EFFECT OF NICOTINE ON BIOFILM FORMATION OF *STREPTOCOCCUS MUTANS* ISOLATES FROM SMOKING VERSUS NON-SMOKING SUBJECTS

by

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DEDICATION

First, I would like to dedicate this work to the Almighty God.

Second, to my amazing and loving parents,

Farouk and Faiza,

Without their prayers and support I would never be able to make it.

To my precious my sons,

Tato and Omar,

Whose love and patience made it possible for me to achieve my work and to continue to be a strong fighter for my family's future.

To my sweet and supportive brothers,

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INTRODUCTION

Tooth decay is a complex dieto-bacterial disease with an association of social, behavioral and biological factors. *Streptococcus mutans* plays a major role in tooth decay. This endogenous oral microorganism adheres to tooth surfaces, and grows and develops into micro-communities that mature and form dental biofilm. Development of cariogenic biofilm is one of the major factors associated with the tooth decay process. The use of tobacco is considered a great risk factor for oral diseases. Several studies demonstrated the association of tooth decay and the use of tobacco as effects of first hand or second hand smoking. Nicotine has been reported to increase the biofilm growth and metabolism of *S. mutans* in a dose-dependent manner up to 16 mg/ml of nicotine. However, its effects on biofilm formation of *S. mutans* strains isolated from smokers are not known and should be investigated. Therefore, we proposed the use of an *in-vitro* model to better understand the effects of nicotine on the biofilm formation of strains of *S. mutans* isolates from smokers versus non-smoking subjects.

Objectives: To investigate the effects of nicotine on biofilm formation of *S. mutans* isolates from oral washes of smoker and non-smoker human subjects.

Null Hypotheses: 1) Nicotine will not increase biofilm formation in both smoker and non-smoker *S. mutans* isolates. 2) An increase in nicotine concentrations will not increase biofilm *f*ormation in both smoker and non-smoker *S. mutans* isolates in a dose-dependent manner. 3) Nicotine will not produce statistically significant differences in biofilm formation between smoker and non-smoker *S. mutans* isolates.

Alternative Hypotheses: 1) Nicotine increases the growth of biofilm formation in both smoker and non-smoker *S. mutans* isolates. 2) An increase in nicotine concentrations will increase biofilm formation of both smoker and non-smoker *S. mutans* isolates in a dose-dependent manner. 3) Nicotine increases biofilm formation of smoker *S. mutans* strains more than non-smoker *S. mutans* isolates.

REVIEW OF LITERATURE

ETIOLOGY OF DENTAL CARIES

Tooth decay is a complex dieto-bacterial disease with an association of social, behavioral, and biological factors.[1] This complex disease is still considered one of the major chronic diseases worldwide and affects 90 percent of the population in the US.[2,3] It is the most common childhood disease and occurs five times more than bronchial asthma, which is considered the second most common childhood disease.[4]

Tooth decay is an infectious disease that develops over time involving a complex interaction of oral microflora, specifically S. *mutans*, dietary carbohydrates, and a susceptible tooth surface.[5]

Tooth decay is mainly associated with *S. mutans* colonization. *S. mutans* has been consistently isolated from caries-active subjects.[6] Previous data strongly suggest high levels of *S. mutans* in occlusal fissure lesions.[7] It has been well defined that *S. mutans* and tooth decay are closely related given that *S. mutans* can adapt very well in a high carbohydrate environment under acidic conditions (utilizing the aciduric enzymes lactic dehydrogenase and acid phosphatase). *S. mutans* has the ability to metabolize these sugars forming organic acids that bathe tooth surfaces and cause progressive mineral loss. In addition, a water insoluble extracellular polysaccharide substance (matrix) is formed that adheres to hard tooth structures developing cariogenic biofilm.[8-12]

S. mutans is an endogenous member of the oral microflora that thrives in specific oral conditions with unique characteristics. These gram-positive facultative anaerobic organisms grow in the oral cavity as biofilms closely adhered to smooth tooth structures.[9] Adherence of *S. mutans* to hard tooth structures is considered one of the major characteristics that enables it to proliferate and microcolonize, establishing a mature cariogenic biofilm. Numerous cariogenic factors of *S. mutans* are involved in its ability to adhere and to aggregate to form cariogenic biofilms, including initial sucrose-independent adherence in which antigen I/II is involved, and sucrose-dependent adherence based on the function of glucosyltransferases (Gtfs) and other glucan-binding proteins (Gbps).[10] In addition to biofilm formation, *S. mutans* has the ability to produce organic acids that cause tooth demineralization, and the ability to survive in acidic oral conditions allowing it to thrive and colonize in the oral cavity. [11]

ORAL MICROBIAL BIOFILM

Dental biofilm is a multi-dimensional complex structure of microbial colonies embedded in an extracellular polysaccharide matrix commonly formed by the microorganisms themselves and attached to tooth surfaces. Biofilms are characterized by physical and biochemical properties that promote adherence of microorganisms, localize a source for sugars and energy, and act as a protective barrier from external unfavorable stimuli.[12] Oral microbial biofilm (dental plaque) formation involves four stages including salivary acquired pellicle formation, microorganism adherence, growth and maturation of the bacterial microcolonies, and lastly, detachment to form a new biofilm.[13] In the first stage, if the tooth surface is clean, salivary molecules can adsorb to hydroxyapatite on enamel tooth surfaces by electrostatic interactions forming the acquired enamel pellicle.

Salivary acquired pellicle is an acellular, bacterial-free interface with the major constituents being salivary proteins and glycoproteins.[1,14] Initial microorganism adherence is the second stage that occurs when early colonizing bacteria attach to salivary acquired pellicle through a weak reversible attachment in the absence of sucrose-utilizing specific receptors and ligands.[13] *S. mutans* has an important role in the initial sucrose-independent adherence involving a bacterial surface protein adhesin called antigen I/II that interacts specifically with a high molecular weight salivary agglutinin glycoprotein (SAG) found in the acquired enamel pellicle.[15,16] This surface protein adhesin exhibits exceptional functional and immunological properties that have a significant role in *S. mutans* thriving in the human oral cavity.[17,22] It is known that *S. mutans* strains that lack surface protein antigen I/II are unable to initially adhere and to aggregate on the tooth surface.[16,18] An early study investigating inactivation of the gene for antigen I/II surface protein on the adherence and cariogenicity of *S. mutans* strains in germ-free Fischer rats that were on a 5.0-percent sucrose diet demonstrated significantly lower levels of dental carious lesions in the *S. mutans* deleted gene-infected rats compared with wild type infected rats six weeks post-infection. [19]

The third stage involves formation of an extracellular polysaccharide matrix and establishment of cariogenic biofilm attached to tooth surfaces, which is contributed by an important cariogenic factor of *S. mutans* known as sucrose-dependent adherence involving glucosyltransferase (Gtfs) enzymes and glucan-binding proteins. *S. mutans*-associated glucosyltransferases primarily produce both water-soluble and insoluble glucans by metabolizing sucrose to glucose and fructose, subsequently polymerizing glucose to an extracellular adhesive insoluble glucan that binds bacterial cells together

and attaches the cells to the enamel tooth surface.[20,26] Gtfs are a group of enzymes present on the *S. mutans* bacterial cell surface including GtfB, GtfC, and GtfD each with a different but interrelated function to one another.[49] Their protein expression is more significant in planktonic than biofilm *S. mutans* cells.[49] Gtfs have two functional domains, including an N-terminal catalytic and a C-terminal glucan-binding domain.[21,28]

