Nicotine Upregulates Coaggregation of *Candida albicans* and *Streptococcus mutans*

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Abstract:

Purpose: Denture stomatitis is a condition of painless inflammation of denture-bearing mucosa. Reports indicated that nicotine, the major psychoactive ingredient in tobacco, increases growth of *S. mutans* and *C. albicans* in denture biofilm. The purpose of this study was to determine the *in vitro* effects of nicotine on co-aggregation of *Candida albicans* with *Streptococcus mutans*.

Material and Methods: *C. albicans* strain ATCC 10231, *S. mutans* strain UA159 (ATTC 700610), and nicotine dilutions (ranging from 0-32 mg/ml) were used for this study. Both microorganisms were grown for 24 h in dilutions of nicotine (0-32 mg/ml) made in Tryptic Soy Broth (TSB) or TSB supplemented with 1% sucrose (TSBS; *S. mutans*) or Yeast Peptone Dextrose broth (YPD; *C. albicans*). Suspensions of the nicotine-treated cells were prepared, mixed together and incubated for up to 24 h in order to determine if there was an increase in coaggregation of nicotine-treated cells compared to the no nicotine control cells. Qualitative analysis of coaggregation was performed using a visual aggregation assay and light microscopic observation. A spectrophotometric assay was used to provide a quantitative analysis of the coaggregation.

Results: The visual aggregation assay indicated a significant increase in coaggregation between *C. albicans* and *S. mutans* with increasing incubation time (0 to 24 h) and nicotine concentrations (0 to 4 mg/ml). Microbial growth in nicotine at 4 mg/ml demonstrated a significant increase in coaggregation after 24 h of incubation. The numbers of coaggregated *S. mutans/C. albicans* cells exhibited a significant increase with incubation time and nicotine concentrations when the samples were examined microscopically. More coaggregation of *S. mutans* and *C. albicans* was observed with incubation time and increased nicotine compared to the 0 mg/ml nicotine group. There was a noticeable increase of coaggregation when cells were grown in TSBS compared to TSB. Absorbance of nicotine-treated cells (0.25-4 mg/ml) exhibited a decrease in values compared to 0 mg/ml at 0 h of incubation confirming increased coaggregation.

Conclusion: These results demonstrated the effect of nicotine in increasing the coaggregation of *S. mutans* with *C. albicans*. Coaggregation increased with incubation time and nicotine concentration. Coaggregation was increased with *S. mutans* grown in TSBS compared to TSB, suggesting that growth in sucrose media leads to an increase in receptors responsible for coaggregation.

Keywords: Coaggregation; nicotine; denture stomaitits; *C. albicans*; *S. mutans*.

1. Introduction:

Denture stomatitis is a condition where there is a painless inflammation and erythema of denture-bearing mucosa and the reported prevalence is 11-67% of complete denture wearers. Denture stomatitis is the most common oral manifestation of oral candidiasis.[1-3] It is usually symptomless, but a few patients may complain of a burning sensation, bleeding of the mucosa, dryness, halitosis, and frequently associated with angular cheilitis.[1,3,4] The cause of denture stomatitis is multifactorial that include denture trauma, denture cleanliness, dietary factors, allergy, candidal infection, systemic factors, and miscellaneous factors.[3] *C. albicans* is believed to be the primary microbial etiologic factor, but other yeast species such as *Candida glabrata* and *Candida tropicalis* have been found in patients with denture stomatitis.[1-3] Smoking, systemic diseases, poor oral hygiene, age, and lack of dexterity are known as predisposing factors that result in the accumulation of plaque on dentures.[3,6] Adherence to surface irregularities of the denture material is the mechanism that *Candida* species use to colonize denture surfaces and form biofilm. The denture biofilm consists mostly of Gram-positive bacteria, *Lactobacillus*, and *C. albicans*. Exchanging molecular signals or using their own metabolic products are the mechanisms typically used by microorganisms to interact in the oral cavity.[3,4,6] Candidal adherence to denture material increases when there is an increase in surface hydrophobicity.[7,8]

The use of tobacco has shown a significant effect on oral and systemic health. All forms of tobacco increase periodontal diseases, implant failures, peri-implant diseases, soft tissue changes, caries, tooth loss, and oropharyngeal cancer.[9-14] Nicotine, an alkaloid that is the main active ingredient in tobacco is present in cigarettes (1 mg/cigarette[10]), and has an effect on oral microorganisms including *C. albican*s and *S. mutans*.[15,16] Studies in our laboratory indicate that nicotine upregulates certain *S. mutans* cell surface receptors that may be responsible for coaggregation with *C. albicans*. These studies indicated a positive relationship between nicotine concentration and an increase in biofilm formation and metabolic activity of *S. mutans* and the occurrence of *C. albicans* in the oral cavity.[15,16]

Over the last 30 years, fungal infections have become a major problem among immunosuppressed and otherwise medically compromised patients. Diseases of fungal origin can vary from a superficial mucosal lesion to a life-threating systemic form. The pathogenesis of the infection involves host-factors and yeast.[17-19] Fungal infections can be classified as opportunistic or primary infections. Opportunistic fungal infections are aggressive, occur in

immunocompromised patients, and spread quickly to other organs. Examples of opportunistic fungal infections are: Aspergillosis, Candidiasis, and Mucormycosis. Primary fungal infections occur slowly in individuals with normal immune systems and do not spread to organs deep in the body. Examples of primary fungal infections are: Histoplasmosis, Blastomycosis, Coccidioidomycosis, and Paracoccidioidomycosis.[17-19]

