Effects of casein phosphopeptide-amorphous calcium phosphate crème on nicotine-induced *Streptococcus mutans* biofilm in vitro

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Abstract

Objectives: The aim of this study was to test the effects of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) crème, or MI Paste™ (MIP), on nicotine-induced *Streptococcus mutans* biofilm. The experiment utilized *S. mutans* biofilm assays with varying concentrations of nicotine and MIP aqueous concentrate levels. First hand exposure to nicotine has been demonstrated to significantly increase *S. mutans* biofilm formation, while the active component, CPP-ACP, in MIP has been shown to reduce *S. mutans* biofilm formation.

Materials and Methods: A 24-h culture of *S. mutans* UA159 in microtiter plates were treated with varying nicotine concentrations (0-32 mg/ml) in Tryptic Soy broth supplemented with 1% sucrose (TSBS) with or without MIP aqueous concentrate. A spectrophotometer was used to determine total growth absorbance and planktonic growth. The microtiter plate wells were washed, fixed and stained with crystal violet dye and the absorbance measured to determine biofilm formation.

Results: The presence of MIP aqueous concentrate inhibits nicotine-induced *S. mutans* biofilm formation at different concentrations of nicotine (0-32 mg/ml).

Conclusion: The results demonstrated nicotine-induced *S. mutans* biofilm formation is decreased in the presence of MIP. This provides further evidence about the cariostatic properties of CPP-ACP, the active soluble ingredient in the MIP, and reconfirms the harmful effects of nicotine.

Clinical significance: Smokers may gain dual benefits from the use of MIP, as a remineralization agent and as a cariostatic agent, by inhibiting nicotine-induced *S. mutans* biofilm formation.

Keywords: Tobacco, Oral Bacteria, Caries, Caries Treatment, CPP-ACP, Plaque/Plaque biofilms.

Introduction

In 2003, the World Oral Health published a report stating that 60 – 90 % of school children and the majority of adults, living in industrialized countries, are affected by dental caries. Furthermore, caries still remains a major public health concern in other regions, such as Latin American and Asian countries [1]. According to the National Health and Nutrition Examination Survey (NHANES) conducted in the United States between 1999 - 2002, 42% of children and adolescents aged 6 -19 years, and 90% of adults had dental caries [2]. Dental caries is caused by cariogenic bacteria present in plaque, the biofilm covering the teeth. One of the most significant contributors to caries is *Streptococcus mutans*. *S. mutans* metabolizes carbohydrates, such as sucrose, and excretes lactic acid into plaque which can demineralize the dental hard tissues. As an additional consequence, this acidic environment will provide favorable conditions for the growth of *S. mutans* [3].

Another national epidemic concern is the continued use of tobacco. In the latest World Health Organization (WHO) global report on trends in prevalence of tobacco smoking 2000-2015, published in 2015, it estimates there are six million deaths each year related to tobacco, either by smoking or smokeless tobacco [4]. In 2014, survey results disclose that there are approximately forty million current cigarette smokers among adults in the United States [5] which results in significant systemic and oral effects [6,7]. Tobacco smoke has 7,357 chemical compounds from many different classes. Of these chemicals, nicotine is the most abundant of the alkaloid chemicals found in the tobacco leaf [8]. Nicotine is the main drug in tobacco causing addiction, and this the primary reason behind habitual tobacco use [9]. Furthermore, recent studies

demonstrated nicotine has a positive influence on *S. mutans* proliferation. There was a remarkable increase in biofilm formation and *S. mutans* activity with increasing nicotine concentrations which followed a dose-response pattern up to 16.0 mg/ml of nicotine [10]. This may explain the higher caries prevalence among smokers [11].

Among the non-fluoride agents for caries prevention, casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) has demonstrated promising anticariogenic effects [12]. CCP-ACP is a bioactive medium with a milk product base, prepared from two components: casein phosphopeptides (CPP) and amorphous calcium phosphate (ACP) [13]. CPP has the ability to stabilize calcium phosphate. By binding to ACP and forming CPP-ACP complexes, it has the potential to assist in remineralization and inhibit demineralization of enamel surfaces [14]. CPP-ACP has also been shown to reduce bacterial adherence in addition to bacteriostatic and bactericidal effects [15]. The addition of CPP to acquired salivary pellicle decreases the adherence of *S. mutans* significantly [16]. Also, the high extracellular free calcium concentrations dispensed by the CPP-ACP complexes can increase streptococcal membrane permeability and cause partial lysis [17]. Furthermore, CCP-ACP has the ability to bind to plaque, providing a considerable calcium reservoir within plaque, and reduces diffusion of free calcium. In return, this restricts mineral loss during a cariogenic process and supplies a possible source of calcium for remineralization [18]. Overall, CPP-ACP is a promising anticaries agent. Unlike fluoride, CPP-ACP can be included in food, and can therefore could be considered an important functional ingredient in food, toothpastes and mouthwashes for dental caries control [19]. CPP-ACP has been shown to exert anticariogenic benefits in different application mediums such as sugar-free sorbitol- or xylitol-based chewing