The N-terminal domain of Gtfs has a major role in catalyzing the synthesis of glucan in the presence of dietary sucrose to enable *S. mutans* to adhere and to aggregate on the tooth surface. An *in-vivo* study demonstrated that water-insoluble glucan is associated with initial tooth decay by enhancing the adhesion and aggregation of *S. mutans* in dental biofilm in 12-month to 30-month old children.[20] Moreover, it was observed that alteration of the Gtfs genes remarkably decreases the cariogenic potential of *S. mutans* strains.[22]

The synthesis of extracellular glucan enhances the adherence of *S. mutans* through a cell-to-cell interaction where the C-terminal domains of streptococcal Gtfs bind to glucan that successively attaches them to smooth tooth surfaces.[23] Molecular data studying the effects of an oolong tea polyphenol on the cariogenicity of *S. mutans* in rats demonstrated a decrease in glucan binding to the C-terminal glucan binding domain of Gtfs. It was suggested that an oolong tea polyphenol strongly affects the function of the glucan-binding domain by attaching or combining to it.[24]

Additionally, *S. mutans* synthesizes Gbps that have a significant role in establishing a mature biofilm by adhering bacteria to the extracellular glucan. An *in-vitro* study by Lynch et al., indicated that engineered *S. mutans* with deleted Gbps genes affected the adherence and aggregation of these organisms resulting in a decrease in the

biofilm mass and change in its architecture.[25] In another study, where the researchers deleted genes coding for glucan binding proteins of *S. mutans* in rats, the results showed a reduction in the caries activity of these strains. The researchers concluded that glucan-binding proteins have a significant role in biofilm formation and are strongly associated with the cariogenicity of *S. mutans*.[26]

In addition to adherence and biofilm formation, *S. mutans* has the ability to produce large amounts of organic acids and tolerate acid environments that contribute to the cariogenicity and virulence of these organisms. Tooth decay is a dynamic process including several remineralization and demineralization cycles depending on pathological and protective factors. Acid production is one of the pathological factors that leads the dental caries process in favor of demineralization. Acidogenic bacteria produce large amounts of organic acids including lactic, acetic, formic, and propionic acids as byproduct of dietary carbohydrate fermentation resulting in a remarkable decrease in pH and the establishment of an aciduric biofilm.[27] These acids diffuse through the tooth enamel and cause demineralization of tooth tissue and development of tooth decay.[28]

SMOKING AND DENTAL CARIES

In the US, tobacco still remains a major cause of disease and death since decades ago, when the first Surgeon General report on smoking and health was released.[29] Tobacco use is a behavioral risk factor that adversely affects oral health and is directly linked to the most common life threatening diseases such as cancer, and cardiovascular and respiratory diseases.[30,31,32] The oral cavity is the first place in the human body to get exposed to either chewing tobacco or tobacco smoke and their chemical components. Therefore, tobacco not only affects systemic organs, but it also has a significant influence

on periodontal and other oral tissues.[33] Nicotine is the major alkaloid in tobacco, forming 90-percent of the total alkaloid pool.[34] This active chemical has a toxic effect on alveolar bone and clinical attachment loss. The amount of nicotine absorbed in blood is about 1 mg to 1.5 mg per single cigarette that on average contains 12 mg to 20 mg nicotine.[35] Nicotine is significantly associated with periodontal infectious disease by up-regulating microbial virulence factors causing more alveolar bone and clinical attachment loss.[37] In addition, nicotine delays the disease-healing process by inhibiting the proliferation, migration and differentiation of mesenchymal cells.[36] The effects of nicotine on periodontal disease have been elucidated, but nicotine's exact effects associated with tooth decay have not been fully investigated at present. Recently, data from a cohort study concluded that subjects smoking 20 cigarettes daily had two times more in number of carious lesions compared with non-smoking subjects after four years. In a cross-sectional epidemiological study by Axelsson et al., the relation between dental status and smoking behavior of four age groups of Swedes was investigated.[36] The authors concluded that smoking is a significant risk factor for both periodontal disease and dental caries.[37] In another cross-sectional study conducted to assess the association between periodontitis, dental caries and smoking among patients obtaining periodontal treatment in a dental teaching institute in Jordan were observed. Smoking patients with various types of periodontal disease except aggressive periodontitis had significantly greater DMFT scores compared with non-smoker patients.[38] A study conducted on 824 male Mexican truck drivers found a remarkable association between tobacco use and dental caries experience. Drivers who smoked more than 10 cigarettes per day had twice as many carious lesions than non-smokers. The study also found that older subjects

exhibited poor oral hygiene and greater tobacco use, and that they had a greater number of large caries and missing teeth.[39] In an Italian military population, it was determined that heavy smokers had twice the number of decayed teeth than a general population. Therefore, the authors concluded that an increase in smoking habits results in an increased caries risk.[40]

In addition, much interest has been focused on second-hand smoke. It was found that second-hand smoke is responsible for serious general disease and oral health problems in thousands of non-smokers including children (US Surgeon General, 2010). It has been indicated that children living in homes with smokers had a much higher caries experience than children from non-smoking homes.[41] Another study investigated the effects of mothers that smoked during and after pregnancy on the dental caries experience of their children at the age of 3 years. The authors concluded that mothers that smoked during and after pregnancy were remarkably associated with increased caries experience in their children.[42] Saliva is the main factor that significantly contributes to oral immunity. Secretory immunoglobulin A (sIgA) is the major constituent of saliva that plays an important protective role against periodontal disease and dental decay. It was observed that individuals with low caries rates had both high total salivary sIgA levels and sIgA anti-*S. mutans* antibody levels.[43] Recently, a study determined the association of dental caries in children and adults with the number of *S. mutans* and the level of salivary sIgA in saliva. It was found that the number of *S. mutans* was significantly higher in caries-active subjects of both populations compared with caries-free subjects. Additionally, sIgA concentrations were significantly lower in caries active adults and children.[44] More interesting, it was found that smokers with a high prevalence of dental

caries exhibited lower concentrations of sIgA compared with non-smokers.[45] In another study, data demonstrated significantly lower concentrations of sIgA linked to smokeless tobacco subjects compared with high concentrations of sIgA in non-smoking subjects.[46,47] Studies by our laboratory and another lab investigated the *in-vitro* effects of cigarette smoke on the growth of *S. mutans* and *Streptococcus sanguis*. These studies concluded that nicotine has dose-dependent effects on the growth of *S. mutans*; as the nicotine concentration derived from cigarettes increased, there was an increase in *S. mutans* growth.[48,49] Recently, we also determined that nicotine stimulates *S. mutans* planktonic cell Gtfs and GbP gene expression significantly more than *S. mutans* biofilm Gtfs and GbP gene expresion as a mechanism to increase planktonic cell attachment to a biofilm matrix. This attachment process leads to an increased number of cells in the biofilm and to the development of more carious lesions in smokers.[50] Our rationale for the up-regulation of Gtfs of planktonic cells at 2 mg/ml and 4 mg/ml nicotine concentration is that planktonic and biofilm cells are physiologically different.[49] The intercellular signaling and the sensing system that is present in *S. mutans* biofilm cells regulates and controls Gtfs gene expression of *S. mutans* biofilm cells and down-regulates it with nicotine treatment. *S. mutans* in the planktonic state is not involved in the same intercellular signaling and surface sensing system and thus the nicotine effect was clearly demonstrated by the significant increase of Gtfs of planktonic cells.[49] In this same study, the authors studied the effect of nicotine on *S. mutans* lactate dehydrogenase. There was no evidence that nicotine stimulates *S. mutans* lactate dehydrogenase activity to produce lactic acid other than by increasing the number of bacterial cells that lead to increased total lactic acid formation.[49]

