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Original Article

Nicotine is a potent extracellular polysaccharide inducer in *Fusobacterium nucleatum* biofilms

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Aim: The purpose of this in vitro study was to analyze the influence of nicotine on the extracellular polysaccharides in *Fusobacterium nucleatum* biofilm. **Methods:** *F. nucleatum* (ATCC 10953) biofilms supplemented with different concentrations of nicotine (0, 0.5, 1, 2, 4, and 8 mg/mL) were grown in two different BHI broth conditions [no sucrose and 1% sucrose]. Extracellular polysaccharides assay, pH measurements, and a spectrophotometric assay were performed. Data were submitted for ANOVA and Tukey honestly significant difference analyses (HSD) tests (α =.05). **Results:** Extracellular polysaccharides synthesis was influenced by an interaction between nicotine concentrations and growth medium solution containing sucrose (P<.05). The pH values declined in the sucrose-exposed biofilm were greater than in the group exposed only to nicotine (P<.05). The biofilm exposed to sucrose and nicotine had a higher total biofilm growth (P<.05) than the nicotine-treated biofilm without sucrose. **Conclusions:** Regardless of sucrose exposure, biofilms exposed to different nicotine concentrations influenced the amount of extracellular polysaccharides.

Keywords: Biofilms. Nicotine. Polysaccharides, bacterial.

Introduction

Periodontitis is a bacteria-induced persistent inflammatory disease characterized by the progressive destruction of the tooth-supporting tissues. In particular, *Fusobacterium nucleatum* (*F. nucleatum*), a gram-negative anaerobe, is considered an important pathogen in periodontitis, due to its enhanced prevalence within the subgingival biofilm[1]. The major avoidable risk factor in the initiation and progression of periodontal diseases is smoking, which has been shown to increase susceptibility to destructive disease and promocolonization by several pathogens, including *Porphyromonas gingivalis* and *F. nucleatum*[2,3].

Nicotine is the main bioactive component of tobacco products. It is a potent chemical component with strong tobacco-dependence development properties[4]. Therefore, users of tobacco products are subject to exposure to high concentrations of nicotine, which can lead to the development of a variety of disorders[4,5]. Studies have also demonstrated the destructive effects of smoking on oral health. In a clinical study, Albandar et al. observed that smokers had a higher prevalence of moderate and severe periodontitis and a higher prevalence and extent of attachment loss and gingival recession than non-smokers, suggesting poorer periodontal health in smokers[6]. In another recent clinical study, Kanmaz et al. observed that smokers with periodontitis stage III and IV, Grade C respond well to non-surgical periodontal treatment during the 6-month follow-up. However, smokers exhibit faster repopulation of Gram-negative bacteria[7].

Furthermore, previous studies demonstrated nicotine is also associated with increased metabolism and biofilm formation[8-10]. In addition, the extracellular matrix components are essential to building and maintaining the bacterial community. At the same time, extracellular polysaccharides (EPS) are crucial compounds in the biofilm matrix[11].

During biofilm formation, bacteria produce EPS to form a matrix for the bacteria. EPS is essential for bacterial adhesion–cohesion[12], and is responsible for the structural and functional integrity of commonly termed "mature" biofilms[13]. EPS also serves as a local sugar reservoir that can be fermented to acids[12,14]. In addition, EPS essentially determines the pathogenicity and coherence of biofilms[15]. Therefore, the increased EPS synthesis induced by nicotine may increase smokers' caries risk and periodontal diseases.

Although the link between nicotine and increased biofilm development has been extensively documented, there have been no publications on the influence of nicotine on EPS generation in *F. nucleatum* biofilms. For this reason, the present study investigated the influence of different nicotine concentrations on EPS synthesis within *F. nucleatum* biofilm formation grown with and without sucrose.

Materials and methods

Bacterial Growth Conditions

F. nucleatum (ATCC 10953) was grown in 5 mL of brain-heart infusion broth (BHI) supplemented with yeast extract, 5% vitamin K, and hemin under anaerobic conditions

overnight at 37°C. After 24 h of culture, bacteria were collected by centrifugation at 6000 *g* for 10 min at 4°C, washed twice, and resuspended in 0.9% NaCl. The absorbance of a final suspension of cells/mL was adjusted to 1.0 using a spectrophotometer (SpectraMax 190; Molecular Devices, Inc., Sunnyvale, CA) at 600 nm[16]. Afterward, 300 µL of the *F. nucleatum* cell suspension was grown in two different supplemented BHI broth conditions. Primarily in 2.7 mL of fresh BHI broth. Secondly, in 2.7 mL of fresh BHI broth supplemented with 1% sucrose. Finally, in both BHI broth conditions, different concentrations of nicotine (0, 0.5, 1, 2, 4, and 8 mg/mL) were added to the growth media[17]. The inoculated growth media were transferred to different 6-well plates and incubated under anaerobic conditions at 37º C for 48 h.