Candidal infections, which are a yeast overgrowth, caused primarily by *C. albicans*, produce several types of infections such as: orophayryngeal candidiasis (oral candidiasis), vulvovaginal infection, and invasive candidiasis or candidemia.[20-22] Oral candidiasis is a common opportunistic infection and is under diagnosed especially among the elderly who wear dentures.It is the most common fungal infection associated with HIV infection patients. In recent years, invasive candidiasis has become a leading cause of mycosis-associated mortality and mortality among patients with health care-associated infections and the prevalence has increased worldwide.[7,23] Oral candidiasis has a variety of manifestations and is divided into primary and secondary oral candidiasis. Primary oral candidiasis is confined to candidal infections of the oral and peri-oral tissues, whereas secondary oral candidiasis is associated with generalized systemic candida infections. Examples of primary oral candidiasis are: pseudomembranous candidiasis, erythematous candidiasis, denture stomatitis (which is the focus of our study), angular cheilitis, median rhomboid glossitis, leukoplakia, lichen planus, and lupus erythematosus. Examples of secondary oral candidiasis include diseases such as athymic aplasia and candidiasis endocrinopathy syndrome.[20-22] *C. albicans* is the most common opportunistic fungus of the candidal species found within the oral cavity. *C. albicans* can be a normal commensal organism, but also can be pathogenic and cause chronic infections. It is a pleomorphic fungus that colonizes many parts of the human body such as mucosal tissue, the gastrointestinal tract, and epithelial tissue.[24,25] *C. albicans* can be found mainly on the dorsum of the tongue, while plaque-covered teeth and mucosa are secondarily colonized sources. *C. albicans* exhibits dimorphism under the microscope, which is a transition from ovoid budding blastospores (yeast cells) to parallel-sided hyphae. The hyphal form of *C. albicans* is known for its invasiveness, which can cause serious damage to human tissue. *C. albicans* has the ability to invade and form biofilm on every site on the human body, as well as biomaterials.[1,2,24,25]

Coaggregation is a protective mechanism where genetically distinct microorganisms can adhere to each other in suspension and autoaggregation is described as the same process in

genetically identical microbial cells.[26] Coaggregation can be detected as clumps of different types of microorganisms when mixed together. A complex biofilm will develop when structural and metabolic codependence occurs between the microorganisms in candidal infections and dental plaque.[27] *C. albicans* has the demonstrated ability to adhere and colonize denture surfaces in coaggregation with *S. mutans*.[7,28] However, there are no reports of the effect of nicotine on coaggregation of these two species.

S. mutans is a gram-positive, facultative anaerobic coccus that occupies hard surfaces in the oral cavity and plays a major role in dental plaque formation.[29] It is cariogenic and grows at temperatures between 18-40^{o}C. The metabolic system of *S. mutans* produces an acidic environment in the oral cavity that can exceed the buffering capacity of saliva leading to changes in the mineral layer of the outer enamel surfaces.[29,30] *S. mutans* has been shown to induce the adherence of microorganisms such as *C. albicans* to both dental and acrylic surfaces.[7] This study suggests that there may be symbiosis between the organisms when *S. mutans* are cultivated with *C. albicans*. Protein-carbohydrate interactions are typically involved in the process of coaggregation of microorganisms.[1,7] Blastopores are the most common type of yeast-like form of *C. albicans* when associated with *S. mutans*.[7]

Previous studies demonstrated that there is an increase in the growth of *S. mutans* and *C. albicans* when the two microorganisms were grown separately in different concentrations of nicotine.[15,16] In addition, other studies indicated that nicotine stimulates synthesis of *S. mutans* GtfB exoenzyme, which binds tightly to *C. albicans* yeast cell surfaces leading to more coaggregation between the two microorganisms.[31] The purpose of this study was to examine the effect of different physiologically-relevant nicotine concentrations on the coaggregation of *C. albicans* and *S. mutans* over time when compared to the 0 nicotine control group.

2. Materials and Methods:

2.1 Fungal and Bacterial Strains, Nicotine, and Media:

S. mutans strain UA159 (ATCC 700610) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Stock cultures of *S. mutans* were stored at -80°C in tryptic soy broth (TSB; Anaerobe Systems, Morgan Hill, CA, USA) containing 10% glycerol. Mitis Salivarius Sucrose Bacitracin (MSSB; Anaerobe Systems) agar plates were used initially for culture of these bacteria; TSB or TSB supplemented with 1% sucrose (TSBS) was used to grow the bacteria and the bacterium was cultured in an atmosphere of 95% air/5% CO_2 at 37°C for 24 h. Both TSB and TSBS were used in order to determine the effect of sucrose on coaggregation.[15,16]

C. albicans strain ATCC 10231 was stored at -80°C, passaged on tryptic soy agar or blood agar plates and incubated in a 95% O_2/5% CO_2 incubator at 30°C prior to use. *C. albicans* cells were inoculated in 5 ml of yeast-peptone-dextrose (YPD) broth medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose; Fisher Scientific, Newark, DE, USA) and cultured for 48 h. Nicotine dilutions (ranging from 0-32 mg/ml) in TSB, TSBS or YPD were prepared from a 1 g/ml nicotine stock solution (Sigma Chemical Co., St. Louis, MO).[15,16]