In another study by Huang and Gregory from this laboratory, seven *S. mutans* strains were treated with different nicotine concentrations (ranging from 0 mg/ml to 16 mg/ml). The MIC (minimum inhibitory concentration), MBC (minimum bactericidal concentration), MBIC (minimum biofilm inhibitory concentration), planktonic cell growth, biofilm formation, and metabolic activity of the strains were determined. Biofilm formation and metabolism of all seven *S. mutans* strains increased in a dose-dependent manner up to 16.0 mg/ml of nicotine. Planktonic cell growth exhibited the highest values between 2 mg/ml to 8 mg/ml of nicotine. The majority of the *S. mutans* strains provided an MIC of 16 mg/ml of nicotine, MBC of 32mg/ml nicotine, and MBIC of 16 mg/ml nicotine.[51] Because of these significant effects of nicotine on *S. mutans*, it is possible that there may be a difference in the manner that *S. mutans* responds to nicotine *in vivo* in smokers. To date there is no information on the effects of nicotine on the biofilm formation of *S. mutans* isolates from smokers.

Therefore, the aims of this study proposed the use of an *in-vitro* model to better understand the effects of nicotine on biofilm formation of *S. mutans* isolates from smoker and non-smoker subjects.

MATERIALS AND METHODS

BACTERIAL STRAINS AND MEDIA

Ten oral washes collected from smoking subjects (subjects 09, 010, 011, 170, 020, 021, 220, 024, 025 and 850) and 10 oral washes from non-smoking subjects (subjects 800, 840, 860, 870, 880, 890, 920, 900, 910 and 960) were used in this study (see Table I and Table II for subject demographics). Three *S. mutans* isolates were cultured from each oral wash. Therefore, a total of 30 *S. mutans* smoker isolates and 30 *S. mutans* non-smoker isolates were used. The oral washes were collected as part of a large multicenter NIH-funded microbiome grant (HL098960) and were obtained under appropriate IRB approval (IRB number 1401371742). The oral wash samples were stored at -80^{o}C until used. Selective agar plates (MSSB; Mitis Salivarius Sucrose Bacitracin; Anaerobic Systems, Inc., Morgan Hill, CA, USA) were used for culturing the oral wash samples in 5.0-percent CO_2 at 37^{o}C as an initial isolation step, and then three different colonies representative of *S. mutans* from each oral wash sample were separated and grown on different MSSB plates. The isolates were subcultured in tryptic soy broth (TSB, Acumedia, Baltimore, MA, USA) for 24 hours in 5.0-percent CO_2 at 37^{o}C. The cultured isolates were stored in TSB with 20-percent glycerol at -80^{o}C until used. Mannitol and raffinose carbohydrate fermentation assays were used to confirm the identity of *S. mutans* isolates (Setterstrom et al., 1979). Briefly, phenol red base (Difco) were prepared with 1.0-percent mannitol or 1.0-percent raffinose and each isolate was grown in 5 ml of the media for 1 day to 14 days at 37^{o}C in 5.0-percent CO_2. Color changes from red to yellow indicated fermentation of the carbohydrate. *S. mutans*

isolates should be able to ferment mannitol and raffinose (Setterstrom et al., 1979). Thirty-four isolates were confirmed from the fermentation assays (12 from smokers and 22 from non-smokers). Nicotine from Sigma-Aldrich (St. Louis, MO, USA) was used.

BIOFILM FORMATION

Overnight cultures of each *S. mutans* strain (10 µl representing approximately 10^6 bacteria) grown in TSB were incubated with 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 of nicotine in TSB containing 1.0-percent sucrose (TSBS; 190 µl) for 24 hours at 37ºC in 5.0-percent CO_2 in sterile (8 x 12) 96-well microtiter plates (Fisher Scientific, Newark, DE, USA). The total absorbance of each well was measured at 595 nm in a microplate spectrophotometer (SpectraMax 190; Molecular Devices, SunnyVale, CA, USA) to assess the total bacterial growth (planktonic + biofilm cells). One hundred twenty µl (120 µl) of the planktonic cells from each well was transferred to another microplate and the planktonic cell absorbance was determined at 595 nm. The biofilm plates were washed two times with deionized water, fixed with 200 µl of 10-percent formaldehyde (Sigma) for 30 minutes at room temperature, and washed two times with water again. Two hundred µl (200 µl) of 0.05-percent crystal violet were used to stain biofilm cells for 30 minutes, the wells washed two times with water and 200 µl of isopropanol (Fisher, Pittsburg, PA, USA) added for 60 minutes to extract the crystal violet from the biofilm cells. The absorbance values were measured at 490 nm.

STATISTICAL METHODS

The experimental stage was conducted in quadruplicate over three trials. Each of the 34 *S. mutans* fermentation-confirmed strains were tested three times in quadruplicate.

Summary statistics (mean, standard deviation, standard error, range) of the absorbance values (total absorbance, planktonic and biofilm) were calculated for each of the 34 strains. The effects of nicotine concentration, smoker vs. non-smoker *S. mutans* strain, and their interaction on biofilm formation were analyzed using ANOVA. The ANOVA included fixed effects for the two factors and their interaction and a random effect of absorbance values were examined. A transformation of the data (e.g., natural logarithm) was necessary to satisfy the ANOVA assumptions. Based on the results of previous studies, a log-normal distribution with a coefficient of variation of 1.0 was expected. With a sample size of 10 samples per treatment combination, this study had 80-percent power to detect a three-fold difference between any two treatment combinations, assuming two-sided tests were each conducted at a 5.0-percent significance level.

Spearman correlation coefficients were calculated to evaluate the association of pack years smoked with biofilm formation. Correlations were calculated for each experiment separately and then averaged across experiments before computing an overall correlation.

In confirming the identity of *S. mutans* strains by the carbohydrate fermentation assay we started with 60 strains, and by completing the carbohydrate assay, we ended up with 34 definitive *S. mutans* strains.

RESULTS

Due to non-normality of the data, a rank transformation was used on the data prior to the analyses. A two-way ANOVA with a random effect for the multiple experiments was used for the analysis. There were significant effects for both nicotine concentrations and smoking on the growth of biofilm, planktonic cells, and total absorbance, for all strains of *S. mutans* ($p < 0.0001$; Figure 1 to Figure 3). For biofilm, there was a significant interaction of nicotine concentrations and smoking for smoker *S. mutans* smoking strains (Figure 1). For planktonic and total absorbance, there was a significant interaction of nicotine concentration and non-smoking *S. mutans* isolates ($p < 0.0001$; Figure 2 and Figure 3).