EPS assay

For the quantification of polysaccharide concentration, the phenol-sulfuric acid colorimetric method was used[18]. Biofilm was scraped from the bottom of each well into 1 mL of sterile saline, and the biofilm suspension was mixed; then, an aliquot of 50 µL of the concentrated biofilm of each well was transferred to a 96-well microtiter plate. For each sample, 150 µL of concentrated sulfuric acid was added. Immediately after that, 30 µL of a 5 % phenol solution was added to the mixture and heated to 90°C in a hot water bath for 5 min. After cooling the plate at room temperature for 5 min, the absorbance was measured at OD 750nm[19].

pH measurements

At various times during the incubation period, biofilm was scraped from the bottom of each well into 1 mL of sterile saline, and the solution was mixed. Following, 1 mL of the concentrated biofilm of each well was transferred to 24-well plates for the pH measurement. The pH of the culture medium was measured inside each well with a pH meter (Fisher Scientific, accumet AB15 pH meter, San Diego, CA, USA) after each incubation period. Before each test session, the pH electrode was calibrated using a buffer solution at pH 7[20].

Spectrophotometric assay

At various times during the incubation period, biofilm was scraped from the bottom of each well into 1 mL of sterile saline, and the suspension was mixed. Immediately after that, 100 µL of the concentrated biofilm of each well was transferred to wells of a 96-well flat-bottom microtiter plate. The total growth of *F. nucleatum* biofilm was measured using a multi-well spectrophotometer plate reader (SpectraMax 190; Molecular Devices, Inc., Sunnyvale, CA) at 600 nm[10].

Statistical analysis

For the statistical analysis, the normality of errors (Shapiro-Wilk test) and homogeneity of variances (Levene test) were evaluated for each response variable. Two-way ANOVA was used to evaluate the effect of growth media type (2 levels) and nicotine concentration (6 levels) in the dependent variables. Tukey Honestly Significant Difference (HSD) posthoc test was used to determine the significance level between groups at a 95% confidence interval (SPSS v. 20.0; SPSS Inc).

Results

Measurement of EPS

Regarding the EPS assay, the type of growth medium influenced the biofilm formation. There were significant effects of all nicotine concentrations and BHI sucrose medium on the biofilm growth of *F. nucleatum*. There was a significant interaction between nicotine concentrations for biofilm containing sucrose in the BHI solution as well as BHI without sucrose. EPS analysis exhibited significantly more EPS at 0-8 mg/mL nicotine with 1% sucrose compared to those without sucrose ($p<0.5$). However, EPS synthesis at 2 mg/mL of nicotine concentration showed no difference between the biofilm solutions with or no sucrose. In relation to the BHI growth medium without sucrose, cultures demonstrated increased EPS values with increasing nicotine concentrations at 0 to 2 mg/mL of nicotine. On the other hand, at 4 and 8 mg/mL of nicotine, EPS values decreased with the increased nicotine concentration. Nonetheless, EPS concentrations at 1 and 2 mg/mL of nicotine were similar and differed from other nicotine concentrations. As regards the BHI with 1% sucrose, 0 and 0.5 mg/ml were similar and exhibited differences from other nicotine concentrations (Figure 1).