2.2 Aggregation Assays:

Both microorganisms were grown separately for 24 h in dilutions of nicotine (0-32 mg/ml) made in 40 ml of TSB or TSBS (*S. mutans*) or YPD (*C. albicans*). The cells were washed in sterile saline three times by centrifugation at 3,000 g for 10 min. Cell pellets of both microorganisms were resuspended in sterile saline, killed with 1% formaldehyde and the $OD_{600\ nm}$ was adjusted to 1.0 (working stock concentration) using a spectrophotometer. Because formaldehyde treatment may affect coaggregation, fresh live washed nicotine-treated cultures were also examined in a preliminary experiment. Working stocks of each cell preparation were prepared and used to determine the aggregation properties of the bacterial coaggregation. Visual aggregation assays (naked eye and microscopic) were used as qualitative analyses of coaggregation. A spectrophotometric assay was used to quantitatively determine coaggregation.[32] All assays were conducted at least three times with triplicate samples for each group/experiment.

Visual Aggregation Assay: Three 1 ml replicates of the working stock suspensions of both organisms were transferred to a 15 ml glass test tube, mixed together and examined for aggregation

visually. The tubes were incubated for 0, 1, 2, and 24 h at 37ºC. Separate tubes were prepared for each time interval. At each time interval, after incubation, each tube was assigned a score based on the aggregation and compared with the mixed suspensions at 0 h of incubation. Pictures of the tubes were taken at each time interval. The scoring ranged from "1" to "4" as follows; "1" shows turbid supernatant: "2" shows definite turbid supernatant with finely dispersed coaggregates; "3" indicates slightly turbid supernatant with formation of small precipitating aggregates; "4" represents clear supernatant and large coaggregates which precipitate immediately.[32,33]

Microscopic Analysis: Twenty µl of the mixed suspensions from the above visual aggregation assay of the microorganisms was placed on glass microscope slides, stained with Gram's crystal violet and observed with a light microscope at 1000X under oil immersion.. Coaggregation was observed as the smaller blue *S. mutans* cells bound to the larger blue *C. albicans*. The number of coaggregated *S. mutans* cells/*C. albicans* cells bound together was enumerated in 10 fields for each suspension.[32]

Spectrophotometric Assay: Mixed suspensions prepared in triplicate as described above in the visual aggregation assay.[32,34] were used. After 0, 1, 2, and 24 h of incubation, the test tubes were briefly shaken, allowed to remain undisturbed for 5 min and 100 µl of the aqueous phase was transferred to wells of a 96-well flat bottom microtiter plate. Separate tubes were prepared for each time interval. It is important to note that transferring 100 µl of the aqueous phase measured only cells that were not aggregated. Therefore, lower absorbances indicate more coaggregation. The OD was measured using a multi-well spectrophotometer plate reader (SpectraMax 190; Molecular Devices, Inc., Sunnyvale, CA) at 600 nm.[32,34]

2.3 Statistical Analysis:

Visual aggregation was summarized using counts and percentages in contingency tables. The effects of nicotine concentration, incubation time, and growth media on visual aggregation were analyzed using cumulative logistic regression models for ordered categorical responses. Microscopic aggregation was summarized using the mean, standard deviation, standard error, and range of the number of each cell type bound together. The effects of nicotine concentration, incubation time, and growth media on microscopic aggregation were analyzed using a three-way ANOVA with a random effect for experiment. Spectrophotometric aggregation was summarized using the mean, standard deviation, standard error, and range of aggregation absorbance. The

effects of nicotine concentration, incubation time, and growth media on aggregation absorbance were analyzed using ANOVA. A 5% significance level was used for all comparisons.

3. Results:

Visual aggregation assay: There were no differences between live and dead cells in the degree of coaggregation in each of the assays used in this study. Most samples demonstrated an increase in the visual aggregation assay in TSBS (Fig. 1A) and TSB (Fig. 1B) with time. Nicotine at 4 mg/ml provided a significant increase in coaggregation when *S. mutans* was grown in TSBS and TSB compared to the 0 mg/ml groups (Figs. 1A and 1B). *S. mutans* grown in nicotine at 4 mg/ml in TSBS demonstrated the highest value after 24 h. Also, 1 and 2 mg/ml exhibited a significant increase in coaggregation after 24 h (Fig. 1A). Nicotine at 0.25 mg/ml did not provide a significant change in coaggregation in TSB. Nicotine at 1 and 2 mg/ml increased coaggregation noticeably after 2 h of incubation (Fig. 1B). There were no differences in the preliminary coaggregation experiment using the visual, microscopic or spectrophotometric analyses between live and dead cells. In addition, nicotine concentrations above 4 mg/ml were not used because higher levels of nicotine significantly inhibited growth of both *S. mutans* and *C. albicans* (results not shown).

Microscopic analysis: The number of *S. mutans* attached to *C. albicans* increased with increasing nicotine concentrations (0-4 mg/ml) in both TSBS and TSB media (Fig. 2 and Figs. 3A. and 3B, respectively). *S. mutans* grown in nicotine at 4 mg/ml in TSBS exhibited a significant increase in coaggregation among all the other groups after 24 h (Fig. 3A). *S. mutans* grown in TSBS at 0.5, 1, and 2 mg/ml of nicotine demonstrated an increased coaggregation of *S. mutans*/*C. albicans* compared to the 0 mg/ml nicotine group (Fig. 3A). The 0 nicotine control group grown in TSB demonstrated the least coaggregation after 24 h amongst all groups grown in both media. *S. mutans* grown in TSB at 4 mg/ml of nicotine provided a significant increase in coaggregation of *S. mutans*/*C. albicans* compared to *S. mutans* grown in the other nicotine concentrations in the same media. In addition, coaggregation increased significantly with *S. mutans* grown in 1 and 2 mg/ml of nicotine over time (Fig. 3B).

