time, this acid production may cause demineralized non-cavitated enamel to become cavitated enamel.[10] The cariogenicity of these bacteria arises from the ability to form a biofilm on the hard surfaces and utilize carbohydrates to produce lactic acid, which can initiate the carious process.[45, 46] Through the salivary pellicle that coats the enamel, *S. mutans* can attach to the tooth structure by two main mechanisms: sucrose-dependent and sucrose-independent attachment.[47, 48] In the sucrose-dependent mechanism, *S. mutans* produces glucosyltransferase to form glucans from sucrose. The glucans facilitate bacterial attachment to the tooth structure and to each other.[47, 49] In addition, glucan-binding proteins are involved in this process as it is produced by *S. mutans* to facilitate the binding between bacteria and glucans.[50] On the other hand, the sucrose-independent mechanism is a unique interaction between saliva

agglutinins and I/II antigen that is found on the cell wall of *S. mutans*.[51] The interaction between *S. mutans* and salivary proteins and agglutinins, leading to bacterial attachment to the tooth structure, has been confirmed in many studies.[52, 53] It can be seen clearly that the presence of carbohydrates is essential to start the sucrose-dependence mechanism, and sucrose has been identified as the most cariogenic carbohydrate.[54] Using the two previously explained mechanisms, biofilm formation begins either as an interaction between microorganisms and salivary components or between many species such as *Actinomyces*, *Streptococcus*, *Lactobacillus* and *Candida* species.[55]

SALIVARY DYSFUNCTION

Saliva plays an important role in the remineralization process. According to Humphrey and Williamson, the main five functions of saliva are: a) lubrication and protection; b) the capability of buffering environmental acidity; c) protect tooth structure integrity; d) antimicrobial action; and e) facilitate mastication and digestion.[56] Mucins are considered one of the main lubricating agents in the oral cavity; they help with eating, talking and swallowing. Also, mucins limit the colonization of fungal and bacterial species. Salivary glands produce two types of mucins, high molecular weight, highly glycosylated mucin (MG1) and a lower molecular weight, single-glycosylated peptide chain mucin (MG2). MG1 tightly adheres to the tooth structure and forms dental pellicle, while MG2 could be removed easily from the enamel and enhance the clearance of the bacteria. Benign commensal oral flora can attach to a heterotypic complex that is produced by MG1 and other salivary proteins (such as amylase, proline-rich proteins, statherin, and histatins). It was found that MG1 is produced more in caries-infected individuals, while MG2 is produced more in caries-free individuals. Dental pellicle and

attached oral flora act as a barrier against acid attack and limit mineral loss from enamel.[57, 58] Saliva has many components such as bicarbonate, phosphate and urea that are able to buffer and neutralize acids. It was found that after 5 minutes of eating, the oral pH drops to under six. Saliva needs 15 minutes or longer after food intake to restore the normal pH of the oral cavity.[59] Saliva protects the tooth structure by inhibiting demineralization through the salivary buffering effect. In addition, saliva acts as scaffold that regulates calcium and phosphate. Many salivary proteins incorporate calcium and phosphate to the tooth structure to increase tooth maturation and limit mineral loss from the tooth structure.[60, 61] In addition, saliva has many antimicrobial agents that interfere with the cariogenicity of bacteria such as IgA, lactoferrin and lysozyme. IgA interferes with bacterial attachment and binds to bacterial antigens. Lactoferrin has the capability to bind ferric iron, a main nutrient for cariogenic bacteria. So, it limits nutrient availability to these microorganisms. Also, lactoferrin is considered a sensitive material for *S. mutans*. Lysozyme has a destructive effect on the bacterial cell limiting bacterial growth in the host tissues.[60, 62-64] In people with salivary gland impairment, buffering capacity decreases and the risk of acidity and demineralization increases.[65, 66] So, in patients with a high caries risk, monitoring salivary flow is very important. Dental practitioners may consider increasing the salivary flow either by using some medications or hydration tools.

THE RISK OF SMOKING

In 2012, the World Health Organization reported that about 23 percent of people older than fifteen in the world are currently smokers. Also, in 2015, the World Health Organization stated that tobacco kills around six million people every year; six hundred thousand of these deaths are due to second hand smoke.[67, 68] In 2012, approximately 42.1

million adults in the United States were smokers.[69] Smoking or tobacco use is considered one of the biggest health problems in the world. Many studies that were based on daily use of tobacco stated that smoking can cause serious health problems.[70] Smoking contributes to cardiovascular diseases. And it has been proven that light smoking had the same risk as intermittent smoking. It was found that heart disease from light smoking, which is about four to seven cig/day, is about 70 percent of heart disease found in heavy smokers (>23 cig/day).[71, 72] Light smoking is three times higher in causing ischemic heart disease and twice as high in causing myocardial infarction than non-smokers.[71, 73] In addition, light smoking can cause aortic aneurysm three times higher than non-smokers.[74] Generally speaking, over a long-term period, 11% of light smokers were found to have cardiac problems when compared to 3.7 percent for non-smokers.[75] Also, it is known that heavy smoking is associated with higher percentages of cardiac problems. In addition, it was found that heavy smokers, both men and women, were 23 and 13 times, respectively, greater in developing lung cancer than non-smokers. Because smoking and lung cancer have a dose-response relationship, these numbers are lower in light smokers, 3-5 times higher than non-smokers.[70, 71, 74] Also, smoking was found to cause other types of cancer such as stomach, pancreas and esophagus cancers. In addition, other conditions are associated with smoking such as respiratory infections, reproductive problems, ectopic pregnancy and physical disability.[76] Smoking has been recognized as one of the risk factors of many oral health problems. Many studies show that first and secondhand smokers are at higher risk of edentulism than non-smokers.[77-79] In addition, a strong association is seen between smoking and oropharyngeal cancer. It was found that heavy tobacco users are 5-25 times higher risk for oropharyngeal cancer than non-tobacco users.

For those people who are heavy tobacco users and drink heavily, this rate rises to 35 times higher.[80,81] Moreover, smoking has been recognized as a risk factor for periodontal diseases, implant failure and peri-implantitis.[82,83] Many studies have investigated the relationship between smoking and dental caries, and it was found that smoking is correlated with high caries incidence.[84-86] Nicotine load is higher in the oral cavity of smokers,[87] and this concentration ranges from 0-2.27 mg/ml in saliva.[87-89] In 2012, Huang et al. conducted a study to investigate the effects of nicotine on seven *S. mutans* strains, UA159, UA130, 10449, A32-2, NG8, LM7, and OMZ175. They found that the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for nicotine was 16 and 32 mg/ml^{-1}, respectively. Also, they illustrated that the minimum biofilm inhibitory concentration (MBIC) was 16 mg/ml^{-1} for all strains except for strains 10449 which was 8 mg/ml^{-1}.[90] The same conclusion was reported by Li et al. who concluded that nicotine could also increase the growth of *S. mutans* over *Streptococcus sanguinis* in dual species experiments.[91] The same authors investigated the specific mechanism of nicotine that allows *S. mutans* biofilm to increase. They found that nicotine is able to increase the number of *S. mutans* and extracellular polysaccharide (EPS) synthesis, the main component of biofilm matrix. Also, they stated that nicotine increases GbpA expression up to 4.0 fold, Gtfs expression up to 2.2 fold, and no change in GbpB expression. While this study demonstrated that nicotine has nothing to do with lactate dehydrogenase (LDH) activity, it can be concluded that increasing the number of *S. mutans* cells leads to more LDH production.[92] A recent metabolomic study indicated that nicotine increases lactate concentration of *S. mutans* by two fold.[93] In addition, Tanaka et al. suggested that smoking during pregnancy or in the postnatal period could increase the

risk of caries in primary dentition.[94] For those people who cannot stop smoking or those who spend time with smokers, an important question should be answered regarding how the effects of nicotine on the growth of biofilm could be diminished.

How to Limit The Cariogenicity of Nicotine?

