Comparison of bacterial growth in the oral cavity between tobacco consumers and non-tobacco users

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Abstract

Consumption of tobacco increases the growth of bacteria in the oral cavity. Oral bacteria cause oral diseases, such as gingivitis, which later lead to periodontitis if left untreated. If treatment is administered too late, precancer and cancer may develop. In this study I hypothesized that there would be an increase of bacteria in the mouths of tobacco consumers compared to non–tobacco users. Oral bacteria in smokers, tobacco chewers, and non–tobacco users were measured using a spectrophotometer after incubation at 37°C for a 12 hour, 24 hour, and 36 hour period. There were 15 participants per group, who were selected from the Saint Martin's University (SMU) student population and non SMU students ages 18-35. Saliva samples were obtained by swabbing the inside of the mouth. Two individual sterilized swabs were used to separately swab either the left or the right cheek. One swab was placed in a test tube with nutrient broth, and the other swab was spread over agar in a Petri dish. Both test tube and Petri dish were incubated at 37°C and the growth of bacteria was measured by recording the optical density with a spectrophotometer, and individual colonies were counted. According to the results obtained from the optical density there was a significant difference between non–tobacco users and smokers or tobacco chewers (P = 0.005) at a 12 hour period. Tobacco consumers exhibited an increase of bacteria during its 12 hour period compared to non–tobacco users. After measurements were obtained at 24 hours and 36 hours there was no significant difference between all three groups. Growth of lawns appeared on Petri dish after 24 hours, therefore colonies were not quantified.

Introduction

Smoking and chewing tobacco are serious health risks that can cause severe oral diseases and introduce pathogenic bacteria into the oral cavity. If left untreated, or if treatment is administered too late, the increase in bacteria or development of disease(s) can result in tooth loss due to unhealthy gums and can lead to costly surgical procedures. The high price that people pay for an addiction can be alarming because tobacco consumers may not realize the many negative effects that tobacco has on their lives.

Tobacco is an addictive drug that is harmful to one’s health and its use could possibly lead to death. According to Wang and Fitzhugh (1994), tobacco consumption has increased among teens throughout the United States. Tobacco is consumed in various forms, such as smoking and chewing tobacco. Consumption of tobacco leads to halitosis, stained teeth, dental restorations, halitosis, and serious diseases such as oral cancer (Patel et al., 2004), gingivitis, periodontitis (Scott and Singer 2004), melanosis (increased pigmentation on the cheeks and gums), and leukoplakia (white patches or plaques on the lining of the oral mucosa) (Srinivasan and Jewel, 2001). The use of tobacco in several forms, such as inhaled and non-inhaled (smokeless tobacco) is considered etiological (the cause or origin...
of a disease or disorder as determined by medical diagnosis) for 2.65% of all cancer cases worldwide (Patel et al., 2004). The effects of tobacco not only damage one’s oral cavity, but the respiratory tract as well.

Tobacco consumption mainly triggers oral and respiratory cancers, such that a combination of tobacco and oxygen generates free radicals and causes damage to the oral cavity and respiratory tract (Patel et al., 2004). Functional normal cells within the human physiology that contains multiple antioxidant defense systems are able to counteract these lethal effects (Patel et al., 2004). Since the incidence of oral cancer has risen, Patel et al. (2004) conducted research to study biological parameters, like antioxidant enzyme systems, that could be beneficial in early detection of oral cancer.

Patel et al.'s (2004) study of the biological parameters and the investigation of tobacco carcinogenesis (substance that causes cancer) was executed and the investigation lead to the discovery of N–nitroso (a carcinogen in tobacco) compounds as well as tobacco–specific nitrosamines, an important class of genotoxic carcinogens. An in vitro study has provided sufficient evidence that free radicals were produced during a process when the removal (from a tobacco consumer’s mouth) of smokeless tobacco contained with human oral epidermal carcinoma was incubated (Patel et al., 2004). The development of cancer varies with the form of consumption of tobacco, i.e., inhaled or non-inhaled. The effect of tobacco consumption is the production of heat during smoking, as well as the changes in pH in body fluids during chewing, thus affecting the formation and stabilization of free radicals (Patel et al., 2004). Therefore, continuous use of tobacco causes internal damages to cellular DNA by free radicals and the accretion of damages in the internal region of the oral cavity causes carcinogenesis (Patel et al., 2004). There is a precancerous disease called oral leukoplakia, which damages the oral cavity as well. In this case tobacco consumers reach this stage that leads to cancer.