Spectrophotometer Assay: A lower absorbance in this assay indicated more coaggregation. The absorbance of most samples demonstrated a decrease in absorbance when compared to the 0 nicotine control group (Figs. 4A and 4B). *S. mutans* and *C. albicans* grown in 0.25 mg/ml of nicotine had the lowest absorbance after 24 h of incubation in both TSBS and TSB. *S. mutans*

and *C. albicans* grown in nicotine at 0.25, 1, and 2 mg/ml in TSBS exhibited absorbances less than the 0 nicotine control group after 24 h of incubation (Fig. 4A). Nicotine at 4 mg/ml provided the highest absorbance at 0 h of the experiment, andn exhibited a significant decrease compared to the 0 nicotine controls in both TSBS (Fig. 4A) and TSB (Fig. 4B). Nicotine at 0.25, 0.5, 1, and 2 mg/ml in TSB demonstrated less absorbance compared to the 0 nicotine control group after 24 h of incubation. In general, the absorbance decreased the longer the cells were incubated together.

Overall, the three way ANOVA analysis indicated significant interactions between the concentration of nicotine, the length of time the microorganisms were co-incubated and the presence or absence of sucrose in the *S. mutans* cultures. As the nicotine concentration and incubation time increased, the amount of coaggregation increased. Furthermore, sucrose significantly increased coaggregation at all nicotine concentrations and incubation times.

4. Discussion:

To our knowledge, this is the first study to examine the correlation between growth in nicotine and coaggregation of *C. albicans* and *S. mutans.* In the present study, the visual aggregation assay of *S. mutans* and *C. albicans* cells grown in 4 mg/ml of nicotine in TSBS illustrated the highest coaggregation compared to the 0 nicotine control group and all other groups. On the other hand, the 0.25 mg/ml nicotine group in TSB did not demonstrate a significant difference and we believe the reason is that the *S. mutans* cells were grown in sucrose. In the microscopic analysis, the coaggregation of *S. mutans* with *C. albicans* was higher when the cells were grown in TSBS and the 4 mg/ml of nicotine-treated group provided the highest value (Fig. 2). In general, all nicotine treated samples resulted in an increase in both visual aggregation score and microscopic numbers of *S. mutans* attached to *C. albicans*. Comparing coaggregation when *S. mutans* was grown in TSBS to TSB, TSBS-grown cells had more coaggregation when compared to TSB and the reason appears to be due to sucrose in the media.

An increase in the absorbance values in the spectrophotometer assay indicates less coaggregation and more microorganisms remaining as individual cells in the media. All nicotine treated groups exhibited a decrease in absorbance over time. *S. mutans* grown in 4 mg/ml of nicotine in TSBS exhibited the most change in absorbance over time which indicated that more coaggregation occurred in this group. Nicotine-treated TSBS groups demonstrated decreased absorbance when compared to TSB-grown cells. We believe that samples might have demonstrated more decreased absorbance if the experiment was extended 24 h more.

Previous studies indicated an increase in the growth of *C. albicans* and *S. mutans* with nicotine. According to Huang *et al.*[15] a positive relationship was observed between nicotine and biofilm formation and metabolic activity of *S. mutans.* At 2-8 mg/ml of nicotine concentrations, both biofilm formation and metabolic activity increased in the UA159 *S. mutans* strain. In addition, Huang *et al.*[15] reported that nicotine stimulated planktonic cell Gtfs and Gpb of *S. mutans.*

GtfB, a streptococcal exoenzyme, adheres effectively to the *C. albicans* yeast cell surface in an enzymatically active form, converting the *C. albicans* cells to *de facto* glucan producers, which contributes to *S. mutans* coaggregation to the yeast cells and may increase its cariogenicity.[7,31] The C terminus of GtfB is highly selective and differs from other exoenzymes. The cell wall of *C. albicans* is composed of glucan, chitin, and mannan. The selective binding of GtfB and changes in the glucan products suggest highly specific and C terminus-mediated

interactions with the cell wall components of *C. albicans.*[7,31] Gregoire *et al.*[31] proposed that the glucans formed on the yeast cell surface by GtfB act as binding sites for *S. mutans*, forming a bacterial-fungal-EPS complex. They also stated that the glucan presence on the *C. albicans* cell surface greatly enhanced the adhesive interactions between the two microorganisms.[31]

Studies indicated that *C. albicans* grown with nicotine exhibited an increase in total growth and biofilm formation.[15] Dubois *et al.*[16] reported that there is an increase in *C. albicans* growth when grown with nicotine. The total growth of *C. albicans* was inhibited in 8 mg/ml of nicotine and biofilm formation was enhanced significantly in 4 and 8 mg/ml.[16] The above mentioned reports support our study indicating absorbance, visual aggregation score, and microscopic analysis exhibited increases in coaggregation over time. A model illustrating the coaggregation of the two microorganisms was developed (Fig. 5).