NaCl, in the form of saline, has been used in the medical field for many decades.[95] It is used as an intravenous infusion, in wound cleaning, and nasal irrigation procedures to treat bronchiolitis.[96, 97] Hypertonic saline, saline with a higher concentration of NaCl, is another form of saline and is used to manage intracranial pressure and traumatic brain injury.[98] NaCl is considered one of the antibacterial tools that can be used in dentistry, and its potency varies in different situations. Hirasawa and Takada found that adding NaCl to aztreonam and carumonam can inhibit the growth of *S. mutans*.[99] Another study found that the presence of NaCl can decrease glucosyltransferase activity of *S. mutans.*[100] In addition, it has been found that the combination of sodium salts with certain materials such as $NaHCO_3$ or human salivary lysosome causes *S. mutans* lysis.[101, 102] Mutanolysin is a bacteriolytic enzyme that was discovered in 1972 by Yokogawa and Yoshimura.[103] When this enzyme was applied to *S. mutans*, no significant reduction in optical density was noticed. But after the addition of 1 M of NaCl to mutanolysin, significant *S. mutans* lysis was observed.[104] The adaptive response is the ability of an organism to adapt to unusual conditions leading to resistance to possible damage. It was found that *S. mutans* is able to resist the acidity of the surrounding environment by a gene called ClpP, which encodes for serine protease.[105, 106] Deng et al. investigated the induction of ClpP after exposing *S. mutans* to many antimicrobial agents including NaCl. They found that 0.25 and 0.5 M of NaCl stimulated a significant induction of ClpP that means NaCl has some

antimicrobial effect on *S. mutans*.[107] The ability of NaCl and KCl to inhibit the demineralization process and acid production has been studied.[108, 109] In addition, it was found the *S. mutans* attachment to salivary agglutinin could be inhibited by using NaCl or KCl salts.[110] Both NaCl and KCl has been used in many studies to suspend growth of *S. mutans* [111-115] or as buffering agents.[115-118] However, some studies indicated that NaCl and KCl were not effective in reducing the cariogenicity or the growth of *S. mutans*.[119-121] KI has been used in different medical fields such as dermatology, treatment of hyperthyroidism and fungal infections.[30, 122, 123] In addition, it has been found that KI could reduce the growth of the *S. mutans*. In a study done by Hamama et al., it was found that when KI is combined with silver diamine fluoride (SDF), a synergistic effect could be developed to diminish *S. mutans* growth.[124] Also, Knight et al. demonstrated that the combination of KI and SDF could increase fluoride uptake of dentin.[125] In another study, the same authors found that this combination is able to inhibit caries progression *in vitro* and prevent biofilm formation.[126]

MATERIALS AND METHODS

PRELIMINARY EXPERIMENT

Before starting the main study, a preliminary experiment was done to investigate the most effective inhibitory concentration of sodium chloride (NaCl), potassium chloride (KCl) and potassium iodide (KI). According to many researchers, the most commonly used antimicrobial concentrations of NaCl, KCl and KI range from 0.075 to 0.35 M.[99, 101, 102, 108, 127, 128] This preliminary experiment was designed to investigate the MIC of 0.0035, 0.007, 0.014, 0.028, 0.056, 0.113, 0.225, 0.25, 0.3, 0.35, 0.4, 0.45, 0.72 and 0.9 M of NaCl, KCl and KI on the growth of *S. mutans*. Ten µl of an overnight culture of *S. mutans* [approximately 10^6 colony-forming units (CFU), determined by spiral plating] in Tryptic Soy Broth (TSB) was treated with 0.0035 M, 0.007 M, 0.014 M, 0.028 M, 0.056 M, 0.113 M, 0.225 M, 0.25 M, 0.3 M, 0.35 M, 0.4 M, 0.45 M, 0.72 M and 0.9 M of NaCl, KCl or KI, which were diluted in TSB supplemented with 1.0-percent sucrose (TSBS), for 24 h in sterile 96-well flat-bottom microtiter plates (Fisher Scientific, Newark, DE). Each salt was investigated separately in different 96-well flat-bottom microtiter plates. The optical density (OD) values of the bacterial cultures were measured at 595 nm in a spectrophotometer (SpectraMax 190; Molecular Devices, Sunnyvale, CA) The MIC was determined by the concentration where there was an obvious clear-cut decrease in the absorbance. After incubation, the unbound planktonic cells were gently aspirated and transferred to a new 96-well plate and the OD at 595 nm was determined in order to calculate the effect on planktonic cells. The remaining planktonic cells were discarded from the biofilm microtiter plate wells (leaving the attached biofilm cells), and

200 μl of 10-percent formaldehyde was added to each well for 30 min to fix the cells. After 30 min, the formaldehyde was removed and the biofilm cells were washed 3 times with deionized water. 200 μl of 0.5-percent crystal violet dye was added to each well and the cells stained for 30 min. The wells were rinsed 3 times and 200 μl of 2-isopropanol was placed into each well for 1 h to lyse the cells and extract the crystal violet. The plates were read in a spectrophotometer at 490 nm to measure biofilm formation.[129]

FIRST-HAND NICOTINE EXPOSURE

After determining the MIC for the three salts, a culture of *S. mutans* UA159 (American Type Culture Collection, Rockville, MD; ATCC 700610) was grown in TSB at 37°C in 5.0 percent CO_2 and stored with 10-percent glycerol at -80°C. Stock solutions of NaCl, KCl, and KI were dissolved in water, (4.2 g/40 ml; 1.8 M) for NaCl, (5.4 g/ 40 ml; 1.8 M) for KCl, and (11.95 g/40 ml; 1.8 M) for KI. All were stored in a -20°C freezer until used. Then, an overnight culture of *S. mutans* UA159 was grown in TSB. NaCl, KCl and KI salts were diluted in TSBS to obtain the MICs determined above, 0.45 M for NaCl, 0.225 M for KCl, and 0.113 M for KI, using the following measurements:

1) NaCl:

- 2.5 ml of 1.8 M of NaCl was mixed with 7.5 ml of TSBS ➔ 10 ml of 0.45 M NaCl/TSBS

2) KCl:

- 1.28 ml of 1.8 M of KCl was mixed with 8.72 ml of TSBS ➔ 10 ml of 0.225 M KCl/TSBS

3) KI:

- 0.63 ml of 1.8 M of KI was mixed with 9.37 ml of TSBS → 10 ml of 0.113 M KI/TSBS

The dilutions of each salt with TSBS were arranged to have 0, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0 mg/ml nicotine (Sigma-Aldrich Chemical Co., St. Louis, MO). Another set of TSBS dilutions was arranged to have 0 mg/dl, 0.25 mg/dl, 0.5 mg/dl, 1.0 mg/dl, 2.0 mg/dl, 4.0 mg/dl, 8.0 mg/dl, 16.0 mg/dl and 32.0 mg/ml nicotine (Sigma-Aldrich Chemical Co., St. Louis, MO) without the NaCl, KCl and KI salts. In addition, each salt was investigated alone. Each nicotine concentration was aliquoted by pipetting 190 μl of TSBS containing the nicotine/salt into wells of a sterile 96-well flat bottom microtiter plate. 10 μl of the fresh overnight TSB culture of *S. mutans* was added. The microtiter plates were kept in 5.0-percent CO_2 at 37^oC for 24 h. The following day, total absorbance (biofilm & planktonic growth) was measured in a spectrophotometer (SpectraMax 190; Molecular Devices Inc., Sunnyvale, CA) at 595 nm. Then, 120 μl from each well was moved to the corresponding well of a new microtiter plate. The absorbance of each well was measured at 595 nm to investigate planktonic growth. The remaining planktonic cells were discarded from the biofilm microtiter plate wells (leaving attached biofilm cells), and 200 μl of 10-percent formaldehyde was added to each well for 30 min to fix the cells. After 30 min, the formaldehyde was removed and the biofilm cells were washed 3 times with deionized water. 200 μl of 0.5-percent crystal violet dye was added to each well and the cells stained for 30 min. The wells were rinsed 3 times and 200 μl of 2-isopropanol was placed into each well for 1 h to lyse the cells and extract the crystal violet. The plates were read in a spectrophotometer at 490 nm to measure biofilm formation.

CONTROLS

Controls included biofilms of *S. mutans* without nicotine and with or without NaCl, KCl and KI salts.

STATISTICAL ANALYSES

Each experiment was repeated three times and each group consisted of 4 wells. Two-way ANOVA was utilized to compare the effects of NaCl, KCl and KI salt exposure (individually or all three combined) and nicotine and their interaction on planktonic, biofilm, and total growth. Pair-wise comparisons were achieved using Fisher's Protected Least Significant Differences method to control the overall significance level at 5 percent. The primary focus of the study hypotheses are the comparisons involving NaCl, KCl and KI salt exposure. The distribution of the measurements was investigated and found to be non-normal, therefore a rank transformation was used to satisfy the ANOVA assumptions prior to analysis.

SAMPLE SIZE CALCULATIONS

Based on prior studies, the within-group standard deviation of the absorbance measurements for biofilm formation is estimated to be 0.15. With 4 samples in each of 3 trials of the study, the study will have 80-percent power to detect a difference of 0.2 between salts for each nicotine concentration, assuming two-sided tests conducted at an overall 5-percent significance level.