There were significant differences between biofilm, planktonic, and total bacterial cell growth at 16 mg/ml and 32 mg/ml of nicotine and biofilm, planktonic, and total bacterial growth at 0 mg/ml, 1 mg/ml, 2 mg/ml, 4 mg/ml, and 8 mg/ml nicotine concentrations of all *S. mutans* strains (Table VI).

There were significant differences between biofilm formation of smoker and non-smoker isolates at 0.25 mg/ml, 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 4 mg/ml, 8 mg/ml, and 32 mg/ml nicotine concentrations (Figure 1). There were significant differences between biofilm formation of smoker isolates with 1 mg/ml, 4 mg/ml, and 8 mg/ml of nicotine and the zero nicotine concentration (Figure 1). The significant differences between biofilm formation of non-smoker isolates and the zero nicotine concentration were observed with 4 mg/ml, 8 mg/ml, 16 mg/ml, and 32 mg/ml nicotine concentrations. Biofilm formation of smoker isolates had dose-dependent effects up to 8 mg/ml. Isolates from smokers had

significantly more biofilm at 0 mg/ml to 16 mg/ml nicotine compared with those from non-smokers (p-value < 0.0001; Table V and Figure 1).

For planktonic and total absorbance there were significant differences between smoker and non-smoker isolates at all nicotine concentrations (Figure 2 and Figure 3). Specifically, there were significant differences of the planktonic cells between smoker and non–smoker isolates (Figure 2). Non-smoker isolates had significantly higher planktonic cell absorbances at nicotine concentrations 0 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 4 mg/ml, and 32 mg/ml compared with smoker isolates ($p < 0.0001$; Table V and Figure 2). Non-smoker isolates had significantly more total absorbance at all nicotine concentrations compared to smokers ($p < 0.0001$; Table V and Figure 3).

The smoking history of the smokers was also considered. While the biofilm formation of smoker isolates was significantly higher compared with the biofilm formation of non-smoker isolates at all nicotine concentrations, interestingly, smoker biofilm formation was dose-dependent up to 8.0 mg/ml of nicotine and smoking subjects had an average of smoking 8 cigarettes per day for an average of 34 years with a 14.5 pack years (Table I.). This indicates that biofilm formation increases with increases in nicotine concentration and the smoking history of subjects. However, only one correlation was statistically significant – a negative correlation for the 0.25 mg/ml nicotine concentration for experiment 3. Several other correlations showed some relation, although they did not reach statistical significance: 0 nicotine concentration for experiment 1; 0.25 mg/ml nicotine concentration for the average across experiments; 0.5 mg/ml nicotine concentration for experiment 1; 4 mg/ml nicotine concentration for

experiments 2 and 3, and the 16 mg/ml nicotine concentration for experiment 3 (Table V).

TABLES AND FIGURES

TABLE I

Average demographic factors of smoking and non-smoking human subjects

Demographic Factors	**Smokers n=10**	**Non-Smokers n=10**
Gender	F=1 M=9	F=4 M=6
Average Age	40.5 years old	42.0 years old
Race	White=1 African American=9	White=5 African American=5
Average: **Pack Years**	(8.5 cigarette per day/20) x 34.3 year smoking history = 14.5 pack years	

TABLE II

Individual demographic factors of oral washes
from smoking and non-smoking human subjects

IUPUI- ID #	Race	Sex	Age	Smoker	Smoking History	Pack Years
891084OR01	W[1]	M	52	No		
891080OR02	W	M	32	No		
891086OR01	AA[2]	F	38	No		
891090OR01	AA	M	37	NO		
891087OR01	AA	M	40	No		
891085OR01	W	M	22	Yes	½ PPD[3]~4 years	3 years
891091OR01	W	M	52	No		
891088OR01	AA	F	42	No		
891089OR01	W	M	35	No		
891092OR01	W	F	56	No		
005017OR01	AA	M	54	Yes	1 PPD~36 years	36 years
005022OR01	AA	M	43	Yes	3 cig PD[4]~42 years	6.3 years
005016OR01	AA	F	46	No		
005009OR01	AA	M	53	Yes	1 PPD~32 years	32 years

[1] White
[2] African American
[3] Pack Per Day
[4] Per Day

(continued)

TABLE II (cont.)						
005010OR01	AA	F	48	Yes	8 cig PD~30 years	12 years
005011OR01	AA	M	51	Yes	1 PPD~37 years	37 years
005020OR01	AA	M	53	Yes	15 cig PD~34 years	25.5 years
005021OR01	AA	M	57	Yes	½ PPD~43 years	22.5 years
005024OR01	AA	M	58	Yes	1 PPD~41 years	41 years
005025OR01	AA	M	59	Yes	½ PPD~44 years	22 years
IUPUI- ID #	Race	Sex	Age	Smoker	Smoking History	Pack Years
891084OR01	W[5]	M	52	No		
891080OR02	W	M	32	No		
891086OR01	AA[6]	F	38	No		
891090OR01	AA	M	37	NO		
891087OR01	AA	M	40	No		
891085OR01	W	M	22	Yes	½ PPD[7]~4 years	3 years
891091OR01	W	M	52	No		
891088OR01	AA	F	42	No		
891089OR01	W	M	35	No		
891092OR01	W	F	56	No		

[5] White
[6] African American
[7] Pack Per Day

(continued)

TABLE II (cont.)						
005017OR01	AA	M	54	Yes	1 PPD~36 years	36 years
005022OR01	AA	M	43	Yes	3 cig PD[8]~42 years	6.3 years
005016OR01	AA	F	46	No		
005009OR01	AA	M	53	Yes	1 PPD~32 years	32 years
005010OR01	AA	F	48	Yes	8 cig PD~30 years	12 years
005011OR01	AA	M	51	Yes	1 PPD~37 years	37 years
005020OR01	AA	M	53	Yes	15 cig PD~34 years	25.5 years
005021OR01	AA	M	57	Yes	½ PPD~43 years	22.5 years
005024OR01	AA	M	58	Yes	1 PPD~41 years	41 years
005025OR01	AA	M	59	Yes	½ PPD~44 years	22 years

[8] Per Day

TABLE III

.

Significance between biofilm/planktonic/total growth absorbances and nicotine concentrations/smoking/both smoking and nicotine concentrations of *S. mutans* isolates from smokers and non-smokers treated with nicotine concentrations

Analysis		
Measurement	**Effect**	**Rank p-value**
Biofilm **Measurement**	Nicotine Dilution	[9]<0.0001
	Smoking	<0.0001
	Smoking*Nicotine Dilution	<0.0001
Planktonic	Nicotine Dilution	<0.0001
	Smoking	<0.0001
	Smoking*Nicotine Dilution	<0.0001
Total Absorbance	Nicotine Dilution	<0.0001
	Smoking	<0.0001
	Smoking*Nicotine Dilution	<0.0001

[9] Biofilm/planktonic/total growth of all strains are significantly affected by different nicotine dilutions and smoking, and both different nicotine dilutions and smoking.