5. Conclusion:

The present study demonstrated the effect of nicotine in increasing the coaggregation of *S. mutans* and *C. albicans.* Previous studies indicated that growing *C. albicans* and *S. mutans* in nicotine-treated media would increase the growth of each individual microorganism.[15,16] In addition, reports indicated that nicotine stimulates GtfB, which is a streptococcal exoenzyme that plays a role in the coaggregation process between *C. albicans* and *S. mutans.*[7,15,16,31] The previously mentioned studies supports and agrees with the results of our study. Coaggregation was increased with *S. mutans* grown in nicotine-treated TSBS compared to nicotine-treated TSB, suggesting that growth in sucrose media leads to an increase in receptors such as GtfB responsible for coaggregation. Clinically, this suggests that smokers will have more coaggregation of *C. albicans* and *S. mutans* increasing the likelihood of both denture stomatitis and caries.

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Figure 1A.

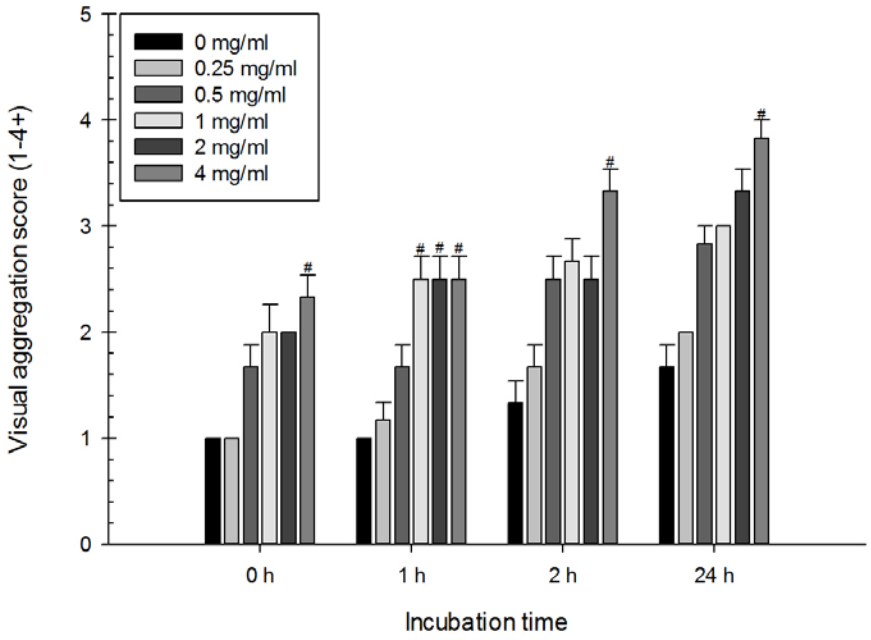

Figure 1B.

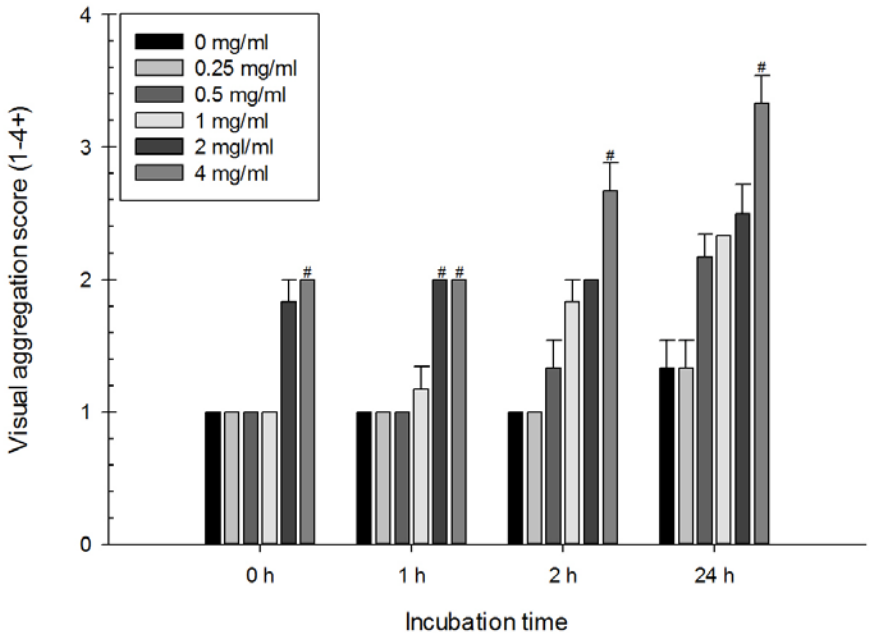

Figure 2.