RESULTS

RESULTS OF THE PRELIMINARY EXPERIMENT

From the results of the preliminary experiment, the MIC of these salts was determined as follows: 0.45 M for NaCl, 0.23 M for KCl, and 0.113 M for KI. Results indicated NaCl, KCl, and KI were able to inhibit biofilm formation significantly ($p<0.05$). Starting with NaCl, it was found that most of the concentrations of NaCl tested were capable of inhibiting biofilm formation. There was significant reduction ($p<0.05$) in biofilm formation between 0.45 M and 0.72 M. It was seen clearly that 0.72 M of NaCl is bactericidal, while 0.45 M NaCl exhibited minimum biofilm formation inhibition. For this reason, 0.45 M of NaCl was recognized as the MIC for *S. mutans* biofilm formation. Regarding KCl, most of the concentrations assayed were capable of inhibiting biofilm formation. However, there was significant biofilm inhibition between two consecutive concentrations, 0.225 and 0.3 M, indicating 0.225 M could be recognized as the MIC while 0.3 M could be recognized as the MBC. Therefore, 0.225 M of KCl was determined as the MIC. Finally, KI results demonstrated the same results regarding biofilm formation. 0.113 M was determined as the MIC as any higher concentration than that was bactericidal.

RESULTS OF THE MAIN EXPERIMENT

Overall, there was a significant effect for salt presence, nicotine concentration, and their interaction for all salts (NaCl, KCl, and KI) and measures (biofilm, planktonic, total absorbance), with the exception of KCl and planktonic growth. Regarding total

absorbance, there was a significant effect for NaCl presence, nicotine and their interaction to reduce *S. mutans* total absorbance when the nicotine concentration was 0, 4 or 8 mg/ml. Also, the NaCl/nicotine interaction demonstrated a significant effect inhibiting *S. mutans* biofilm formation with all concentrations in comparison to the no NaCl group except when the nicotine concentration was 32 mg/ml. On the other hand, NaCl presence increased *S. mutans* planktonic growth significantly except when the nicotine concentration was 4, 8 or 32 mg/ml.

KCl exhibited significant interaction with nicotine as *S. mutans* total absorbance was reduced when the nicotine concentration was 4 or 8 mg/ml. With the exception of the 32 mg/ml nicotine concentration, KCl/nicotine interaction significantly decreased *S. mutans* biofilm formation when it was compared to the no KCl group. Regarding planktonic growth, no significant effect was found between the KCl/nicotine and nicotine groups.

Also, the KI/nicotine interaction significantly reduced *S. mutans* total absorbance except when the nicotine concentration was 0, 0.25, 0.5 and 32 mg/ml. The KI/nicotine interactions significantly diminished *S. mutans* biofilm formation with all nicotine concentrations except the 32 mg/ml concentration. Finally, the KI/nicotine interaction significantly reduced *S. mutans* planktonic growth when the nicotine concentration was 0.25, 0.5, 2 and 16 mg/ml.

FIGURES AND TABLES

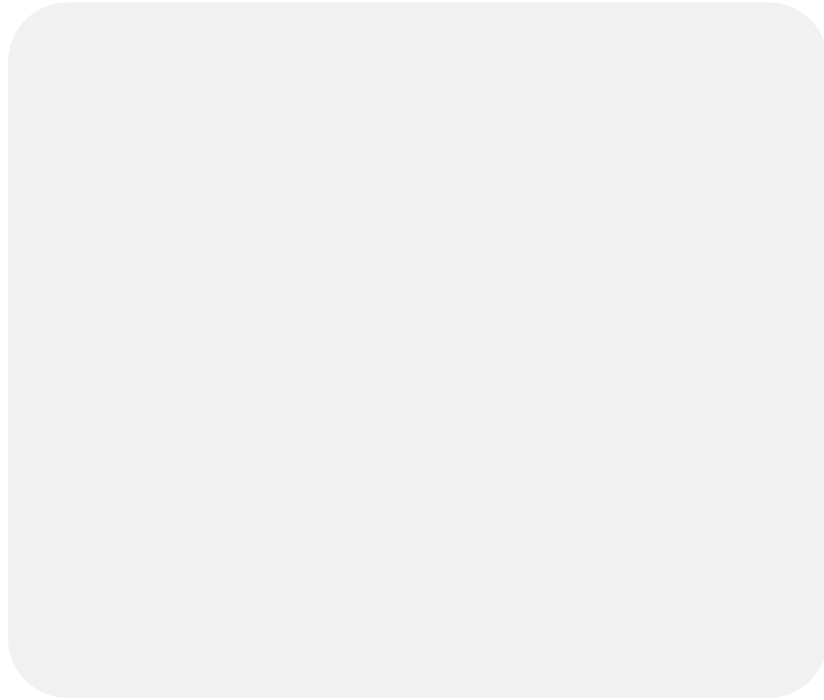

FIGURE 1. *S. mutans* UA159 was grown in TSB at 37°C in 5% CO_2 for 24 hours and stored with 10% glycerol at -80°C.

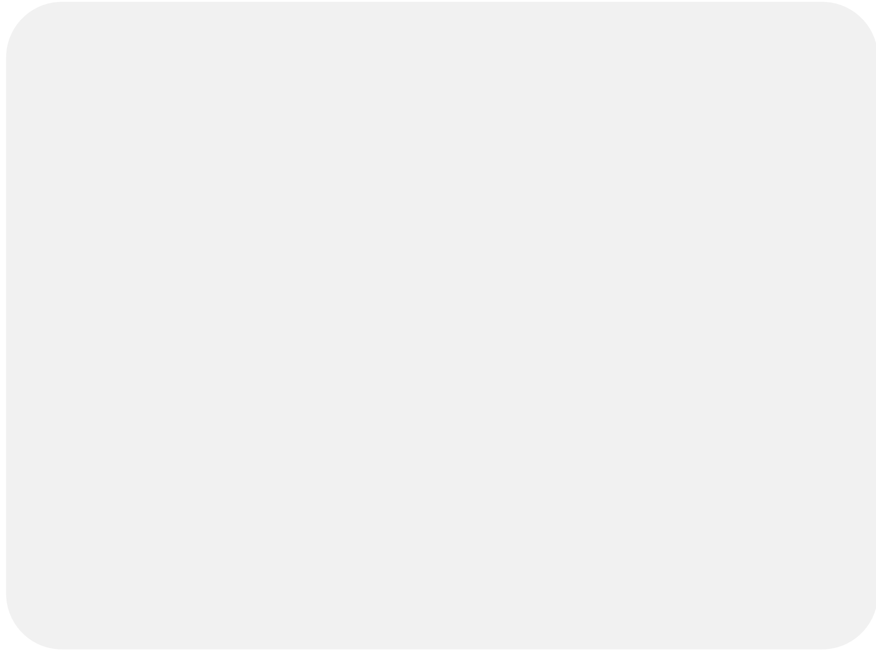

FIGURE 2. Each salt (NaCl, KCl and KI) was dissolved in TSBS.

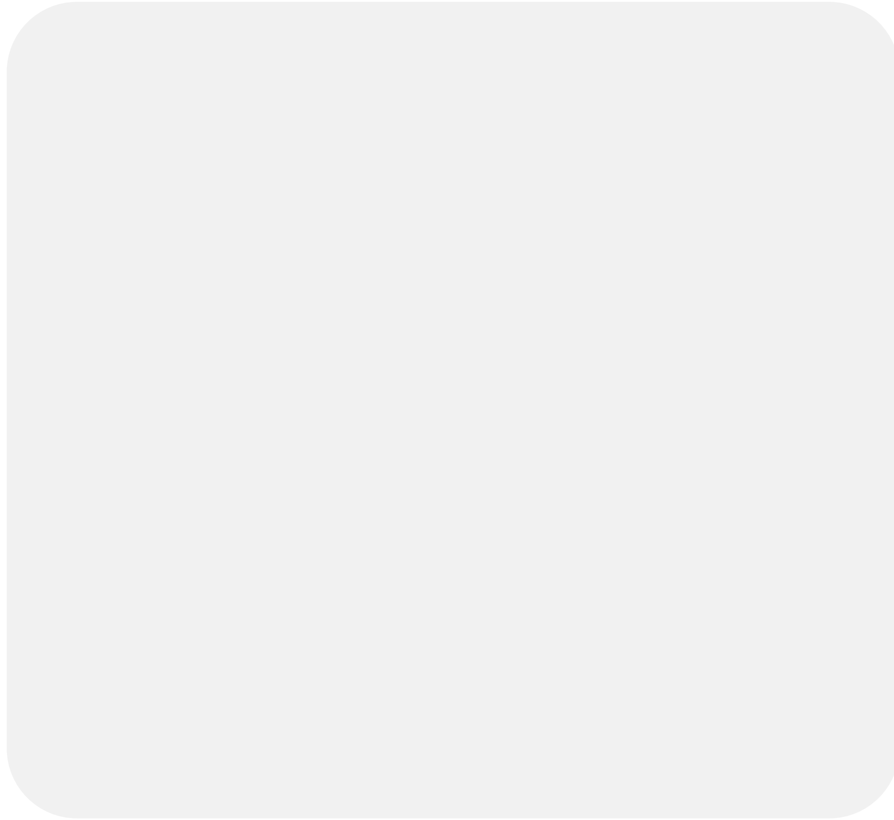

FIGURE 3. Dilutions of 0 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 2.0 mg/ml, 4.0 mg/ml, 8.0 mg/ml, 16.0 mg/ml and 32.0 mg/ml nicotine in TSBS with and without the salts.