TABLE IV

Significance (p-value) between biofilm/planktonic/total growth at different nicotine concentrations

Biofilm	0	0.25	0.5	1	2	4	8	16	32
0		NSD	NSD	[10]0 < 1	0 < 2	0 < 4	0 < 8	0 > 16	0 > 32
0.25	0.4406		NSD	NSD	NSD	0.25 < 4	0.25 < 8	0.25 > 16	0.25 > 32
0.5	0.1656	0.5382		NSD	NSD	NSD	NSD	0.5 > 16	0.5 > 32
1	[11]0.0152	0.0972	0.2969		NSD	NSD	NSD	1 > 16	1 > 32
2	0.0373	0.1894	0.4859	0.7291		NSD	NSD	2 > 16	2 > 32
4	0.0013	0.0149	0.0686	0.4365	0.2609		NSD	4 > 16	4 > 32
8	0.0011	0.0127	0.0604	0.4035	0.2373	0.9543		8 > 16	8 > 32
16	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		16 > 32
32	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0311	

Planktonic	0	0.25	0.5	1	2	4	8	16	32
0		0 < 0.25	0 < 0.5	0 < 1	0 < 2	0 < 4	0 < 8	NSD	0 > 32
0.25	0.0332		NSD	0.25 < 1	0.25 < 2	0.25 < 4	0.25 < 8	NSD	0.25 > 32
0.5	0.0019	0.3250		NSD	NSD	0.5 < 4	0.5 < 8	0.5 > 16	0.5 > 32
1	<0.0001	0.0126	0.1309		NSD	NSD	1 < 8	1 > 16	1 > 32
2	<0.0001	0.0239	0.2021	0.8142		NSD	2 < 8	2 > 16	2 > 32
4	<0.0001	0.0001	0.0044	0.1798	0.1150		NSD	4 > 16	4 > 32
8	<0.0001	<0.0001	<0.0001	0.0083	0.0040	0.1934		8 > 16	8 > 32
16	0.4014	0.1967	0.0229	0.0002	0.0004	<0.0001	<0.0001		16 > 32
32	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	

Total Absorbance	0	0.25	0.5	1	2	4	8	16	32
0		0 < 0.25	0 < 0.5	0 < 1	0 < 2	0 < 4	0 < 8	0 > 16	0 > 32
0.25	0.0004		NSD	0.25 < 1	0.25 < 2	0.25 < 4	0.25 < 8	0.25 > 16	0.25 > 32
0.5	0.0005	0.9721		0.5 < 1	0.5 < 2	0.5 < 4	0.5 < 8	0.5 > 16	0.5 > 32
1	<0.0001	0.0473	0.0435		1 < 2	1 < 4	1 < 8	1 > 16	1 > 32
2	<0.0001	<0.0001	<0.0001	0.0362		2 < 4	NSD	2 > 16	2 > 32
4	<0.0001	<0.0001	<0.0001	<0.0001	0.0127		NSD	4 > 16	4 > 32
8	<0.0001	<0.0001	<0.0001	0.0006	0.1828	0.2456		8 > 16	8 > 32
16	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		16 > 32
32	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	

[10] Biofilm/planktonic/ total growth of all strains had significantly more growth at 1, 2, 4, and 8 mg/ml nicotine concentration compared to zero nicotine.

[11] Biofilm.planktonic/total growth of all strains had significant differences in growth between all nicotine concentrations.

TABLE V

Significance (p-value) between of *S. mutans* isolates from smokers and non-smokers treated with various nicotine concentrations

Smokers Versus Non-Smokers (Rank p-values)			
Nicotine Dilution	**Biofilm**	**Planktonic**	**Total Absorbance**
0	[12]<0.0001	[13]<0.0001	<0.0001
0.25	0.0001	0.0002	<0.0001
0.5	<0.0001	<0.0001	<0.0001
1	<0.0001	0.0013	<0.0001
2	<0.0001	0.0003	<0.0001
4	<0.0001	0.0002	<0.0001
8	<0.0001	<0.0001	<0.0001
16	0.0086	<0.0001	<0.0001
32	0.7353	0.0016	0.0493

[12] Smoker isolates had significantly higher biofilm formation than non-smoker isolates.

[13] Non-smoker isolates had significantly higher planktonic and total absorbance growth than smoker isolates.

TABLE VI

Pairwise comparisons of dilution, by smoking status

	Non-Smokers				Smokers			
Measurement	**Dilution Comparison**			**Rank p-value**	**Dilution Comparison**			**Rank p-value**
Biofilm	0	>	32	<0.0001	0	>	32	<0.0001
	0	>	16	<0.0001	0	>	16	<0.0001
	0	>	8	0.0079	0	>	8	0.0355
	0	>	4	0.0092	0	>	4	0.0386
	0	NSD*	2	0.1853	0	NSD*	2	0.1069
	0	NSD*	1	0.2924	0	>	1	0.0251
	0.25	>	32	<0.0001	0.25	>	32	<0.0001
	0.25	>	16	<0.0001	0.25	>	16	<0.0001
	0.25	NSD*	8	0.1263	0.25	>	8	0.0487
	0.25	NSD*	4	0.1392	0.25	NSD*	4	0.0528
	0.5	>	32	<0.0001	0.5	>	32	<0.0001
	0.5	>	16	<0.0001	0.5	>	16	<0.0001
	1	>	32	<0.0001	1	>	32	<0.0001
	1	>	16	<0.0001	1	>	16	<0.0001
	2	>	32	<0.0001	2	>	32	<0.0001
	2	>	16	<0.0001	2	>	16	<0.0001
	4	>	32	<0.0001	4	>	32	<0.0001
	4	>	16	<0.0001	4	>	16	<0.0001
	8	>	32	<0.0001	8	>	32	<0.0001
	8	>	16	<0.0001	8	>	16	<0.0001
	16	NSD*	32	0.9617	16	>	32	0.0085
Planktonic	0	>	32	<0.0001	0	>	32	0.0119
	0	>	8	<0.0001	0	>	8	0.0001
	0	>	4	<0.0001	0	>	4	0.0001
	0	>	2	0.0008	0	>	2	0.0027
	0	>	1	0.0010	0	>	1	0.0009
	0	>	0.5	0.0063	0	NSD*	0.5	0.0620
	0	NSD*	0.25	0.1509	0	NSD*	0.25	0.1119
	0.25	>	32	<0.0001	0.25	>	32	<0.0001
	0.25	>	8	<0.0001	0.25	>	8	0.0139
	0.25	>	4	0.0009	0.25	>	4	0.0193
	0.25	NSD*	2	0.0567	0.25	NSD*	2	0.1583
	0.25	NSD*	1	0.0635	0.25	NSD*	1	0.0825
	0.5	>	32	<0.0001	0.5	>	32	<0.0001
	0.5	NSD*	16	0.2323	0.5	>	16	0.0002
	0.5	>	8	<0.0001	0.5	>	8	0.0291
	0.5	>	4	0.0441	0.5	>	4	0.0391
	1	>	32	<0.0001	1	>	32	<0.0001
	1	NSD*	16	0.5247	1	>	16	<0.0001
	1	>	8	0.0005	1	NSD*	8	0.4697
	2	>	32	<0.0001	2	>	32	<0.0001
	2	NSD*	16	0.5575	2	>	16	<0.0001
	2	>	8	0.0005	2	NSD*	8	0.2943
	4	>	32	<0.0001	4	>	32	<0.0001
	4	NSD*	16	0.4124	4	>	16	[14]<0.0001