gums [20], milk [21], lozenges [22], dental creams [23] and mouthwashes [24]. In the United States, CPP-ACP can only be found in the prescribed topical, water-based, sugar-free dental cream, MIP (GC America, Alsip, IL). The CPP-ACP active ingredient in the MIP is marketed by the company under the name of Recaldent™. MIP contains other ingredients; pure water, glycerol, D-sorbitol, CMC-Na, propylene glycol, silicon dioxide, titanium dioxide, xylitol, phosphoric acid, flavoring, zinc oxide, sodium saccharin, ethyl p-hydroxybenzoate, magnesium oxide, guar gum, propyl p-hydroxybenzoate and butyl p-hydroxybenzoate (MI Paste is available in five flavors: melon, mint, strawberry, tutti-frutti and vanilla).

The aim of this study was to identify the effect of nicotine exposure on the inhibitory effects of MIP aqueous concentrate on *S. mutans* biofilm formation. This study demonstrates the results of planktonic, biofilm and total growth assays incubated with the MIC of MIP aqueous concentrate and nicotine levels corresponding with a primary level of nicotine exposure.

Materials and Methods

Determination of MIC, MBIC, & MBC of MIP Aqueous Concentrate

The study investigated the most effective concentration of MIP aqueous concentrate. This initial step determined the minimum inhibitory concentration (MIC), the minimum biofilm inhibitory concentration (MBIC), and the minimum bactericidal concentration (MBC) of MIP aqueous concentrate on the growth of *S. mutans*. MIC is the lowest concentration of MIP aqueous concentrate that inhibits visible growth of *S. mutans*,

MBIC is the lowest concentration that inhibits visible biofilm formation and MBC is the lowest concentration of MIP aqueous concentrate that is able to kill *S. mutans*.

The assays were accomplished by preparing serial dilutions 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024 & 1:2048 of MIP-TSBS and centrifuging (Beckman GS-6R Refrigerated Centrifuge) for 10 min. Ten μ l of an overnight culture of *S. mutans* [10^6 colony-forming units (CFU)/ ml, determined by spiral plating] in Tryptic Soy Broth (TSB) was treated with 190 μ l of the supernatants of the 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024 & 1:2048 of MIP-TSBS dilutions and incubated at 37°C for 24 h in sterile 96-well flat-bottom microtiter plates (Fisher Scientific, Newark, DE, USA). The optical density (OD) values of the bacterial cultures were measured at 595 nm in a spectrophotometer (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA). The MIC was defined as the lowest concentration yielding a change in the OD at 595 nm of less than 0.050 from the negative control.

The MBC was determined by transferring bacterial cultures from the serial dilution wells of MIP-TSBS onto Sheep Blood Agar (Difco, Detroit, MI, USA) plates, and incubated overnight. The MBC was determined as the lowest concentration of nicotine that had no visible bacterial colonies on the agar plates after 24 h incubation.

After the initial incubation, 120 μ l of 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 removed from the biofilm microtiter plate wells (leaving attached biofilm), and 200 μ l of 10% 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 μ l of 0.5% crystal violet dye was added to each well and the cells stained for 30 min. The wells were rinsed 3 times and 200 μ l 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. The MBIC was defined as the lowest concentration yielding a change in OD at 490 nm of less than 0.050 from the negative control.