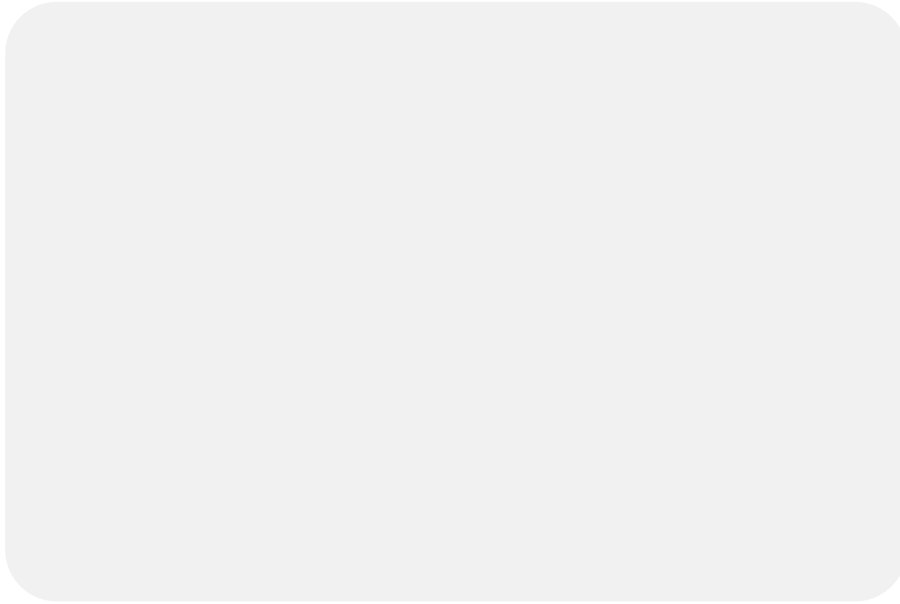

FIGURE 4. Each nicotine/CSC concentration was aliquoted by pipetting 190 µl of TSBS containing the nicotine/CSC/salt into wells of a sterile 96-well flat bottom microtiter plate. Then, 10 µl of the fresh overnight TSB culture of *S. mutans* was added.

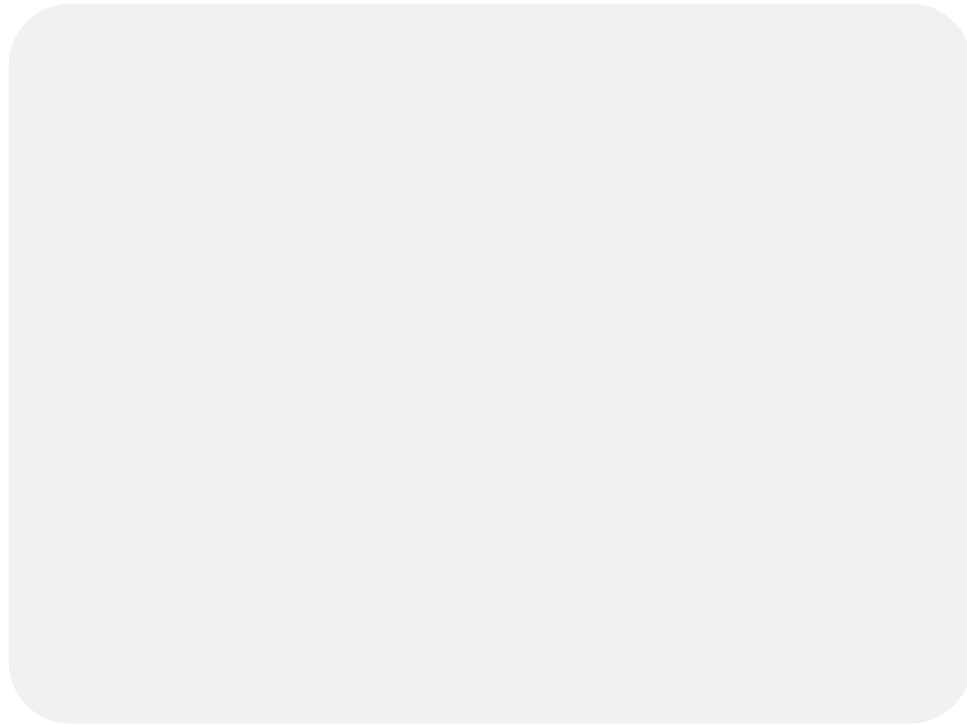

FIGURE 5. Biofilm formation can be seen clearly in the control group (rows E, F, G and H/columns 1-6) after 30 minutes following the application of formaldehyde.

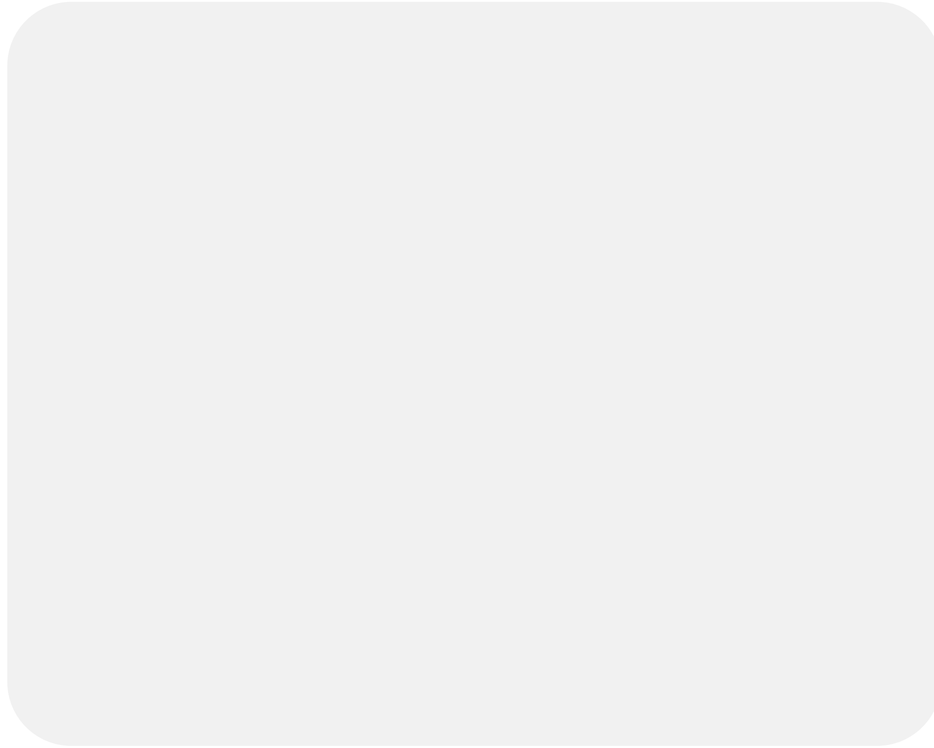

FIGURE 6. 200 μl of 0.5% crystal violet dye was added to each well and the biofilm cells stained for 30 min.