[14] Biofilm/planktonic/total growth of smoker isolates was significantly different between different nicotine concentrations

(continued)

	Non-Smokers				Smokers			
Measurement	**Dilution Comparison**			**Rank p-value**	**Dilution Comparison**			**Rank p-value**
	8	>	32	[15]<0.0001	8	>	32	<0.0001
	8	>	16	0.0041	8	>	16	<0.0001
	16	>	32	<0.0001	16	NSD*	32	0.4766
Total Absorbance	0	>	32	<0.0001	0	>	32	<0.0001
	0	NSD*	16	0.1069	0	>	16	<0.0001
	0	>	8	<0.0001	0	>	8	<0.0001
	0	>	4	<0.0001	0	>	4	<0.0001
	0	>	2	<0.0001	0	>	2	<0.0001
	0	>	1	<0.0001	0	>	1	0.0020
	0	>	0.5	0.0001	0	NSD*	0.5	0.1108
	0	>	0.25	<0.0001	0	NSD*	0.25	0.1570
	0.25	>	32	<0.0001	0.25	>	32	<0.0001
	0.25	>	16	<0.0001	0.25	>	16	<0.0001
	0.25	>	8	<0.0001	0.25	>	8	0.0002
	0.25	>	4	<0.0001	0.25	>	4	0.0001
	0.25	>	2	0.0041	0.25	>	2	0.0027
	0.25	NSD*	1	0.2921	0.25	NSD*	1	0.0927
	0.5	>	32	<0.0001	0.5	>	32	<0.0001
	0.5	>	16	<0.0001	0.5	>	16	<0.0001
	0.5	>	8	<0.0001	0.5	>	8	0.0003
	0.5	>	4	<0.0001	0.5	>	4	0.0003
	0.5	>	2	0.0013	0.5	>	2	0.0047
	0.5	NSD*	1	0.1626	0.5	NSD*	1	0.1331
	1	>	32	<0.0001	1	>	32	<0.0001
	1	>	16	<0.0001	1	>	16	<0.0001
	1	>	8	0.0023	1	>	8	0.0351
	1	>	4	<0.0001	1	>	4	0.0306
	1	NSD*	2	0.0696	1	NSD*	2	0.1857
	2	>	32	<0.0001	2	>	32	<0.0001
	2	>	16	<0.0001	2	>	16	<0.0001
	2	>	4	0.0010	2	NSD*	4	0.4010
	4	>	32	<0.0001	4	>	32	<0.0001
	4	>	16	<0.0001	4	>	16	<0.0001
	8	>	32	<0.0001	8	>	32	<0.0001
	8	>	16	<0.0001	8	>	16	<0.0001
	16	>	32	<0.0001	16	NSD*	32	0.7394

[15] Biofilm/planktonic/total growth of non-smoker isolates was significantly different between different nicotine concentrations.

TABLE VII

Calculations of correlation coefficients and p-values to evaluate the association of pack years smoked with biofilm formation at different nicotine concentrations of smoker *S. mutans* isolates

Nicotine Concentration (mg/ml)	Experiment 1		Experiment 2		Experiment 3		Average	
	CO	p-value	CO	p-value	CO	p-value	CO	p-value
0	-0.31	0.400	-0.24	0.517	0.30	0.410	-0.27	0.462
0.25	-0.18	0.633	-0.28	0.452	-0.76	0.008[16]	-0.55	0.099
0.5	-0.38	0.288	-0.27	0.462	-0.17	0.645	-0.27	0.462
1	-0.20	0.598	-0.22	0.563	-0.17	0.645	-0.20	0.598
2	-0.18	0.633	-0.26	0.473	-0.18	0.633	-0.18	0.633
4	-0.13	0.731	-0.41	0.254	-0.33	0.370	-0.26	0.473
8	-0.14	0.706	-0.28	0.441	-0.06	0.870	-0.13	0.731
16	-0.02	0.948	0.01	0.974	-0.32	0.380	-0.05	0.896
32	-0.20	0.586	-0.18	0.633	-0.07	0.858	-0.16	0.669

[16] There was a significant correlation (p-value) between the number of pack years smoked and biofilm formation of *S. mutans* isolates of smokers at a 0.25 mg/ml nicotine concentration.

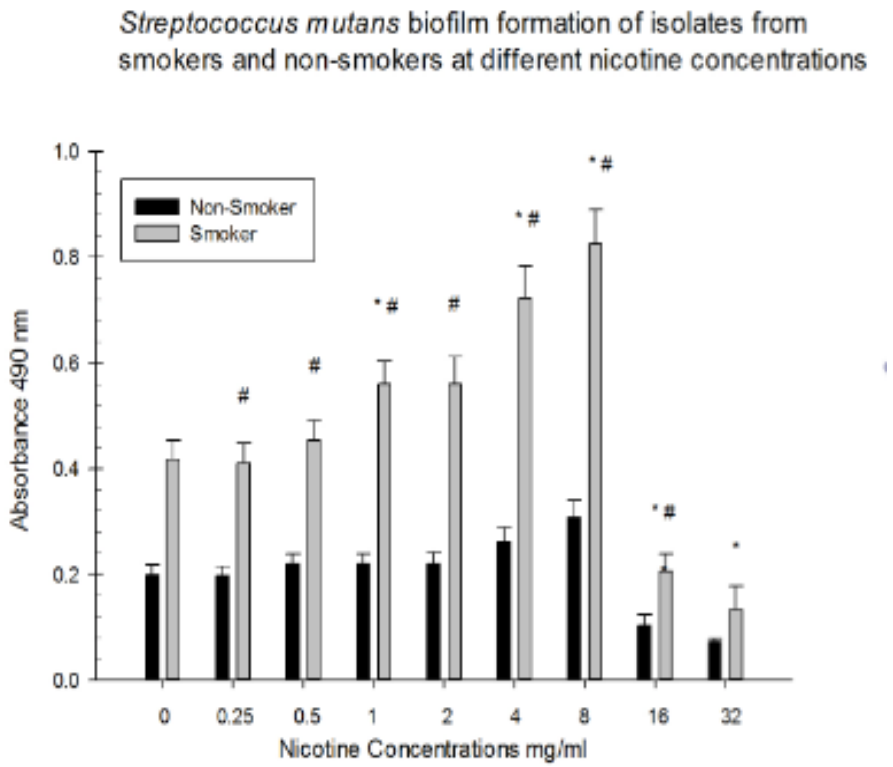

FIGURE 1. Asterisks indicate significant differences between *S. mutans* biofilm formation of isolates from smokers/non-smokers at different nicotine concentrations and the zero nicotine concentration. The # indicates significant differences between *S. mutans* biofilm formation of isolates from smokers and non-smokers at different nicotine concentrations.