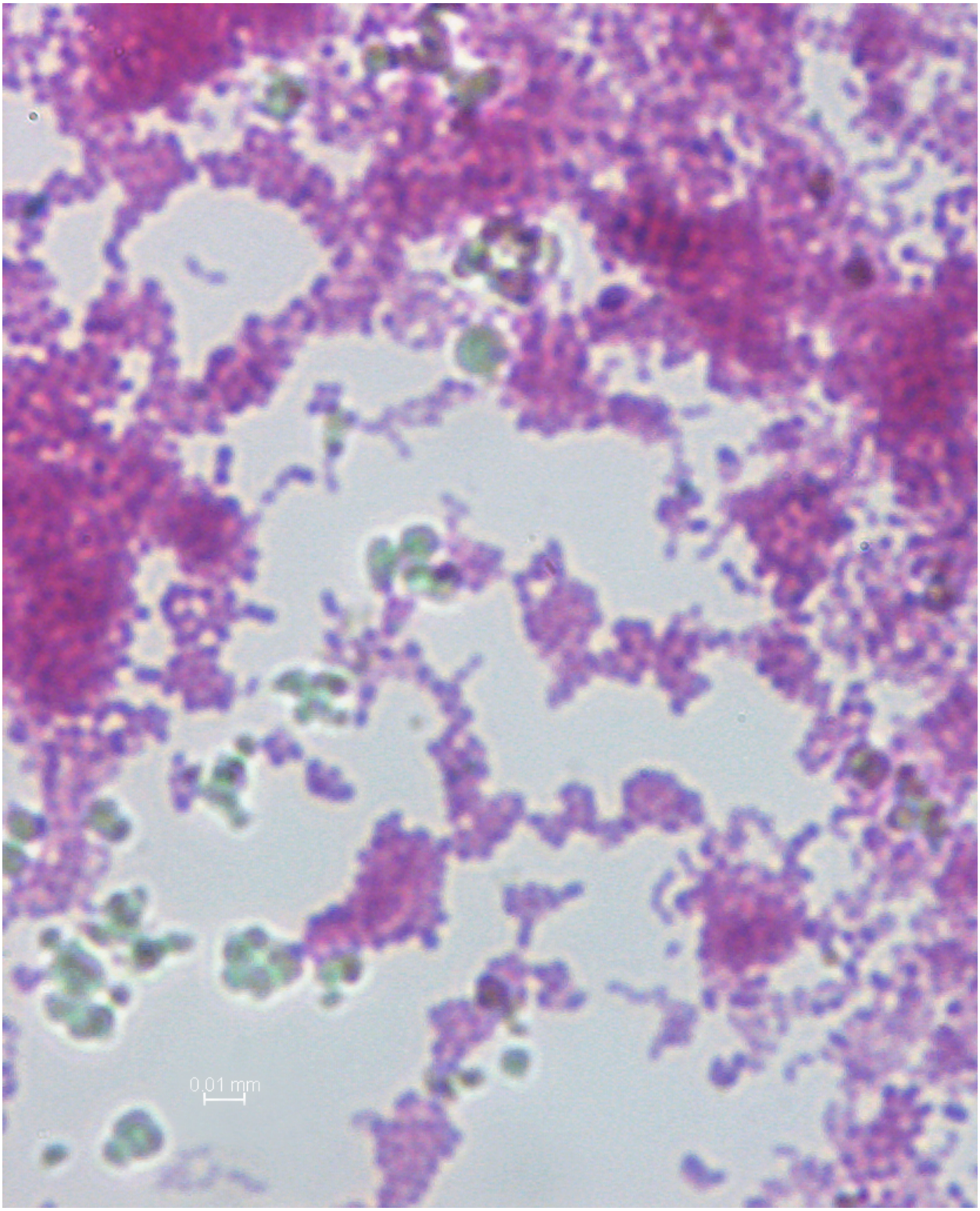

Figure 3A.

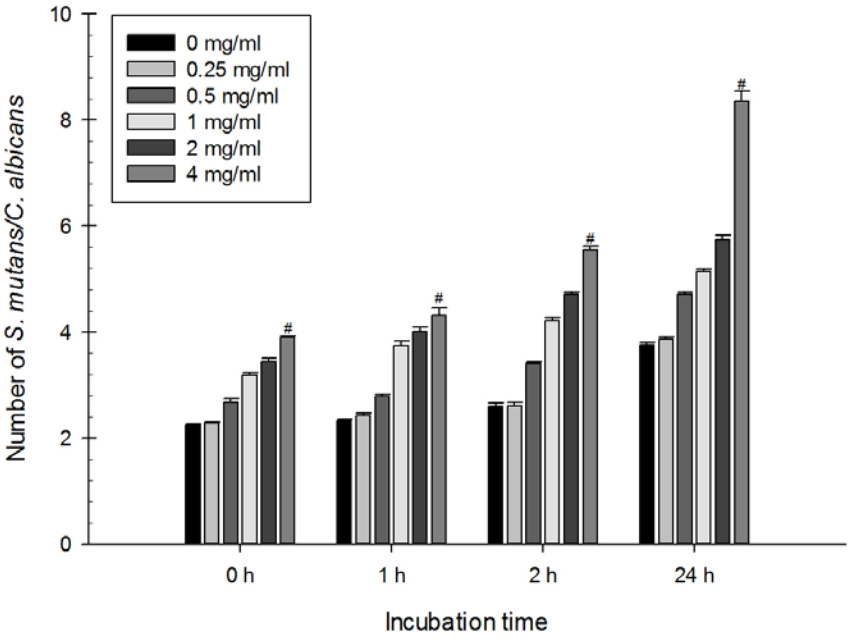

Figure 3B.