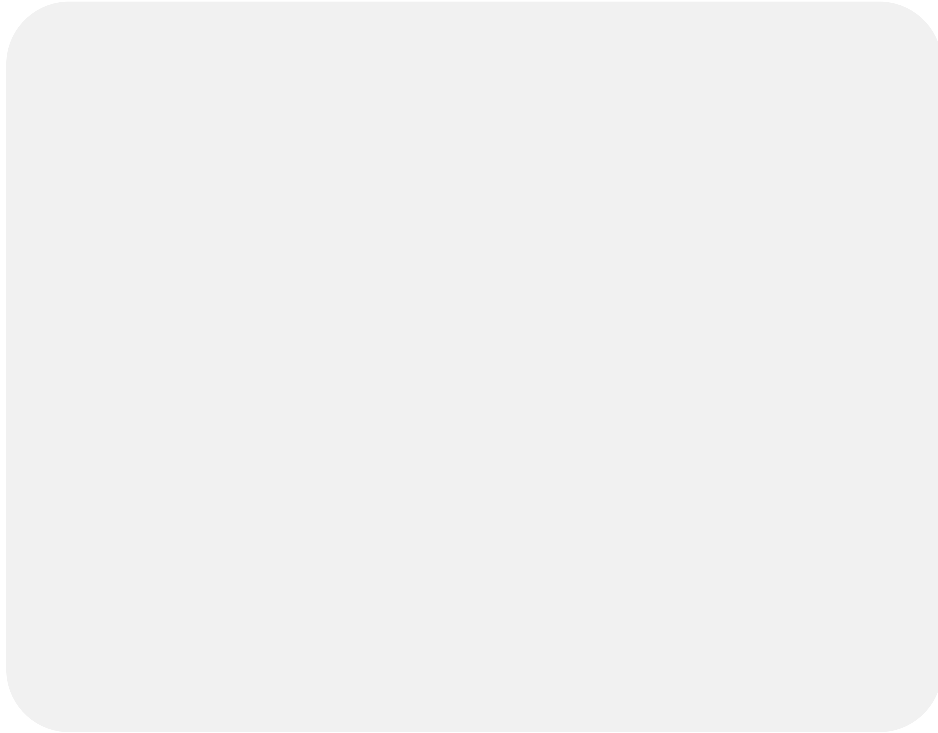

FIGURE 7. After crystal violet dye application, the wells were rinsed 3 times. The heavily stained wells are associated with more biofilm formation.

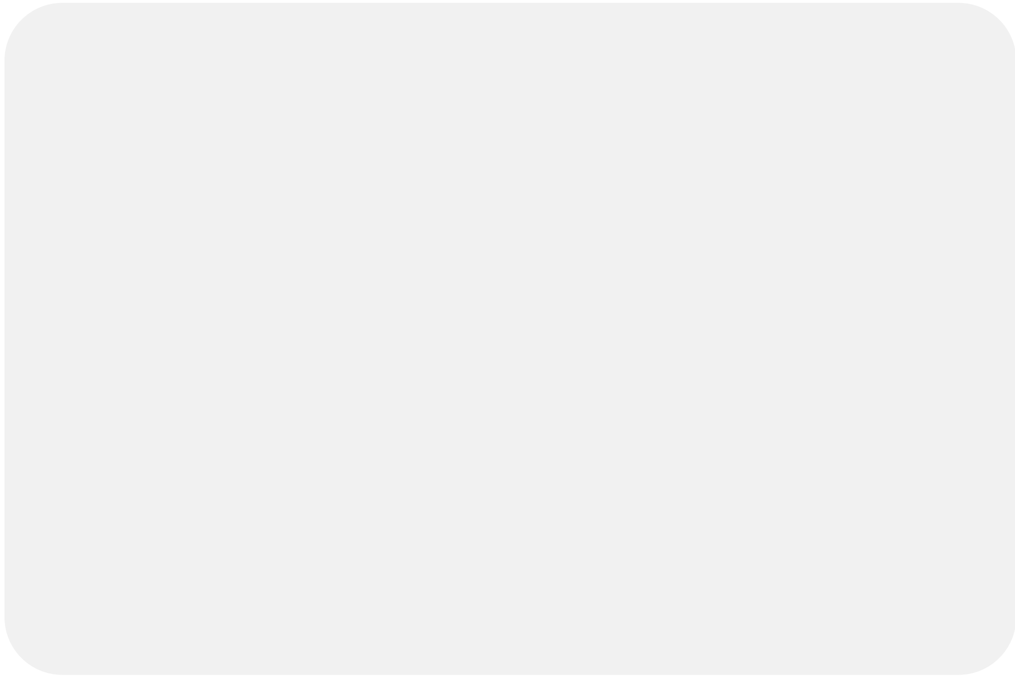

FIGURE 8. Two hundred (200) μl of 2-isopropanol was placed into each well for 1 h to lyse the biofilm cells and extract the crystal violet.

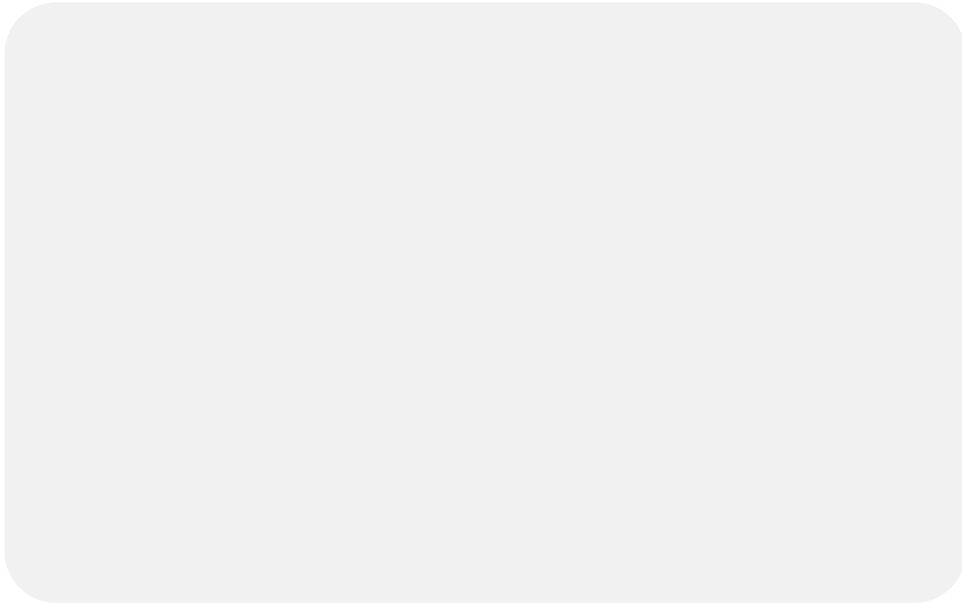

FIGURE 9. Microtiter plate wells were read using a spectrophotometer.

FIGURE 10. Effect of NaCl on *S. mutans* biofilm formation. Asterisks indicate significant differences ($p<0.05$) compared to samples without NaCl.

FIGURE 11. Effect of KCl on *S. mutans* biofilm formation. Asterisks indicate significant differences ($p<0.05$) compared to samples without KCl.

FIGURE 12. Effect of KI on *S. mutans* biofilm formation. Asterisks indicate significant differences ($p<0.05$) compared to samples without KI.

FIGURE 13. Combined effect of NaCl and nicotine on *S. mutans* total absorbance. Asterisks indicate significant differences ($p<0.05$) compared to samples without NaCl.

FIGURE 14. Combined effect of NaCl and nicotine on *S. mutans* planktonic growth. Asterisks indicate significant differences ($p<0.05$) compared to samples without NaCl.

FIGURE 15. Combined effect of NaCl and nicotine on *S. mutans* biofilm formation. Asterisks indicate significant differences ($p<0.05$) compared to samples without NaCl.

FIGURE 16. Combined effect of KCl and nicotine on *S. mutans* total absorbance. Asterisks indicate significant differences ($p < 0.05$) compared to samples without KCl.

FIGURE 17. Combined effect of KCl and nicotine on *S. mutans* planktonic growth. Asterisks indicate significant differences ($p < 0.05$) compared to samples without KCl.

FIGURE 18. Combined effect of KCl and nicotine on *S. mutans* biofilm formation. Asterisks indicate significant differences ($p < 0.05$) compared to samples without KCl.

FIGURE 19. Combined effect of Kl and nicotine on *S. mutans* total absorbance. Asterisks indicate significant differences ($p < 0.05$) compared to samples without Kl.

FIGURE 20. Combined effect of KI and nicotine on *S. mutans* planktonic growth. Asterisks indicate significant differences ($p < 0.05$) compared to samples without KI.

FIGURE 21. Combined effect of KI and nicotine on *S. mutans* biofilm formation. Asterisks indicate significant differences ($p < 0.05$) compared to samples without KI.

TABLE I

Basic statistics for *S. mutans* total absorbance, by salt (NaCl) and nicotine concentration. The type III tests of fixed effects (Table X) show the results of the combined effects of NaCl ($p < 0.0001$), nicotine ($p < 0.0001$), and their interaction ($p = 0.0089$) on *S. mutans* total absorbance.