Oral leukoplakia (OL) are non –removal white patches or plaques (develops into lesions) within the oral mucosa that precede the development of oral cancer (Lee et al., 2003). There are subjective signs of histological features of epithelia dysplasia, which includes basal–cell hyperplasia (abnormal increase in the number of cells in an organ or a tissue with consequent enlargement), bulbous rete ridges, loss of polarity of basal cells, abnormal keratinocyte stratification, increased nucleancytoplasmic, ratio, nuclear hyperchromatism (excessive formation of skin pigment) and pleomorphism (occurrence of two or more structural forms during a life cycle), abnormal keratinization (to produce keratin or become like keratin.), and abnormally increased numbers of mitoses and superbasal mitoses (the entire process of cell division including division of the nucleus and the cytoplasm) (Greenspan and Jordan, 2004). Microscopically diagnosing oral epithelial dysplasia indicates the risk of cancer even though dysplasia does not develop into cancer (Greenspan and Jordan, 2004). According to Sudbo et al. (2003), aneuploidy (an amount of DNA that was not an exact multiple of the diploid number) is the most promising prognostic indicator for the study of oral cancer because patients with aneuploid dysplastic leukoplakia had a low survival rate (Greenspan and Jordan, 2004). Earlier occurrence of aneuploidy (during dysplasia), and the initiation of it results in normal to malignant oral epithelium (Greenspan and Jordan, 2004). Epithelial dysplasia, or carcinomal, are the focuses on assessing oral leukoplakia (Greenspan et al., 2004).
Oral precancers and cancers may develop due to continuous use of tobacco consumption. However, early detection of oral precancer and cancer is possible by dentists. A device a dentist is able to use to detect cancer is known as Oral CDx (Bright, 2001). Oral CDx is a tiny brush used to take tissue samples from mouth lesions (Bright, 2001). The sample is placed under a high tech computer to be scanned and projects images of cancerous cells, if any are detected (Bright, 2001). The presence of cancerous cells allows the dentist to confirm his/her findings by performing a biopsy (Bright, 2001). Oral CDx was used on 945 people at the Johns Hopkins Medical Center in Baltimore, as well as other cancer centers, for detection of oral precancer and cancer, and 100% of the precancer and cancer lesions were detected (Bright, 2001). Another treatment that is being used to detect cancer is called photodynamic therapy, where patients with oral precancer and cancer orally intake a light–sensitive drug that collects the cancerous cells (Matus and Harrar, 2003). A laser then zaps the drug, releasing oxygen that eliminates cancer (Matus and Harrar, 2003). There are other procedures that help in the treatment of oral bacteria, such as gingivitis and periodontitis.

Treatable, if treatment is not too late, yet serious oral disease are gingivitis and periodontitis, which begin with bacteria formation in the oral cavity of the mouth. Inflammation arises from accumulations of oral bacteria in the gingival sulcus (a groove or depression between parts) and the occurrence of tissue changes in the periodontium (gums) (Scott and Singer, 2004). Gingivitis is apparent when redness, inflammation, and bleeding of the gums occur during brushing, or flossing. If left untreated, gingivitis progresses into a serious gum disease called periodontitis. Periodontitis occurs when one has an increased probing depth (measurement of the gums) and bone loss during a dental examination. When taking measurements (in mm) upon probing the gums, 1 – 2mm is normal, 3mm constitutes bleeding gums, and 4 – 6mm means periodontium problems. To reduce or eliminate gingivitis and/or periodontitis from reoccurring, a patient has to perform certain tasks, such as brushing the teeth in a circular motion for three minutes, flossing, and seeing a dentist every six months for cleaning. Those with a severe diagnosis must see a therapist (preferably a dentist or hygienist) to help treat the problem.

One important study that was executed tested how the effects of tobacco smoke lead to gingivitis or periodontitis (Scott and Singer, 2004). Smokers tend to have increased fibrosis and reduced signs of inflammation of the periodontium compared to non-smokers (Scott and Singer, 2004). Several observations of patients who quit after several weeks showed bleeding on probing instead of bleeding on probing while smoking. There was also less probe penetration into the tissues at the base of the pockets in the gums in smokers than non-smokers. During this period, dentists found that patients who smoke had less bleeding during probe penetration, and rare occasions of inflammation (Scott and Singer, 2004). Thus, dentists came to the conclusion that smoking can conceal the clinical signs for gingivitis and periodontitis. Because of this, it is difficult for dentists to make a proper diagnosis and to treat the patient if they have either disease.