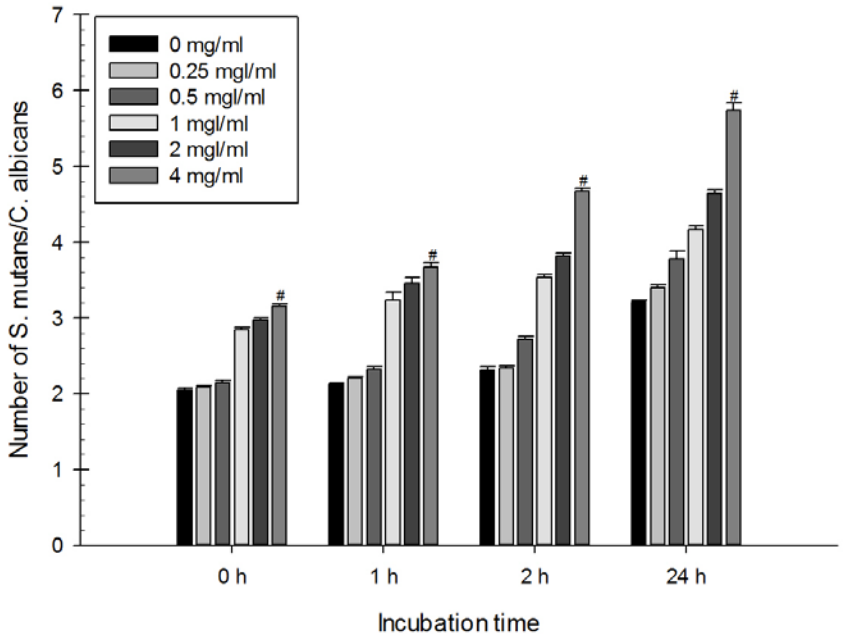

Figure 4A.

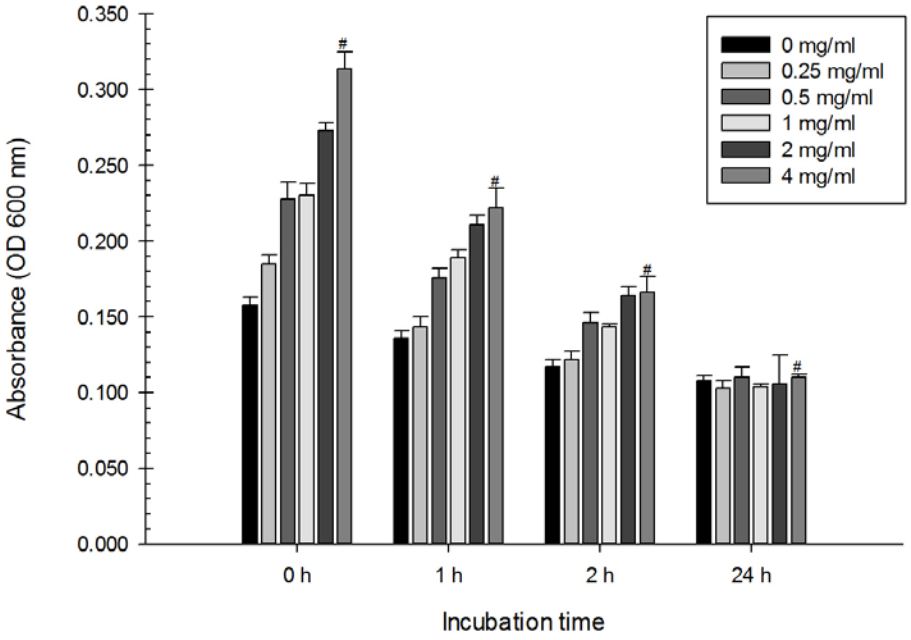

Figure 4B.

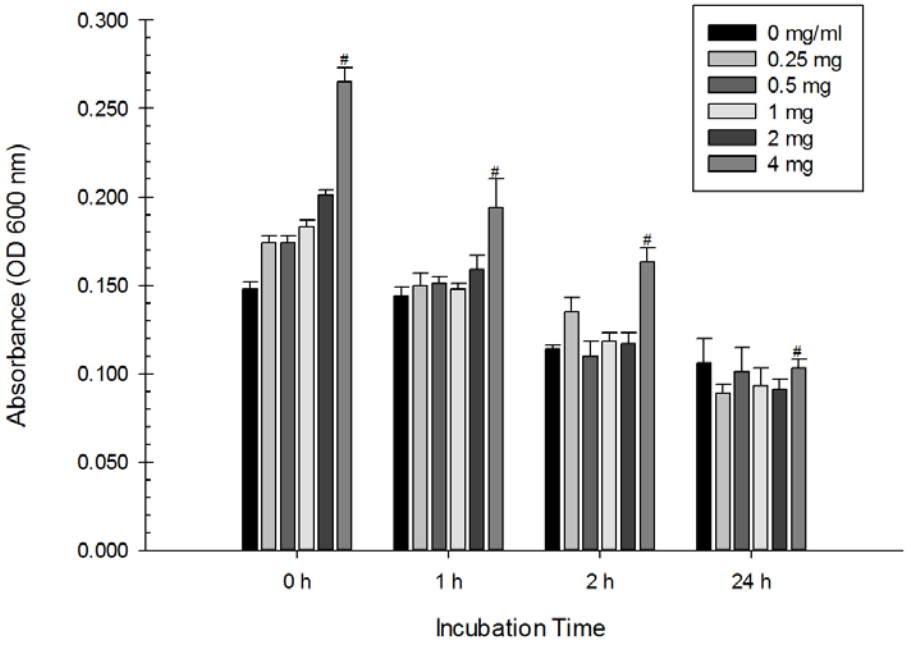

Figure 5.