Measure	**Salt**	**Salt (y/n)**	**Nicotine Concentration (mg)**	**N**	**Mean**	**SD**	**SE**	**Min**	**Max**
Total Abs.	NaCl	No	0	12	0.334	0.092	0.027	0.211	0.450
Total Abs.	NaCl	Yes	0	12	0.267	0.075	0.022	0.195	0.427
Total Abs.	NaCl	No	0.25	12	0.274	0.146	0.042	0.057	0.481
Total Abs.	NaCl	Yes	0.25	12	0.278	0.079	0.023	0.210	0.405
Total Abs.	NaCl	No	0.5	12	0.252	0.186	0.054	0.013	0.474
Total Abs.	NaCl	Yes	0.5	12	0.287	0.086	0.025	0.193	0.413
Total Abs.	NaCl	No	1	12	0.320	0.117	0.034	0.185	0.486
Total Abs.	NaCl	Yes	1	12	0.293	0.085	0.025	0.208	0.421
Total Abs.	NaCl	No	2	12	0.353	0.146	0.042	0.192	0.565
Total Abs.	NaCl	Yes	2	12	0.316	0.117	0.034	0.189	0.470
Total Abs.	NaCl	No	4	12	0.422	0.150	0.043	0.287	0.657
Total Abs.	NaCl	Yes	4	12	0.309	0.120	0.035	0.159	0.502
Total Abs.	NaCl	No	8	12	0.406	0.105	0.030	0.276	0.568
Total Abs.	NaCl	Yes	8	12	0.270	0.242	0.070	0.022	0.620
Total Abs.	NaCl	No	16	12	0.220	0.202	0.058	0.007	0.595
Total Abs.	NaCl	Yes	16	12	0.186	0.262	0.076	0.002	0.578
Total Abs.	NaCl	No	32	12	0.017	0.003	0.001	0.013	0.022
Total Abs.	NaCl	Yes	32	12	0.011	0.003	0.001	0.007	0.015

TABLE II

Basic statistics for *S. mutans* planktonic growth, by salt (NaCl) and nicotine concentration

Measure	Salt	Salt (y/n)	Nicotine Concentration (mg)	N	Mean	SD	SE	Min	Max
Planktonic	NaCl	No	0	12	0.027	0.018	0.005	0.008	0.059
Planktonic	NaCl	Yes	0	12	0.065	0.046	0.013	0.025	0.169
Planktonic	NaCl	No	0.25	12	0.020	0.017	0.005	0.002	0.068
Planktonic	NaCl	Yes	0.25	12	0.064	0.050	0.014	0.016	0.166
Planktonic	NaCl	No	0.5	12	0.017	0.020	0.006	-0.010	0.058
Planktonic	NaCl	Yes	0.5	12	0.050	0.036	0.010	0.013	0.105
Planktonic	NaCl	No	1	12	0.024	0.019	0.005	0.003	0.072
Planktonic	NaCl	Yes	1	12	0.043	0.020	0.006	0.016	0.071
Planktonic	NaCl	No	2	12	0.029	0.022	0.006	-0.001	0.075
Planktonic	NaCl	Yes	2	12	0.054	0.030	0.009	0.016	0.106
Planktonic	NaCl	No	4	12	0.036	0.024	0.007	0.003	0.080
Planktonic	NaCl	Yes	4	12	0.043	0.020	0.006	0.011	0.077
Planktonic	NaCl	No	8	12	0.033	0.041	0.012	-0.001	0.124
Planktonic	NaCl	Yes	8	12	0.044	0.044	0.013	-0.010	0.119
Planktonic	NaCl	No	16	12	0.029	0.034	0.010	-0.012	0.091
Planktonic	NaCl	Yes	16	12	0.011	0.026	0.008	-0.011	0.081
Planktonic	NaCl	No	32	12	0.002	0.006	0.002	-0.008	0.010
Planktonic	NaCl	Yes	32	12	0.000	0.007	0.002	-0.010	0.008

TABLE III

Basic statistics for *S. mutans* biofilm growth, by salt (NaCl) and nicotine concentration

Measure	Salt	Salt (y/n)	Nicotine Concentration (mg)	N	Mean	SD	SE	Min	Max
Biofilm	NaCl	No	0	12	0.871	0.141	0.041	0.676	1.145
Biofilm	NaCl	Yes	0	12	0.352	0.061	0.017	0.257	0.436
Biofilm	NaCl	No	0.25	12	0.615	0.256	0.074	0.186	0.988
Biofilm	NaCl	Yes	0.25	12	0.343	0.108	0.031	0.193	0.507
Biofilm	NaCl	No	0.5	12	0.583	0.466	0.134	0.015	1.249
Biofilm	NaCl	Yes	0.5	12	0.372	0.099	0.029	0.203	0.503
Biofilm	NaCl	No	1	12	0.856	0.285	0.082	0.487	1.366
Biofilm	NaCl	Yes	1	12	0.414	0.104	0.030	0.315	0.647
Biofilm	NaCl	No	2	12	0.835	0.293	0.085	0.458	1.308
Biofilm	NaCl	Yes	2	12	0.332	0.153	0.044	0.105	0.536
Biofilm	NaCl	No	4	12	1.014	0.450	0.130	0.468	1.762
Biofilm	NaCl	Yes	4	12	0.300	0.204	0.059	0.002	0.598
Biofilm	NaCl	No	8	12	0.945	0.747	0.216	0.058	2.074
Biofilm	NaCl	Yes	8	12	0.141	0.186	0.054	-0.028	0.520
Biofilm	NaCl	No	16	12	0.301	0.412	0.119	0.006	1.089
Biofilm	NaCl	Yes	16	12	0.004	0.012	0.003	-0.023	0.025
Biofilm	NaCl	No	32	12	0.003	0.017	0.005	-0.018	0.028
Biofilm	NaCl	Yes	32	12	0.003	0.013	0.004	-0.017	0.017

TABLE IV

Basic statistics for *S. mutans* total absorbance, by salt (KCl) and nicotine concentration

Measure	Salt	Salt (y/n)	Nicotine Concentration (mg)	N	Mean	SD	SE	Min	Max
Total Abs.	KCl	No	0	12	0.331	0.106	0.030	0.195	0.482
Total Abs.	KCl	Yes	0	12	0.320	0.216	0.062	0.043	0.703
Total Abs.	KCl	No	0.25	12	0.284	0.121	0.035	0.187	0.474
Total Abs.	KCl	Yes	0.25	12	0.218	0.215	0.062	0.030	0.486
Total Abs.	KCl	No	0.5	12	0.300	0.127	0.037	0.190	0.495
Total Abs.	KCl	Yes	0.5	12	0.190	0.191	0.055	0.030	0.460
Total Abs.	KCl	No	1	12	0.301	0.133	0.038	0.157	0.511
Total Abs.	KCl	Yes	1	12	0.179	0.207	0.060	0.030	0.477
Total Abs.	KCl	No	2	12	0.346	0.154	0.044	0.186	0.568
Total Abs.	KCl	Yes	2	12	0.255	0.231	0.067	0.026	0.525
Total Abs.	KCl	No	4	12	0.421	0.163	0.047	0.256	0.661
Total Abs.	KCl	Yes	4	12	0.203	0.238	0.069	0.024	0.548
Total Abs.	KCl	No	8	12	0.388	0.125	0.036	0.273	0.584
Total Abs.	KCl	Yes	8	12	0.211	0.291	0.084	0.008	0.649
Total Abs.	KCl	No	16	12	0.260	0.240	0.069	0.001	0.603
Total Abs.	KCl	Yes	16	12	0.226	0.316	0.091	0.006	0.685
Total Abs.	KCl	No	32	12	0.017	0.003	0.001	0.011	0.021
Total Abs.	KCl	Yes	32	12	0.014	0.003	0.001	0.008	0.018

TABLE V

Basic statistics for *S. mutans* planktonic growth, by salt (KCl) and nicotine concentration

Measure	Salt	Salt (y/n)	Nicotine Concentration (mg)	N	Mean	SD	SE	Min	Max
Planktonic	KCl	No	0	12	0.014	0.030	0.009	-0.025	0.081
Planktonic	KCl	Yes	0	12	0.048	0.064	0.019	-0.024	0.167
Planktonic	KCl	No	0.25	12	0.013	0.031	0.009	-0.031	0.057
Planktonic	KCl	Yes	0.25	12	0.013	0.033	0.010	-0.030	0.051
Planktonic	KCl	No	0.5	12	0.012	0.031	0.009	-0.034	0.052
Planktonic	KCl	Yes	0.5	12	0.005	0.033	0.010	-0.037	0.058
Planktonic	KCl	No	1	12	0.010	0.026	0.008	-0.027	0.056
Planktonic	KCl	Yes	1	12	0.008	0.029	0.008	-0.032	0.045
Planktonic	KCl	No	2	12	0.021	0.040	0.011	-0.034	0.089
Planktonic	KCl	Yes	2	12	0.016	0.034	0.010	-0.032	0.060
Planktonic	KCl	No	4	12	0.071	0.147	0.042	-0.025	0.504
Planktonic	KCl	Yes	4	12	0.014	0.033	0.010	-0.028	0.081
Planktonic	KCl	No	8	12	0.081	0.169	0.049	-0.017	0.595
Planktonic	KCl	Yes	8	12	0.011	0.045	0.013	-0.039	0.080
Planktonic	KCl	No	16	12	0.032	0.062	0.018	-0.035	0.139
Planktonic	KCl	Yes	16	12	0.034	0.093	0.027	-0.038	0.275
Planktonic	KCl	No	32	12	-0.006	0.019	0.005	-0.032	0.011
Planktonic	KCl	Yes	32	12	-0.008	0.020	0.006	-0.037	0.008