Inhibition of Nicotine-Induced Biofilm Formation by MIP

An overnight culture of *S. mutans* UA159 (ATCC 700610) was grown in Tryptic Soy Broth (TSB) at 37°C in 5% CO₂ and stored with 20% glycerol at -80° C. The MIC of MIP aqueous concentrate-Tryptic Soy broth supplemented with 1% sucrose (TSBS) determined from the preliminary experiment was used. Serial dilutions of TSBS was prepared to yield 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) without MIP, and 0, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0 mg/ml nicotine with the MIC dilution of the MIP aqueous concentrate (determined by the results of the preliminary experiment). 190 μ l of TSB at each nicotine concentration was aliquoted into wells of a sterile 96-well flat bottom microtiter plate. 10 μ l of a fresh overnight TSB culture of *S. mutans* was added to each well. The microtiter plate was incubated in 5% CO₂ at 37°C 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. Next, 120 μ l from each well was transferred to the corresponding well of a new microtiter plate. The absorbance of each well was read at 595 nm to measure planktonic growth. The remaining planktonic cells

was removed from the biofilm microtiter plate wells (leaving attached biofilm), and 200 μ l of 10% formaldehyde was added to each well for 30 min to fix the cells. After 30 min, the formaldehyde was removed and the biofilm cells was washed 3 times with deionized water. 200 μ l of 0.5% crystal violet dye was added to each well and the cells stained for 30 min. The wells were rinsed 3 times and 200 μ l 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 include biofilms of *S. mutans* without nicotine and with or without MIP aqueous concentrate.

Statistical Analysis

Each experiment was repeated three times. Two-way ANOVA was used to compare the effects of MIP aqueous concentrate exposure (each dilution individually), nicotine concentration (0, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0 mg/ml) and their interaction on planktonic, biofilm, and total growth. Pair-wise comparisons was made using the Sidak method to control the overall significance level at 5%. The comparisons involving MIP aqueous concentrate exposure were of primary interest to test the study hypotheses. The distribution of the measurements was examined and a transformation of the data (natural logarithm, square root, rank, etc.) was used for the analyses to satisfy the ANOVA assumptions. Based on prior studies, the within-group standard deviation of the absorbance measurements for biofilm formation were estimated to be 0.15. With 4 wells of each MIP/nicotine dilution in each of 3 replicates of the study, the study was estimated to have 80% power to detect a difference of 0.25 between samples with and

without MIP for each nicotine concentration, assuming two-sided tests conducted at an overall 5% significance level.

Results

Determination of MIP Aqueous Concentrate MIC, MBIC & MBC.

The MIP aqueous concentration to be used with different nicotine concentrations was determined to be the 1:16 dilution (Figs. 1 and 2). The results indicated that the 1:4, 1:8, 1:16 and 1:32 dilutions had significant reductions ($p < 0.05$) in biofilm formation (Fig. 2). The 1:4 and 1:8 dilutions displayed strong bactericidal effects, while the 1:16 and 1:32 dilutions revealed the MIC and the MBIC (Figs. 1 and 2, respectively). However, for the purpose of this study, the 1:16 dilution was selected to be the MIC and MBIC.

As for the MBC, all the bacterial cultures with serial dilutions of MIP-TSBS on the agar plates provided bacterial colonies. Therefore, no concentrations killed the bacteria and no MIP aqueous concentrate concentration can be defined as the MBC.

Inhibition of Nicotine-Induced Biofilm Formation by MIP

Overall, according to the total absorbance, planktonic and biofilm formation data obtained (Figs. 3-5, respectively), the combined presence of a 1:16 dilution of MIP aqueous concentrate and the various nicotine concentrations demonstrated significant effects on *S. mutans*. Without MIP aqueous concentrate, there was an increased biofilm formation with the presence of nicotine, particularly with the 4 and 8 mg/ml concentrations. However, there was a strong inhibitory effect with the presence of the 16 and 32 mg/ml nicotine concentrations compared to the control group. The MIP

aqueous concentrate – nicotine interaction displayed significant inhibitory effects on *S. mutans* biofilm formation with all nicotine concentrations, in comparison with the no MIP aqueous concentrate, except with the 32 mg/ml (Fig. 5). As for total absorbance, there was a significant increase in growth with the MIP aqueous concentrate – nicotine interaction, except with the 16 mg/ml nicotine concentration as that concentration revealed a decrease in growth (Fig. 3). However, there was a significant increase in planktonic growth with MIP aqueous concentrate – nicotine interactions, except for the 8, 16 and 32 mg/ml nicotine concentrations which exhibited minimum growth, compared to the no MIP aqueous concentrate group.