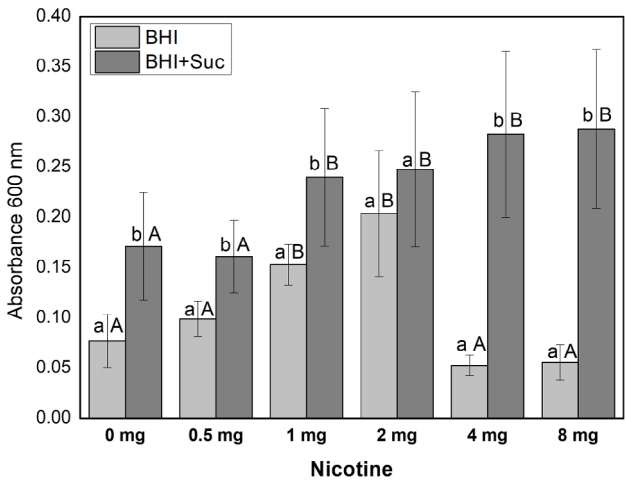

Figure 1. EPS extracted from *F. nucleatum* biofilm grown in two different BHI broth conditions (0 and 1% sucrose) containing different nicotine concentrations. Different media types in the same nicotine concentration are compared using lowercase letters. Different nicotine concentrations within the same medium type are compared using upper case letters. ($p<.05$; 2-way ANOVA and Tukey HSD test).

Measurement of pH

The pH values in the sucrose-exposed nicotine-treated biofilm were lower than in the group exposed only to nicotine without sucrose ($p<.05$). The biofilms exposed to the medium with sucrose had no difference in pH values between them. A higher concentration of nicotine (8 mg/mL) demonstrated the highest pH value among the groups exposed to BHI medium without sucrose than the other groups ($p<.05$) (Figure 2).

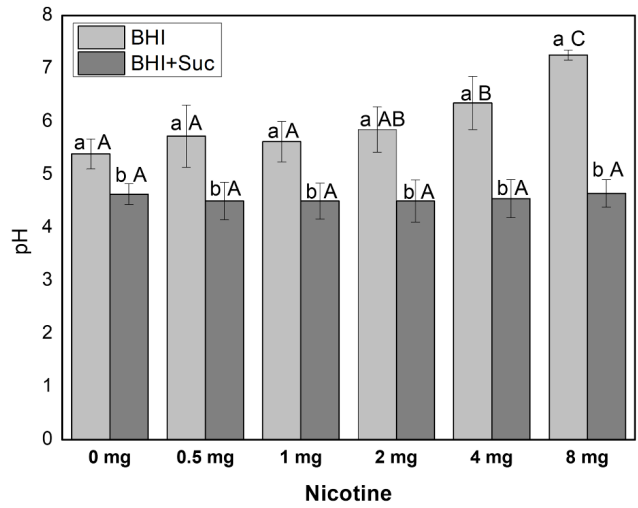

Figure 2. pH values from *F. nucleatum* biofilm grown in two different BHI broth conditions (0 and 1% sucrose) containing different nicotine concentrations. Different medium types in the same nicotine concentration are compared using lowercase letters. Different nicotine concentrations within the same medium type are compared using upper case letters. (p<.05; 2-way ANOVA and Tukey HSD test).

Amount of biofilm

The biofilm exposed to the medium with sucrose had a higher total biofilm growth (p<.05). Within the groups exposed to sucrose, a greater concentration of nicotine (8 mg/mL) stimulated the greatest total biofilm growth than the other groups (p<.05). The biofilm exposed to BHI medium without sucrose supplemented with different nicotine concentrations had no difference between them (Figure 3).

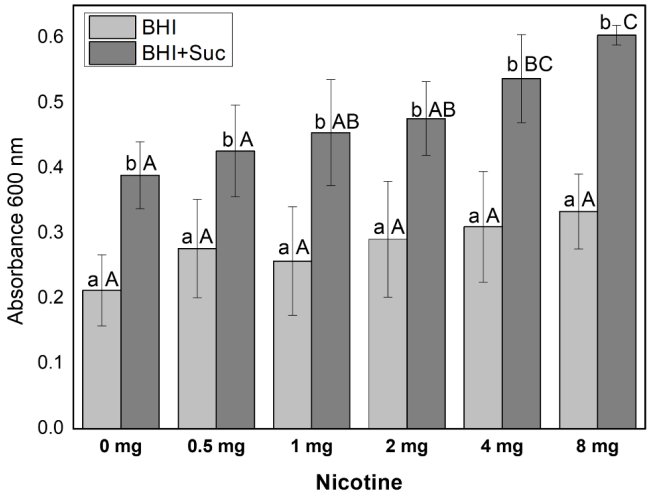

Figure 3. Total growth of *F. nucleatum* biofilm in two different BHI broth conditions (0 and 1% sucrose) containing different nicotine concentrations. Different medium types in the same nicotine concentration are compared using lowercase letters. Different nicotine concentrations within the same medium type are compared using upper case letters. (p<.05; 2-way ANOVA and Tukey HSD test).