TABLE VI

Basic statistics for *S. mutans* biofilm growth, by salt (KCl) and nicotine concentration

Measure	Salt	Salt (y/n)	Nicotine Concentration (mg)	N	Mean	SD	SE	Min	Max
Biofilm	KCl	No	0	12	0.732	0.116	0.033	0.599	0.995
Biofilm	KCl	Yes	0	12	0.220	0.123	0.035	0.098	0.454
Biofilm	KCl	No	0.25	12	0.676	0.133	0.038	0.545	0.938
Biofilm	KCl	Yes	0.25	12	0.163	0.117	0.034	0.052	0.399
Biofilm	KCl	No	0.5	12	0.714	0.132	0.038	0.557	0.973
Biofilm	KCl	Yes	0.5	12	0.132	0.061	0.018	0.045	0.275
Biofilm	KCl	No	1	12	0.693	0.143	0.041	0.561	0.999
Biofilm	KCl	Yes	1	12	0.140	0.162	0.047	0.051	0.638
Biofilm	KCl	No	2	12	0.772	0.142	0.041	0.562	1.080
Biofilm	KCl	Yes	2	12	0.119	0.084	0.024	0.048	0.311
Biofilm	KCl	No	4	12	0.919	0.378	0.109	0.377	1.795
Biofilm	KCl	Yes	4	12	0.067	0.045	0.013	0.026	0.161
Biofilm	KCl	No	8	12	0.788	0.404	0.117	0.150	1.365
Biofilm	KCl	Yes	8	12	0.020	0.023	0.007	0.005	0.081
Biofilm	KCl	No	16	12	0.356	0.482	0.139	-0.005	1.260
Biofilm	KCl	Yes	16	12	0.019	0.044	0.013	-0.009	0.154
Biofilm	KCl	No	32	12	0.005	0.012	0.004	-0.018	0.026
Biofilm	KCl	Yes	32	12	0.008	0.014	0.004	-0.006	0.040

TABLE VII

Basic statistics for *S. mutans* total absorbance, by salt (KI) and nicotine concentration

Measure	Salt	Salt (y/n)	Nicotine Concentration (mg)	N	Mean	SD	SE	Min	Max
Total Abs.	KI	No	0	12	0.335	0.112	0.032	0.208	0.514
Total Abs.	KI	Yes	0	12	0.232	0.127	0.037	0.133	0.442
Total Abs.	KI	No	0.25	12	0.330	0.103	0.030	0.215	0.471
Total Abs.	KI	Yes	0.25	12	0.255	0.160	0.046	0.124	0.502
Total Abs.	KI	No	0.5	12	0.338	0.120	0.035	0.182	0.517
Total Abs.	KI	Yes	0.5	12	0.248	0.167	0.048	0.099	0.489
Total Abs.	KI	No	1	12	0.352	0.123	0.035	0.213	0.537
Total Abs.	KI	Yes	1	12	0.253	0.180	0.052	0.099	0.502
Total Abs.	KI	No	2	12	0.396	0.155	0.045	0.231	0.601
Total Abs.	KI	Yes	2	12	0.268	0.217	0.063	0.095	0.587
Total Abs.	KI	No	4	12	0.457	0.134	0.039	0.277	0.648
Total Abs.	KI	Yes	4	12	0.230	0.228	0.066	0.045	0.582
Total Abs.	KI	No	8	12	0.432	0.122	0.035	0.306	0.616
Total Abs.	KI	Yes	8	12	0.184	0.233	0.067	0.016	0.686
Total Abs.	KI	No	16	12	0.210	0.247	0.071	0.007	0.616
Total Abs.	KI	Yes	16	12	0.013	0.004	0.001	0.004	0.018
Total Abs.	KI	No	32	12	0.019	0.003	0.001	0.015	0.024
Total Abs.	KI	Yes	32	12	0.018	0.004	0.001	0.011	0.023

TABLE VIII

Basic statistics for *S. mutans* planktonic growth, by salt (KI) and nicotine concentration

Measure	Salt	Salt (y/n)	Nicotine Concentration (mg)	N	Mean	SD	SE	Min	Max
Planktonic	KI	No	0	8	0.039	0.019	0.007	0.020	0.079
Planktonic	KI	Yes	0	8	0.038	0.009	0.003	0.025	0.048
Planktonic	KI	No	0.25	8	0.026	0.019	0.007	0.012	0.071
Planktonic	KI	Yes	0.25	8	0.216	0.330	0.117	0.017	0.844
Planktonic	KI	No	0.5	8	0.020	0.015	0.005	0.004	0.053
Planktonic	KI	Yes	0.5	8	0.038	0.022	0.008	0.013	0.063
Planktonic	KI	No	1	8	0.028	0.020	0.007	0.016	0.076
Planktonic	KI	Yes	1	8	0.052	0.037	0.013	0.012	0.104
Planktonic	KI	No	2	8	0.022	0.021	0.007	0.008	0.072
Planktonic	KI	Yes	2	8	0.061	0.050	0.018	0.018	0.171
Planktonic	KI	No	4	8	0.063	0.064	0.022	0.009	0.201
Planktonic	KI	Yes	4	8	0.044	0.032	0.011	0.012	0.094
Planktonic	KI	No	8	8	0.046	0.050	0.018	0.002	0.155
Planktonic	KI	Yes	8	8	0.101	0.127	0.045	0.010	0.353
Planktonic	KI	No	16	8	0.021	0.026	0.009	0.007	0.085
Planktonic	KI	Yes	16	8	0.005	0.004	0.002	-0.002	0.009
Planktonic	KI	No	32	8	0.005	0.003	0.001	0.000	0.012
Planktonic	KI	Yes	32	8	0.007	0.002	0.001	0.003	0.010

TABLE IX

Basic statistics for *S. mutans* biofilm growth, by salt (KI) and nicotine concentration

Measure	Salt	Salt (y/n)	Nicotine Concentration (mg)	N	Mean	SD	SE	Min	Max
Biofilm	KI	No	0	12	0.837	0.156	0.045	0.624	1.195
Biofilm	KI	Yes	0	12	0.286	0.059	0.017	0.167	0.413
Biofilm	KI	No	0.25	12	0.791	0.139	0.040	0.623	0.995
Biofilm	KI	Yes	0.25	12	0.293	0.079	0.023	0.208	0.473
Biofilm	KI	No	0.5	12	0.829	0.124	0.036	0.662	0.995
Biofilm	KI	Yes	0.5	12	0.260	0.062	0.018	0.142	0.371
Biofilm	KI	No	1	12	0.859	0.127	0.037	0.675	1.068
Biofilm	KI	Yes	1	12	0.308	0.058	0.017	0.220	0.384
Biofilm	KI	No	2	12	1.022	0.284	0.082	0.560	1.615
Biofilm	KI	Yes	2	12	0.412	0.215	0.062	0.192	0.837
Biofilm	KI	No	4	12	1.118	0.401	0.116	0.525	1.641
Biofilm	KI	Yes	4	12	0.227	0.088	0.025	0.103	0.383
Biofilm	KI	No	8	12	0.944	0.622	0.180	0.117	1.836
Biofilm	KI	Yes	8	12	0.113	0.076	0.022	0.014	0.209
Biofilm	KI	No	16	12	0.225	0.196	0.056	0.004	0.618
Biofilm	KI	Yes	16	12	0.002	0.013	0.004	-0.023	0.014
Biofilm	KI	No	32	12	0.010	0.020	0.006	-0.021	0.053
Biofilm	KI	Yes	32	12	0.004	0.012	0.003	-0.011	0.019

TABLE X

A two-way ANOVA test comparing the effects of salt presence (either NaCl, KCl, or KI), nicotine (concentrations ranging from 0 – 32 mg), and their interaction on S. mutans biofilm, planktonic cells, and total absorbance