What are the mechanisms of reducing bleeding in the periodontal tissues of smokers? Traditionally, reduced bleeding in smokers has been attributed to gingival vasoconstriction by nicotine (Scott and Singer, 2004). Though Meekin et al. (2000) used laser Doppler flowmetry to measure blood flow in the gums for smokers, before,
during, and after smoking using the Doppler flowmetry. Meekin et al. (2004) tested the blood flow in the gums of heavy smokers, light smokers, and non-smokers. There were no differences in any of his test results, other than that the Doppler flowmetry showed no signs of impaired blood flow in humans.

According to Reibel (2005), dentists play a pivotal role in preventing the effects and use of tobacco. Dentists in developing (poor) countries, created tobacco cessation activities to teach about tobacco awareness, as well as maintaining good oral hygiene. The World Health Organization (WHO) Global Oral Health Programme and the WHO Framework Convention on Tobacco Control emphasize the effects of tobacco and tobacco prevention in schools in developing countries (Reibel, 2005).

Not many people know how to maintain healthy teeth and gums, especially when tobacco usage is habitual. Without oral hygiene, one may develop pathogenic diseases such as gingivitis and periodontitis, and if treatment is administered too late, development of oral precancerous diseases that may evolve into oral cancer can affect the lives of tobacco users. In this research experiment, I compared the oral bacteria growth between three experimental groups, smokers, tobacco chewers, and non–tobacco users, and compared which group, by measuring bacteria growth of their saliva sample, was most susceptible to oral health problems in the future. I hypothesized that tobacco consumers would have more bacterial growth than non–tobacco users.

Methods

Selecting participants

I selected 15 participants per experimental group who were 1) smokers; 2) tobacco chewers; and 3) non–tobacco users from Saint Martin’s University students and non–Saint Martin’s students. For the participants who attended Saint Martin's University, I made a time sheet of times I would be swabbing, and arranged for them to come to the Biology Laboratory to be sampled. I notified them several hours before their scheduled date and reminded them to meet in the Biology Laboratory to have their saliva sample taken. For those who lived outside the Saint Martin's University campus, and were unable to meet me I traveled to their homes and took their saliva sample.

There were two methods I used to obtain and grow oral bacteria: 1) I swabbed the back of the right cheek with an individual sterilized swab and grew the culture from the saliva sample in a test tube and, 2) with a different individual sterilized swab I swabbed the back of the left cheek, and spread the swab onto the Petri dish.

Preparing the nutrient broth and agar

To prepare the nutrient broth, I used a 1 L flask and dissolved 4 g of Bacto™ Luria Broth Base (3g/L Beef Extract; 5g/L Bio-Gel Peptone; pH 6.8) into 500 ml of distilled water. Next, I heated the nutrient broth on a hot plate, used a stirring rod to dissolve the powder, poured the 167 ml of broth into three glass bottles with screw cap, and I sterilized them in the autoclave (Barnstead 14-48823) at 121ºC for 15 minutes at 15 psi. I allowed the nutrient broth to cool to 45ºC–50ºC, and poured 25 ml into 20 test tubes. I then autoclaved the test tubes, and placed them in the 4ºC refrigerator. To prepare the agar, I used a 1 L flask to dissolve 11.5 g of Difco™ Luria Agar Base powder (Pancreatic Digest of Casein 10g/L; Yeast Extract 5g/L; Sodium Chloride 0.05g/L; Agar 15g/L) in 500ml of distilled water. Next, I heated the agar over a hot plate, and used a stirring rod to dissolve the powder. I used a thermometer to check the temperature until it reached
75ºC, and placed the agar over a wire plate to cool to 50 ºC. Then, I poured 167 ml of the agar into three separate glass bottles with screw caps, and autoclaved them at 121ºC for 15 minutes at 15 psi. I allowed the agar to cool to 50ºC and poured the 20 ml of agar on 25 Petri dishes. I allowed the plates to sit overnight, and the next day I wrote my name and date on each of the plates and placed them in a 4 ºC refrigerator. I had to repeat the process of preparing nutrient broth two more times and agar one more time to sample all my participants.

1st trial of swabbing subjects mouths and growing bacterial cultures

While taking samples from my subjects, I wore my protective gear, which consisted of goggles, lab coat, and gloves. When the participants arrived, I told them to rinse their mouth three times using water from the water fountain. This was to ensure the removal of contaminants from their mouths. I also had each person fill out a short anonymous information form (Table 1).

Table 1. Anonymous Information Form.