Discussion

A preliminary experiment was conducted to outline the antimicrobial effect of the MIP aqueous concentrate by measuring MIC, MBIC and MBC. Previous studies report antimicrobial effects of CPP-ACP, the active soluble agent in MIP [12]. Unfortunately, there have been no *in-vitro* studies reported on MIP. In addition, there have been no reports, either *in-vivo* or *in-vitro*, on the effect of MIP on nicotine-induced *S. mutans* biofilm growth. From these results, MIP significantly inhibits *S. mutans* biofilm and nicotine-induced *S. mutans* biofilm formation. However, according to the planktonic results which was confirmed with the MBC data, MIP has no bactericidal effect on the *S. mutans*. Also, the total absorbance reading provides support for its nonbacterial effect, where it shows the MIP groups had higher OD readings compared to the non-MIP groups. Since the critical stage of a cariogenic bacterium is the biofilm phase, and disrupting biofilm will eventually eliminate the caries process [3], then MIP has been

proven to inhibit *S. mutans* biofilm and nicotine-induced *S. mutans* biofilm formation. This supports the role of MIP as an anticariogenic agent [19].

It was observed with increasing nicotine concentrations from 0.25 to 8 mg/ml, there were increases in biofilm formation. Meanwhile, the 16 and 32 mg/ml nicotine concentrations had strong inhibitory effects on *S. mutans* growth and viability, respectively. These results confirm a previous study with seven strains of *S. mutans*, including the UA159 strain tested in this study, exposed to varying concentrations of nicotine [10]. It was theorized previously that any nicotine concentration above 8 mg/ml is toxic to *S. mutans*, and furthermore any concentration above 32 mg/ml is bactericidal.

With the addition of a 1:16 dilution of MIP aqueous concentrate to the nicotine concentrations, biofilm formation was inhibited, compared to nicotine-treated groups with no MIP aqueous concentrate. There was no increase in biofilm formation associated with increasing nicotine concentrations in the presence of the 1:16 dilution of MIP aqueous concentrate. The minimum inhibitory effect on MIP aqueous concentrate-nicotine was observed at 8 mg/ml, which is the highest biofilm formation with the no MIP aqueous concentrate group. There was negligible biofilm formation observed in the 16 and 32 mg/ml MIP aqueous concentrate and nicotine-treated groups. This was interpreted to indicate that any nicotine concentration above 4 mg/ml, when combined with MIP aqueous concentrate can be toxic to *S. mutans* biofilm formation.

As for the MIP inhibition of nicotine-induced biofilm formation, this can be explained by the competition of the MIP main component, CPP-ACP, with *S. mutans* on the attachment of bacteria to the polystyrene surface of the 96 well plate. This concept has been demonstrated in previous studies, where the incorporation of CPP, which is a

component of the CPP-ACP complex, into salivary pellicles significantly reduced the adherence of *S. mutans* [16].

There are limitations to this study. The MIP used was an aqueous concentrate, which contained the soluble active complex CPP-ACP, and did not contain the entire dental crème product. Due to the nature of the patent of the CPP-ACP complex by the manufacturer, the study utilized the MIP aqueous solution, which might have other elements that could have played a role on the effect on *S. mutans* biofilm formation.

Conclusion

In summary, this study confirms the inhibitory effect of MIP on nicotine-induced *S. mutans* biofilm formation. Smokers may gain dual benefits from the use of MIP, as a remineralization agent and as a cariostatic agent, by inhibiting nicotine-induced *S. mutans* biofilm formation. Further clinical studies are needed to confirm the results of this study.

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Compliance with Ethical Standards

Conflict of Interest: Naser B Alawadhi declares that he has no conflict of interest. Frank L Lippert declares that he has no conflict of interest. Richard Gregory declares that he has no conflict of interest.

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Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Figure Legends

Figure 1. Effect of MIP on total absorbance (595 nm; representing the combined growth of planktonic and biofilm cells) and biofilm formation (490 nm) of *Streptococcus mutans* treated with various dilutions of MI Paste (1:4 - 1:2048). Asterisks indicate significant differences ($p < 0.05$) compared to the 0 MI Paste control group.

Figure 2A. Effect of MIP (1:16 dilution) on total absorbance (representing the combined growth of planktonic and biofilm cells) of *Streptococcus mutans* treated with various nicotine concentrations (0-32 mg/ml). Asterisks indicate significant differences ($p < 0.05$) compared to the 0 nicotine control group. Hashmarks indicate significant differences ($p < 0.05$) within the same nicotine concentration groups.

Figure 2B. Effect of MIP (1:16 dilution) on planktonic growth of *Streptococcus mutans* treated with various nicotine concentrations (0-32 mg/ml). Asterisks indicate significant differences ($p < 0.05$) compared to the no MIP control group.

Figure 2C. Effect of MIP (1:16 dilution) on *Streptococcus mutans* biofilm formation treated with various nicotine concentrations (0-32 mg/ml). Asterisks indicate significant differences ($p < 0.05$) compared to the no MIP control group.