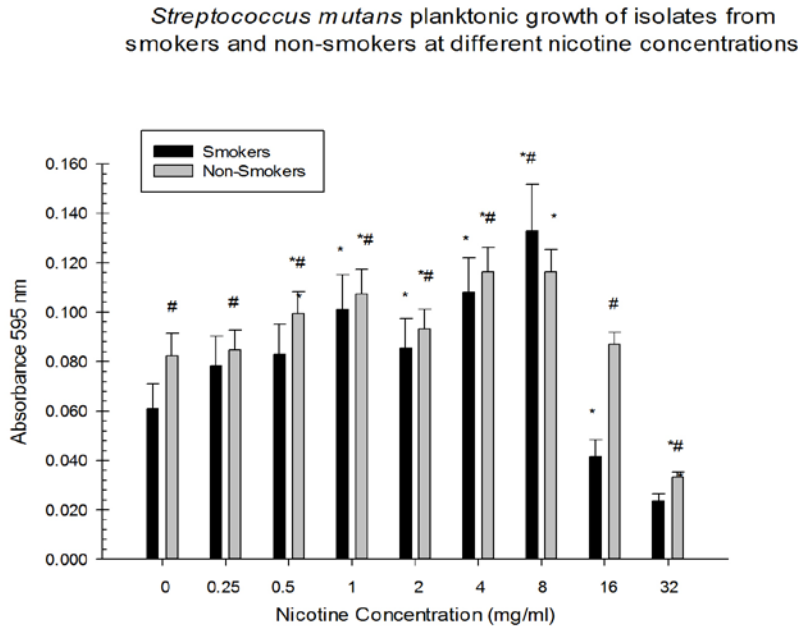

FIGURE 2. Asterisks indicate significant differences between *S. mutans* planktonic growth of isolates from smokers/non-smokers at different nicotine concentrations and the zero nicotine concentration. The # indicate significant differences between *S. mutans* planktonic growth of smokers/ non-smokers at different nicotine concentrations.

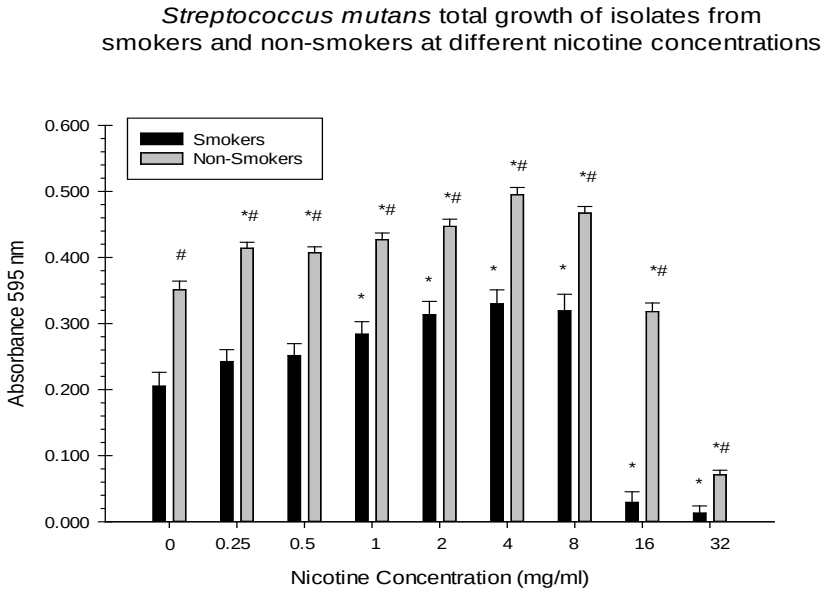

FIGURE 3. Asterisks indicate significant difference between total growth of *S. mutans* isolates from smokers/non-smokers at different nicotine concentrations and the zero nicotine concentration.
#s indicate significant differences between total growth of *S. mutans* isolates of smokers/non-smokers at different nicotine concentrations.

DISCUSSION

To determine the effect of smoking history and the addition of nicotine on the formation of *S. mutans* biofilm, planktonic cells, and total growth *in vitro, S. mutans* isolates from smokers and non-smokers were compared in this present study. To date, this is the first study that compares the effect of nicotine on both smoker and non-smoker isolates.

In this study, nicotine enhanced biofilm growth in both *S. mutans* smoker and non-smoker isolates. Biofilm formation increased in a dose-dependent manner up to 8.0 mg/ml nicotine in both smoking and non-smoking oral strains. Smoker isolates incubated with most of the nicotine concentrations produced significantly more biofilm compared with the non-smoker isolates. However, the total growth of the non-smoking isolates was significantly greater than smoker isolates at 8 mg/ml. The study also indicated that nicotine had an antibacterial effect on both smoking and non-smoking isolates in large concentrations (16 mg/ml to 32 mg/ml).

Tobacco use is a behavioral risk factor that adversely affects oral health and is directly linked to many common life-threatening diseases such as cancer.[30,31] More interestingly, it has been shown that environmental tobacco smoke (second-hand smoking) is responsible for several systemic diseases and the increased risk of dental caries in children.[41] Tobacco is significantly associated with periodontal disease, tooth loss, and dental caries. Also, it has been shown that smokers with a high prevalence of dental caries exhibit lower concentrations of sIgA compared with non-smokers.[46]

In this study, there was a significant effect of the addition of nicotine on the growth of biofilm and planktonic cells, and the total growth of all *S. mutans* isolates. This is consistent with a previous *in-vitro* study from this laboratory suggesting that as the nicotine concentration in the cultures increase, there is an increase in *S. mutans* growth.[48] Another previous *in-vitro* study from this laboratory reported that biofilm formation and metabolism of *S. mutans* increased in a dose-dependent manner up to 16.0 mg/ml of nicotine. Planktonic cell growth was highest between 2 mg/ml to 8 mg/ml nicotine. The majority of *S. mutans* isolates measured a MIC (maximum inhibitory concentration) of 16 mg/ml nicotine, MBC (maximum bactericidal concentration) of 32 mg/ml nicotine, and MBIC (maximum biofilm inhibitory concentration) of 16 mg/ml nicotine.[51] The results of the present study demonstrated that the growth of *S. mutans* smoker and non-smoker isolates increased in a dose-dependent manner up to 8 mg/ml of nicotine. Nonetheless, smoker isolates demonstrated a greater amount of biofilm formation compared with non-smoker isolates particularly at 8 mg/ml of nicotine. On the other hand, the biofilm of both smoker and non-smoker isolates were inhibited at 16 mg/ml nicotine concentration. Also, there was a significant difference in biofilm formation between smoker and non-smoker isolates at almost all nicotine concentrations. There was a more significant increase in biofilm formation of smoker isolates at 1 mg/ml, 4 mg/ml, 8 mg/ml, 16 mg/ml and 32 mg/ml compared with biofilm at the zero nicotine concentration. In this study, it was clear that *S. mutans* isolates from smokers are more influenced by high nicotine concentrations (up to 16 mg/ml) than non-smokers.