Measure	Salt	Effect	Rank p-value
Biofilm	KCl	Concentration	<0.0001
Biofilm	KCl	Salt_yn	<0.0001
Biofilm	KCl	Concentration*Salt_yn	<0.0001
Biofilm	KI	Concentration	<0.0001
Biofilm	KI	Salt_yn	<0.0001
Biofilm	KI	Concentration*Salt_yn	<0.0001
Biofilm	NaCl	Concentration	<0.0001
Biofilm	NaCl	Salt_yn	<0.0001
Biofilm	NaCl	Concentration*Salt_yn	<0.0001
Planktonic	KCl	Concentration	0.0669
Planktonic	KCl	Salt_yn	0.3226
Planktonic	KCl	Concentration*Salt_yn	0.7760
Planktonic	KI	Concentration	<0.0001
Planktonic	KI	Salt_yn	0.0015
Planktonic	KI	Concentration*Salt_yn	<0.0001
Planktonic	NaCl	Concentration	<0.0001
Planktonic	NaCl	Salt_yn	<0.0001
Planktonic	NaCl	Concentration*Salt_yn	<0.0001
Total Absorbance	KCl	Concentration	<0.0001
Total Absorbance	KCl	Salt_yn	<0.0001
Total Absorbance	KCl	Concentration*Salt_yn	0.0123
Total Absorbance	KI	Concentration	<0.0001
Total Absorbance	KI	Salt_yn	<0.0001
Total Absorbance	KI	Concentration*Salt_yn	0.0142
Total Absorbance	NaCl	Concentration	<0.0001
Total Absorbance	NaCl	Salt_yn	<0.0001
Total Absorbance	NaCl	Concentration*Salt_yn	0.0089

DISCUSSION

The preliminary experiment was designed for two main reasons: 1) to confirm the antimicrobial activities of the three salts and 2) to determine the MIC of each salt. Even though the antimicrobial activity of these salts was discussed in many previous studies, there was a lack of evidence regarding the direct effect of these salts on *S. mutans* biofilm growth. In addition, *S. mutans* is considered one of the normal flora species in the oral cavity. So, it is biologically relevant to obtain the MIC to not negatively affect the normal ecology of the oral flora. An overnight culture of *S. mutans* was placed in 96-well microtiter plates with TSBS to stimulate *S. mutans* growth and biofilm formation. Nicotine was added to both study and control groups to increase biofilm formation as this was confirmed in previous studies.[90-93] The results of this study indicate that NaCl, KCl and KI are able to significantly diminish the biofilm formation of *S. mutans*. In addition, these three salts are able to significantly limit the ability of nicotine to increase the growth of *S. mutans*, especially biofilm formation. Each salt was investigated in different microtiter plates. Each plate contained the study group involving the salt/nicotine combination and a control group of nicotine without the salt, as it was better to include both study and control groups in one microtiter plate in order to standardize the environment and preparation conditions. A sterility group was added to assure contamination was not present, because if there was contamination, the sterility wells will exhibit some kind of bacterial growth. A two-way ANOVA was used to compare the effects of salt presence (either NaCl, KCl, or KI) and nicotine (concentrations ranging from 0 – 32 mg) on *S. mutans* biofilm, planktonic cells, and total absorbance. Since the

experimental trial was repeated 3 times (with 4 samples per group per repeat), a random effect for the multiple trials was used. Due to non-normality, a rank transformation was used prior to analysis.

The most important phase of bacterial growth between total absorbance, planktonic and biofilm is the biofilm form as it is the most favorable one for oral bacteria to grow *in vivo* and cause disease. [124] Also, protein expression in biofilm cells differs from the expression that is observed in planktonic cells.[130] So, even though the salts demonstrated minor restriction of nicotine activity when total absorbance and planktonic growth are observed, this restriction was recognized clearly when biofilm formation is observed, with the exception of the 32 mg/ml nicotine concentration. In the nicotine group (with no salt), 0.25-8 mg/ml of nicotine increased biofilm formation. The MIC and MBC concentrations were found with 16 and 32 mg/ml of nicotine, respectively, and this was found also in a study by Huang et al. who investigated the effect of nicotine on seven species of *S. mutans* including the UA 159 strain.[90] This is because nicotine becomes toxic to *S. mutans* above 8 mg/ml of nicotine. The toxicity of nicotine to *S. mutans* UA 159 with > 8 mg/ml of nicotine was described in the Huang et al. study.[90] It is understood that any salt with 16 or 32 mg of nicotine demonstrated more inhibition than nicotine alone and this probably is because of the combined antimicrobial effect of the salt and nicotine. But at a 8 mg/ml nicotine concentration, it was observed that with no salt, there was more biofilm formation than concentrations < 8 mg/ml nicotine. However, when any salt was added, there was less biofilm formation with 8 mg of nicotine than with concentrations < 8 mg/ml nicotine. A reasonable explanation is that a synergistic effect

was developed between each salt and nicotine at this specific concentration of nicotine, 8 mg/ml.

The clinical implication of these results can be related to the amount of nicotine in human saliva of smokers. Feyerabend et al. found that the nicotine level in human saliva ranges from 0-0.31 mg/ml for those people who are not smokers but are affected by secondary or tertiary hand smoke. For light or medium smokers, the range of nicotine in human saliva is between 0-1.33 mg/ml and for heavy smokers is 0-2.27 mg/ml.[88] In another study, it was found that the level of nicotine in human saliva ranges from 0.07-1.56 mg/ml for those people who have smoked for at least ten years.[87] For smokers, if we suppose that the average nicotine level in human saliva is 1 mg/ml, it could be said that the presence of 0.45 M of NaCl, 0.23 M of KCl and 0.113 M of KI significantly inhibited first hand nicotine-induced *S. mutans* biofilm formation by 52 percent, 79.7 percent and 64.1 percent, respectively.

However, the last statement could be refuted because of the fact that the previous mentioned nicotine levels was found in the saliva, and what is needed specifically is to determine the level of nicotine in the biofilm itself. Further research may discuss how NaCl, KCl and KI specifically inhibit *S. mutans* biofilm formation, and what affect these salts may have on extracellular polysaccharide synthesis, glucosyltransferase synthesis, glucan-binding protein synthesis and acid production. Nicotine was found to increase extracellular polysaccharide (EPS) synthesis, GbpA expression, Gtfs expression, and lactic acid production.[92] So, NaCl, KCl, and KI may interfere with EPS synthesis, GbpA, Gtfs expression or lactic acid production. In addition, *in-vivo* studies are needed to confirm the anti-microbial effect of these salts and its biocompatibility with oral tissues.

SUMMARY AND CONCLUSION

This study indicates that the growth of nicotine-induced *S. mutans* could be diminished in the presence of NaCl, KCl and KI salts. NaCl, KCl and KI demonstrated an inhibitory effect on the growth of *S. mutans*, this could be beneficial clinically by asking smokers to rinse with these salts to diminish the cariogenicity of first hand nicotine-induced *S. mutans* biofilm formation. *In-vivo* studies are needed to confirm this benefit.

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ABSTRACT

FIRST-HAND NICOTINE/TOBACCO EXPOSURE ON *STREPTOCOCCUS MUTANS* BIOFILM AND EFFECTS OF SODIUM CHLORIDE, POTASSIUM CHLORIDE AND POTASSIUM IODIDE SALTS

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Objective: The aim of this study is to investigate the effects of first hand nicotine exposure on *Streptococcus mutans* biofilm formation and the inhibitory effects of sodium chloride (NaCl), potassium chloride (KCl) and potassium iodide (KI) salts. This study examined bacterial growth with varying concentrations of NaCl, KCl and KI salts and nicotine levels consistent with primary levels of nicotine exposure.

Design: A preliminary experiment was done to investigate the most likely effective concentration of NaCl, KCl and KI. Then, a 24-hour culture of *S. mutans* UA159 in microtiter plates was treated with nicotine (0-32 mg/ml) in Tryptic Soy broth supplemented with 1.0-percent sucrose (TSBS) simulating first hand exposure with and without 0.45 M of NaCl, 0.23 M of KCl and 0.113 M of KI. A spectrophotometer was used to determine total growth and planktonic growth. The biofilm was fixed, stained with crystal violet dye and the absorbance measured to determine biofilm formation.

Results: The presence of 0.45 M of NaCl, 0.23 M of KCl and 0.113 M of KI significantly inhibited ($p<0.05$) first hand nicotine-induced *S. mutans* biofilm formation by 52 percent%, 79.7 percent, and 64.1 percent, respectively. Similar results were obtained for planktonic growth.

Conclusion: These results provided more evidence regarding the negative effects of nicotine and also demonstrated the positive influence of these salts in reducing nicotine-induced biofilm formation, which needs be confirmed by *in-vivo* studies.

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