<table>
<thead>
<tr>
<th>Gender (circle one)</th>
<th>Male</th>
<th>Female</th>
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</thead>
<tbody>
<tr>
<td>Age:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Are you… (circle one)</td>
<td>Smoker</td>
<td>Tobacco Chewer</td>
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<tr>
<td></td>
<td>1-2 packs per day</td>
<td>1-2 cans per day</td>
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<tr>
<td></td>
<td>Less than 1 pack per week</td>
<td>Less than 1 can per week</td>
</tr>
<tr>
<td></td>
<td>Greater than 1 pack per week</td>
<td>Greater than 1 pack per week</td>
</tr>
</tbody>
</table>

2nd trial of swabbing subject’s mouths and growing bacterial cultures

First, I swabbed the back of the participant’s left cheek. Next, I spread the swab across the Petri dish, labeled the Petri dish, and placed it in an incubator at 37ºC. After 12 hours, I counted the number of colonies by placing the Petri dish upside down and marked each colony with a felt tip pen (Brown, 2005). Every 12 hours for 36 hours, I counted the colonies and recorded my data.

Measuring samples

After 12 hours, I used a spectrophotometer (SPECTRONIC 20s, Bausch and Lomb) to measure the growth of the culture (Brown, 2005). Fifteen minutes before the culture in the test tube reached its 12 hour peak I turned on the spectrophotometer and adjusted the wavelength knob to 686 nm because it is the
required wavelength for the nutrient broth, and set the meter at zero using the left–control knob (Brown, 2005). Next, I rinsed out the cuvette with distilled water, put 10 mL of nutrient broth into a cuvette as a blank, and placed the cuvette into the sample holder. I rotated the right–control knob so that the meter read 100% transmittance, and when I removed the cuvette from sample holder the transmittance went back to zero (Brown, 2005). If it did not, I repeated the process again until there was 100% transmittance, and the meter read zero (Brown, 2005). Next, I shook the test tube to mix the contents inside and transferred approximately 7 ml of the contents from the test tube to a clean cuvette (Brown, 2005). I recorded the absorbance and % transmittance. Higher absorbance indicated a higher concentration of bacteria in the sample used. I then disposed of the sample into a beaker, and rinsed the cuvette with distilled water several times before I used it for the next sample. I then placed the cuvette containing the nutrient broth back to the sample holder to ensure that there was 100% transmittance before using my next sample. I checked the growth of bacteria every 12 hours for 36 hours.

Statistical analysis

I used an ANOVA (analysis of variance) test using Minitab (Minitab Inc, 2005). This test was used to compare the three groups to see if there was a significant difference in bacterial growth among the three groups: smokers, tobacco chewers, and non–tobacco users. I calculated optical density by using the formula O.D = log (100/%T). I also used a Tukey test, which is a direct test to compare the groups to each other to test multiple comparisons.

Photographs

Every 12 hours for 36 hours I took photographs of the turbidity of the cultures in the test tube, to show the growth of bacteria. I also took pictures every 12 hours for 36 hours of the Petri dishes, to show the growth of colonies and the lawns.

Results

Figure 1 shows how, after swabbing the participant’s oral cavity, the swab was placed in a test tube with nutrient broth and then incubated at 37°C for 24 hours. The turbidity of bacterial growth from the saliva sample was measured using a spectrophotometer. Higher bacterial growth resulted in higher turbidity and a higher optical density reading. Lower bacterial growth resulted in lower turbidity and a smaller optical density reading.

At 12 hours the growth of oral bacteria obtained from smokers, tobacco chewers, and non–tobacco users were significantly different (F = 10.50; d.f. = 2; P = 0.005). Since P < 0.05, there was a significant difference and, therefore, the null hypothesis was rejected. The growth of bacteria between tobacco users (smokers and tobacco chewers) and non–tobacco users was significantly different from each other according to the 98.07% Confidence Interval using a Tukey Test. According to the Tukey Test, non–tobacco users had less bacterial growth than either smokers or tobacco chewers.

When samples were measured at 24 hours, the growth of oral bacteria obtained from smokers, tobacco chewers, and non–tobacco users showed no significant difference (F = 2.03; d.f. = 2; P = 0.144). Since P > 0.05, the null hypothesis (there was no difference in bacterial growth between the three groups) was accepted.

The growth of oral bacteria obtained from smokers, tobacco chewers, and non–tobacco users at 36 hours was also not significantly different (F = 2.87; d.f. = 2; P = 0.068). Since P > 0.05, the null hypothesis
(there was no change in bacterial growth, therefore there was no difference) was accepted.