In addition, this study indicated that planktonic cell growth was greater in non-smoking isolates at all nicotine concentration compared with the planktonic cell growth

of smoker isolates at the same nicotine concentrations. Furthermore, there was a significant difference in planktonic cell growth and total growth between smoker and non-smoker isolates at all nicotine concentrations. The possible mechanism of nicotine on enhancement of biofilm growth of all *S. mutans* strains tested in this present study can be explained by a recent study done by Huang et al. that demonstrated the effect of nicotine on the expression of Gbps and Gtfs genes.[50] Interestingly enough, it was found that nicotine up-regulates the expression of Gbps and Gtfs genes of *S. mutans* planktonic cells and down-regulates Gbps and Gtfs of *S. mutans* biofilm cells. Thus, an increase of planktonic cell attachment to a biofilm surface results in an increased growth of biofilm.[50]

In this study, overall there was not a significant relation between the number of pack years smoked and biofilm formation of *S. mutans* isolates at all nicotine concentrations. However, 0.25 mg/ml of nicotine in experiment three demonstrated a significant correlation between the number of years smoked and bacterial biofilm formation.

The present study hypothesized that nicotine produces significant differences in biofilm formation of smoker and non-smoker *S. mutans* isolates. According to the study results, this hypothesis was validated. Biofilm formation was significantly increased in *S. mutans* smoker isolates at large nicotine concentrations compared with non-smoker *S. mutans* isolates.

The rationale for this hypothesis was derived from preliminary data from a study done in our laboratory (personal communication) indicating that *S. mutans* can become tolerant to increased nicotine concentrations and that this tolerance appears to be stable. This may allow smoker isolates to be able to respond more vigorously to higher nicotine

concentrations than non-smoker isolates. This preliminary study suggested that *S. mutans* becomes adapted with stable resistance at high nicotine concentrations by some type of mutation and possible stable up-regulation of antigen I/II. This resistance was maintained after it had been passed at least three times in 0 mg/mL nicotine (unpublished, personal communication).

The use of nicotine products increases the growth of *S. mutans* and may place tobacco users at risk for dental decay. Moreover, we speculate that subjects with a long smoking history may be at more risk of tooth decay than non-smokers. More analysis is required to determine the exact mechanism of nicotine on growth enhancement of *S. mutans* isolates.

In conclusion, nicotine increases biofilm formation of both smoker and non-smoker *S. mutans* isolates in a dose-dependent manner. However, *S. mutans* smoker isolates are more affected by large nicotine concentrations than non-smoker isolates.

SUMMARY AND CONCLUSION

Dental decay is a complex disease associated with the presence of *S. mutans*.[52] This endogenous oral microorganism adheres to tooth surfaces, grows and develops into micro-communities that mature and form dental biofilm.[53] Development of cariogenic biofilm is one of the major factors associated with the tooth decay process.[2] The use of tobacco is considered a great risk factor for oral diseases.[54] Several studies demonstrated the association of tooth decay and the use of tobacco as a first-hand or second-hand smoke. It was found that nicotine increases the metabolism of *S. mutans* and the growth of dental biofilm in a dose-related manner up to 16 mg/ml.[55] The investigation of the effects of nicotine on smoker and non-smoker *S. mutans* isolates provided us with information that high nicotine concentrations can enhance more biofilm formation in smoker isolates more than non-smoker isolates. The understanding of the effects of nicotine on biofilm formation of smoker versus non-smoker *S. mutans* isolates can lead to development of more effective strategies for prevention of dental plaque and tooth decay development in smokers. Also, it can lead to further investigations in the types of mechanisms that these strains use to tolerate high nicotine concentrations.

Conclusion: These results suggest that there may be increased formation of dental caries in smokers than non-smokers because of the significant increase of biofilm formation in the *S. mutans* smoker isolates compared with non-smoker *S. mutans* isolates. The use of nicotine products increases the growth of *S. mutans* and may place tobacco users at risk for dental decay.

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ABSTRACT

EFFECT OF NICOTINE ON BIOFILM FORMATION OF *STREPTOCOCCUS MUTANS* ISOLATES FROM SMOKING VERSUS NON-SMOKING SUBJECTS

by

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Tooth decay is a complex dieto-bacterial disease with an association of social, behavioral and biological factors. *Streptococcus mutans* plays a major role in tooth decay. This endogenous oral microorganism adheres to tooth surfaces and grows and develops into micro-communities that mature and form dental biofilm. Development of cariogenic biofilm is one of the major factors associated with the tooth decay process. The use of tobacco is considered a great risk factor for oral diseases. Several studies demonstrated

the association of tooth decay and the use of tobacco as effects of first-hand or second-hand smoking. Nicotine has been reported to increase the biofilm growth and metabolism of *S. mutans* in a dose-dependent manner up to 16 mg/ml of nicotine. However, its effects on biofilm formation of *S. mutans* strains isolated from smokers are not known and should be investigated. Therefore, we proposed the use of an *in-vitro* model to better understand the effects of nicotine on biofilm formation of strains of *S. mutans* isolates from smokers versus non-smoking subjects.

Objectives: To investigate the effects of nicotine on biofilm formation of *S. mutans* isolates from oral washes of smoker and non-smoker human subjects.

Materials and Methods: This study was conducted using three *S. mutans* isolates collected from oral washes of 10 smoking subjects and 10 non-smoking subjects. The oral wash samples were stored at -80^{o}C before *S. mutans* isolation. *S. mutans* isolates were obtained by plating on Mitis Salivarius Sucrose Bacitracin plates and species identity confirmed by carbohydrate fermentation assays. Nicotine from Sigma-Aldrich (St. Louis, MO, USA) was used. Biofilm was formed by overnight culturing of each *S. mutans* strain (10 μ l) in 190 μ l of tryptic soy broth (TSB) supplemented with 1.0-percent sucrose (TSBS) containing 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 of nicotine for 24 hours in 5.0-percent CO_2 at 37^{o}C in sterile (8 x 12) 96-well microtiter plates (Fisher Scientific, Newark, DE, USA). The absorbance values of biofilm were measured at 490 nm in a microplate spectrophotometer (SpectraMax 190; Molecular Devices, SunnyVale, CA, USA) after crystal violet staining.

Null Hypotheses: 1) Nicotine will not increase biofilm formation in both smoker and non-smoker *S. mutans* isolates. 2) An increase in nicotine concentrations will not increase biofilm *f*ormation in both smoker and non-smoker *S. mutans* isolates in a dose-dependent manner. 3) Nicotine will not produce si*gnificant* differences in biofilm formation between smoker and non-smoker *S. mutans* isolates.

Alternative Hypotheses: 1) Nicotine increases the growth of biofilm formation in both smoker and non-smoker *S. mutans* isolates. 2) An increase in nicotine concentrations increase biofilm formation of both smoker and non-smoker *S. mutans* isolates in a dose-dependent manner. 3) However, nicotine increases biofilm formation of smoker *S. mutans* strains more than non-smoker *S. mutans* isolates. The rationale for this hypothesis is that our preliminary data indicated that *S. mutans* can become resistant to increased nicotine concentrations and that this resistance appears to be stable and may allow the smoker isolates to be able to respond more vigorously to higher nicotine concentrations than the non-smoker isolates.

Results: There was a significant effect ($p < 0.05$) of both nicotine concentrations and smoking on the growth of biofilm, planktonic cells, and total absorbance, for all strains of *S. mutans* ($p < 0.0001$). Isolates from smokers had significantly more biofilm at 0 mg/ml to 16 mg/ml of nicotine compared with those from non-smokers (p-value < 0.0001).

Conclusion: *S. mutans* smoker isolates are more affected by high nicotine concentrations than non-smoker isolates.

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