Discussion

Smoking tobacco is a common practice with serious health risks. Although smoking was originally considered a habit, it is today recognized as a chronic relapsing medical illness and nicotine dependence[21]. Among the pathogenic bacteria, *F. nucleatum* is more common in smokers than in nonsmokers[22]. Smoking has long been recognized as a significant risk factor for periodontal disease, affecting the disease's prevalence, severity, progression, and treatment response[23]. Therefore, the present study used an in vitro model to investigate the influence of nicotine on anaerobic biofilm supplemented with sucrose.

BHI growth media supplemented with sucrose demonstrated the highest EPS values in the anaerobic biofilm in the different nicotine concentrations compared to the BHI growth medium with no sucrose (Figure 1). Growth medium supplemented with sugar carbohydrates can cause changes in biofilm composition, such as an increase in EPS values[24]. EPS also plays a role in bacterial cell adhesion and biofilm formation[25]. In this study, the presence of sucrose in the growth medium impacted EPS values, regardless of nicotine supplementation. Although the growth medium without sucrose presented reduced EPS values for the highest values in nicotine concentration, the association of nicotine and sucrose in the BHI growth medium proved to be a potent inducer of EPS production of a biofilm formed from the *F. nucleatum* microorganism.

It is evident that nicotine can accelerate the development of biofilms, including EPS from *F. nucleatum*. Although more research is necessary to determine the precise mechanism, the findings on the impact of nicotine on *F. nucleatum* biofilm may help to explain the prevalence and close association of this bacterium in cariogenic plaque in smokers.

Glucosyltransferases are responsible for producing EPS. Nicotine has been shown to upregulate glucosyltransferase genes[26]. This could explain why biofilm treated with nicotine increased carbohydrate levels in our investigation. Furthermore, EPS synthesis may have aided the pH drop in the sucrose-exposed groups (Figure 2). Despite this, in the no-sucrose-exposed groups, pH results indicated that the higher the nicotine concentration, the higher the pH value, similar to previous studies[27,28].

Correspondingly, nicotine enhanced the level of *F. nucleatum* biofilm growth with sucrose, as observed in the absorbance values in the spectrophotometric assay when compared with the medium with no sucrose (Figure 3). The presence of more microorganisms is indicated by an increase in the absorbance values[10]. This is in line with prior studies, which demonstrated increased biofilm development of *S. mutans* in the presence of nicotine (concentrations ranging from 0.25 to 8 mg/mL)[29,30], as well as biofilm formation of *A. actinomycetemcomitans* and *P. gingivalis* on epithelial cells[31]. According to previous research, *Haemophilus influenzae* may use nicotine as a nutrition source, causing biofilm formation to be enhanced[32]. In addition, nicotine is a source of nitrogen, carbon, and energy for the growth and development of other microorganisms in the environment[33].

F. nucleatum was chosen for this in vitro study since it can co-aggregate with various plaque bacteria suggesting that it functions as a microbiological bridge between early

and late subgingival plaque invaders[34] allowing pathogenic successors to settle and grow in the gingival sulcus[35,36].

Clinically, sucrose exposure associated with smoking can increase the amount of polysaccharides in biofilm, promoting overall biofilm development and bacterial attachment to the teeth or dental implant surfaces, promoting periodontal disease or peri-implant diseases, and, consequently, marginal bone loss.

Limitations of the present study include the in vitro setting; therefore, clinical studies are needed to confirm these results. Also, this study did not investigate how nicotine increased the biofilm formation of *F. nucleatum* during the course of time. As this study was conducted using a single-specie biofilm, future experiments may investigate *F. nucleatum* interaction with other species, such as other periodontium-related pathogens.

Conclusion

Within the limitations of this in vitro study, extracellular polysaccharides from the biofilm of *F. nucleatum* were affected by nicotine, regardless of sucrose exposure.

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Conflict of interest

None.

Authors contribution

Adaias Oliveira Matos: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original Draft. **Valentim Adelino Ricardo Barao:** Resources, Writing, – Review & Editing. **Richard Lee Gregory:** Conceptualization, Writing – Review & Editing, Supervision, Project administration.

Data availability

Datasets related to this article will be available upon request to the corresponding author.

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