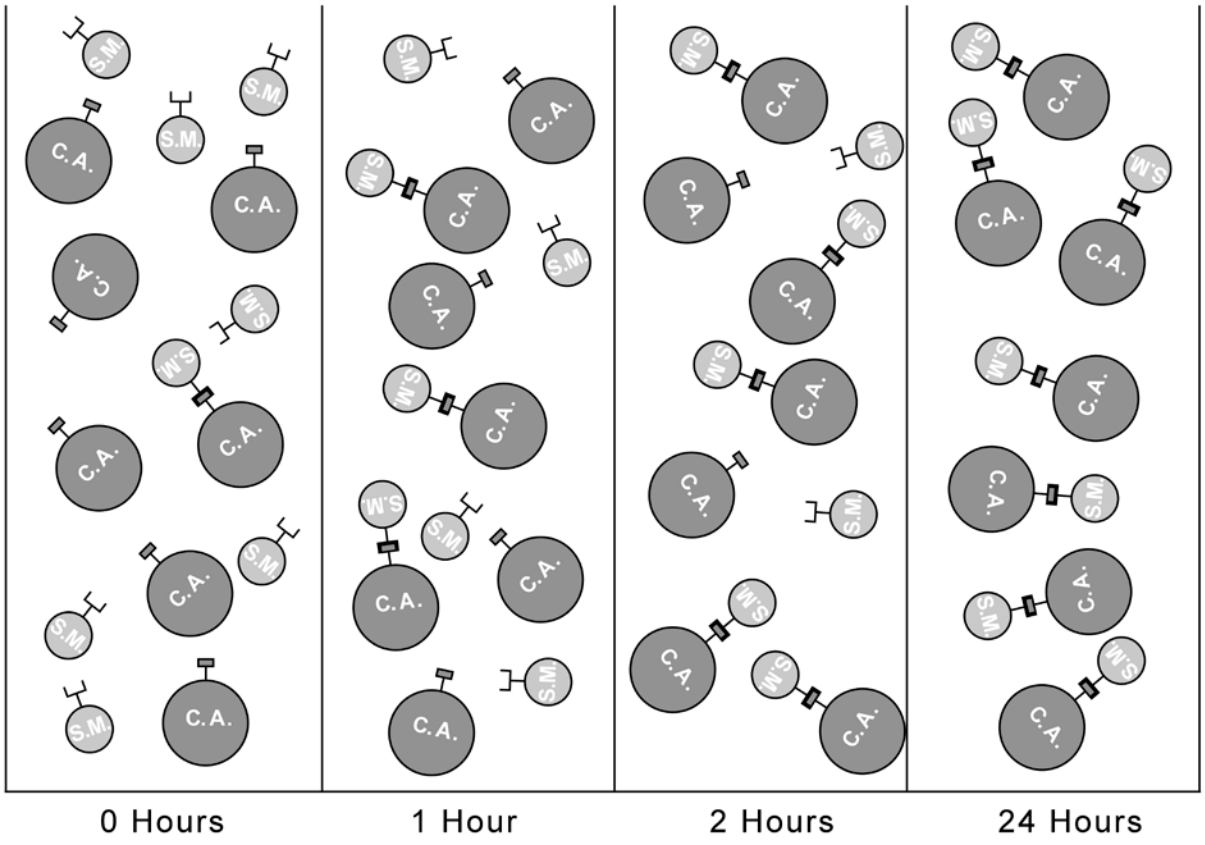

Figure legends

Figure 1. Visual aggregation scores were recorded for *S. mutans* grown in various concentrations of nicotine (0-4 mg/ml) diluted in: (A) TSBS; and (B) TSB. *C. albicans* was grown in YPD. *S. mutans* cells were mixed with *C. albicans* cells, and the scores were recorded after different incubation times (0, 1, 2 and 24 h) at 37 °C. The data are expressed as mean score ± S.E.M. # $P < 0.05$ indicates a significant increase in visual coaggregation compared to the 0 nicotine group

Figure 2. Microscopic image of the coaggregation between *C. albicans* and *S. mutans*. The larger green colored microorganisms are *C. albicans* and the smaller purple colored microorganisms are *S. mutans.* This image is similar to what was used to enumerate the number of *S. mutans* attached to *C. albicans*.

Figure 3. Microscopic analysis for *S. mutans* grown in various concentrations of nicotine (0-4 mg/ml) diluted in: (A) TSBS; and (B) TSB. *S. mutans* cells were mixed with *C. albicans* cells, which were grown in YPD, and the number of *S. mutans/C. albicans* was recorded after different incubation times (0, 1, 2 and 24 h) at 37 °C. The data are expressed as mean score ± S.E.M. # $P < 0.05$ indicates a significant increase in microscopic coaggregation compared to the 0 nicotine group.

Figure 4. Spectrophotometric analysis of coaggregation of *S. mutans* grown in various concentrations of nicotine (0-4 mg/ml) diluted in: (A) TSBS; and (B) TSB with *C. albicans*. *S. mutans* cells were mixed with *C. albicans* cells, which were grown in YPD, and the absorbances were recorded after different incubation times (0, 1, 2 and 24 h) at 37 °C. Lower absorbances indicated more coaggregation. The data are expressed as mean score ± S.E.M. # $P < 0.05$ indicates a significant decrease in absorbance compared to the 0 nicotine group.

Figure 5. Illustration model that demonstrates the possible coaggregation process of *C. albicans* and *S. mutans* with incubation time (0, 1, 2 and 24 h). In addition, it demonstrates the receptor on *S. mutans* (GtfB) that may be responsible for attachment to glucan on the *C. albicans* cell wall.