Figure 3 presents the mean and standard deviation of the bacterial growth optical density among the three groups. Each bar represented the growth of bacteria taken at 12, 24, and 36 hours. The graph depicts that throughout a 36 hour period the mean of optical density for non–tobacco users was slightly lower than for smokers and tobacco chewers.

Figure 1. This swab was used to obtain a saliva sample from the oral cavity of a smoker. It was incubated in a test tube with nutrient broth at 37°C for 24 hours and the concentration of the bacteria was measured using a spectrophotometer.

Figure 3. The measurements of oral bacterial concentrations obtained from smokers, tobacco chewers, and non–tobacco users. The absorbance of the bacteria was measured by a spectrophotometer. There were 15 participants in each group and each bar represents the mean of bacterial growth at 12, 24, and 36 hours. Error bars represent one standard deviation about the mean.
To examine bacterial colony growth on a Petri dish, a swab with the saliva sample obtained from the participant’s mouth was spread across the agar. After being incubated at 37°C for 24 hours colonies were formed, and quantified (Figure 2). However, due to the growth of lawns Petri dishes were not included and were not quantified.

Discussion

My research showed a significantly higher number of bacteria in saliva samples incubated for 12 hours at 37°C from smokers and tobacco chewers as compared to non–tobacco users (P = 0.005). So, during its initial growth period (12 hours), the reproduction of bacteria exhibited a significant difference by producing higher bacterial growth in smokers and tobacco chewers compared to non–tobacco users. This result supported my hypothesis, that tobacco consumers contained more bacterial growth in their oral cavity compared to non–tobacco users. Results indicated that the mean of optical density for smokers at 12 hours was 0.077, and 0.077 for tobacco chewers, compared to non–tobacco users who had a mean of 0.033. According to Reibel (2005), habitual use of tobacco consumption and poor oral hygiene can lead to both harmless (treatable) and life–threatening diseases such as, oral cancer.

At 12 hours the optical density of smokers and tobacco chewers was extremely high, due to the initial growth rate for bacteria to reproduce at an optimal temperature. Contrary to expectation, the measurements obtained at 24 hours and 36 hours showed no difference in bacterial growth between all three groups. After a 24 hour period, bacteria may have grown exponentially, because there were enough nutrients in the nutrient broth for bacteria to feed on, and the environment they grew in was within an optimal temperature. This would result in high growth in all groups, and therefore, would not be as direct of a measure or oral bacteria from a participant.

Bacterial colonies were also grown on a Petri dish. When measurements were taken at 12 hours, there were 0-5 colonies in each Petri dish. However, the growth of lawns after 24 hours made it impossible to identify colonies, therefore colonies were not quantified. Lawns may have reproduced rapidly and successively due to the rich
nutrient contained in the agar, as well as being incubated at an optimal temperature, 37ºC.

Results suggest that saliva samples distributed in non-sterile cuvettes may have slightly disrupted the data collection of optical density because cuvettes may have contained bacteria from the previous sample.

For future experiments, times to collect data could be changed. Instead of measuring bacteria every 12 hours, I would measure the growth of bacteria every hour for 12 hours. This was due in part by my study, and how I found through my results that a significant difference among the three groups appeared at 12 hours. If I checked the rate of bacteria through a 12 hour period I may find a more concrete result. I would also increase the sample size for each group, because a larger sample size would provide a broader spectrum of bacterial growth among tobacco consumers and non-tobacco users. Temperature changes would be interesting to change because I would like to see the rate of bacteria reproduce at different temperatures, and to see the effects on the reproduction stage. Would bacteria reproduce faster, slower, or would there be any changes at all? I would also like to add to my experimental group the different types of tobacco usage, such as cigar smokers, pipe smokers, and those who add other chewing substances (betel nut, lime paste, kava leaf, and snuff) to chewing tobacco.

Poor oral hygiene and tobacco consumption plays a critical role in the development of treatable diseases such as periodontitis and gingivitis (Scott and Singer, 2004). However, if tobacco consumers are often addicted to tobacco usage, non-treatable diseases such as, oral cancer may occur (Patel et al. 2004). There are several ways to maintain good dental hygiene, such as brushing and flossing twice a day, visitation to the dentist every six months for cleaning, and quitting the habit of tobacco use. These habits are easy to follow, and at the same time customarily practicing them plays a vital role in maintaining a functional oral cavity. Even if one is not a tobacco user, practicing good dental hygiene is of great importance. Reducing or even quitting tobacco usage is extremely important when talking about health risks in the long run, as well as subsiding the growth of oral bacteria.

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Literature Cited


