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The Comparison of the in Vitro Effects of Cigar and Cigarette Smoke on the Growth of Streptococcus Mutans and Streptococcus Sanguis

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ABSTRACT

Streptococcus mutans (s. mutans) and *Streptococcus sanguis*(S.sanguis) are two important bacteria of the oral microflora. *S. mutans* is the most important cause of dental caries and *S. sanguis* is one of the major bacteria that play an important role in formation of microbial plaque. The aim of this study is to determine the effect of cigar and cigarette smoke on the growth of streptococcus mutans and streptococcus sanguis. The standard strains of s.mutans (ATCC25175) and sanguis(ATCC10550) were cultured on blood agar and incubated for 48h in different environments: atmospheric air, microaerophillic, carbon dioxide, cigar and three type of cigarette smoke (Winston ,ultralight Winston and kent). Then digital photographs of the colonies were taken and the diameter of the colonies was measured. Data were analyzed using post hoc and general linear model statistical tests. Both cigar and cigarette smoke significantly increased the growth of s.mutans and s.snguis (p=0/000). In almost all environments except cigar smoke, the growth of s.mutans was more than s.sanguis. The diameter of s.mutans colonies in dioxide ,microaerophilic, cigar and cigarette smoke (Winston,ultra light wniston and kent) showed in order: 82%, 65%, 69%, 106%, 92% increase and the diameter of s.sanguis showed in order: 45%, 53%, 137%, 89%, 40%, 55% increase. These findings indicate that cigarette smoke increases the s.mutans/s.sanguis ratio but cigar smoke decreases this ratio.

Introduction

Oral cavity contains one of the most variable microflora of the human body. Approximately there are 400 different species of microorganisms in the oral cavity. In each mililiter of saliva, more than 106 microorganisms can be found (Bagg 1999). *Streptococcus mutans* (s. mutans) and *Streptococcus sanguis*(S.sanguis) are two important bacteria of the oral microflora. *S. mutans* is the most important cause of dental caries. *S. sanguis* is considered as one of the major bacteria that play an important role in formation of microbial plaque. The higher ratio of *S. mutans* to *S. sanguis* increases the possibility of dental caries. Actually the non-cariogenic Streptococci which are a part of normal flora of the oral cavity may be useful in the control or restriction of infectious microorganisms. (Roberson and Lundeen 2002).

Cigar and cigarette smoke can affect the microbial ecosystem of the oral cavity (Marcotte and Lavoie 2002). Some authors consider them as a cause of bacterial growth (Väänänen 1994, Heintze 1984) while some others have a contrary opinion (Marsh 1999, Ertel 1991).

The process of combustion of cigarette and cigar is quite complicated, and there is no clear correlation between the inhaled smoke and the ingredients of cigar or cigarettes themselves (Carmines 2002) but generally the composition of cigar and cigarette smoke depends on the type of tobacco, the ingredients added to the tobacco and the design of fabricator (Talhout 2006).

The cigar and cigarette smoke has many carcinogenic and poisoned substances, like carbon monoxide, ammonia, nicotine, benzene, arsenic, hydrogen cyanide, Cadmium, etc. These substances are more concentrated in cigar combustion than in cigarette (Rustemeier 2002). It is believed that the cigar may increase the probability of the formation of oral cancer (Seri 1999), decrease the saliva quantity as well as the igA secretion of Saliva (Van 2001, Barton 1990), change in tooth color, delay in wound reconstruction, decrease in the capacity of distinguishing different odors and tastes and can also create stomatitis, leuckoplakia, gum loss, hairy tongue, gum diseases, melanotic pigmentations and oral candidiasis (Seri 1999).

There is a huge controversy in the literature about the effect of smoking on dental caries. The early studies suggested that tobacco smoking decrease the dental caries (Sgan-Cohen 2000, Gibbs 1952, Zitterbart 1990, Reibel 2003), but Ludwick et al. reported that smoking increases the cariogenic process of oral microorganisms (Ludwick 1952). Some other studies confirmed this relationship (Heng 2007, Ainamo 1971, Hirsch 1991, Heng 2006). Most of investigations agreed that there is a correlation between cigar and cigarette smoking and the increase of dental caries, but to reach a direct etiological relationship, further studies are needed (Vellappally et al. 2007, Vellappally et al. 2008, Aguilar-Zinser 2008, Locker 1992).

Some studies suggest a direct relationship between cigar smoking and increase in cariogenic microorganisms in passive smokers (Lindemeyer 1981, Williams 2000, Aligne 2003, Shenkin 2004, Aligne 1997, Leroy 2008, Tanaka 2005).

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However, in a recent study in Japan, authors couldn't establish a direct correlation between increase of dental caries and passive cigar smoking among the Japanese children (Vellappally 2007). The purpose of this study is to determine the in vitro effect of cigar and cigarette smoke on the growth of *S. mutans* and *S. sanguis* and compare them with each other.

Materials and Methods:

For this experimental study, standard strains of *S. mutans* (ATCC 25175) and *S. sanguis* (ATCC 10556) were obtained from the Iranian Organization of Scientific and Technical Researches and propagated in our laboratory.

The sample size for each strain was determined at least 61 colonies according to Cohen table by using the effect size of 0.5 and (1-B = 0.99) α=0.5; however in this study 420 colonies of each strain (a total of 840 colonies) were used in order to have a more precise study.

The materials and equipments used in this study:

1- Blood agar as the non selective culture environment for most of the m.o.40 gr. of the base blood agar (KFG-Media 1323-p 200) was mixed in 1 Liter of distillated water and heated until complete dissolution. Then it was autoclaved at 121˚c of temperature and under the pressure of 15 pounds. After that, it was cooled until 70 – 80˚c of temperature. Then sterile defibrinated blood (5 – 8 %) was added to it. The resulted solution was stored in sterile plates (4 mm of thickness in each plate). To prevent any contamination, plates were kept in the refrigerator, until the main steps started.

2- Seven equi-size crystal jars. By having a lightened candle in each one approximately 5 to 10 % of Co2 was created.

3- Incubator: An apparatus to keep the microorganisms. under a suitable temperature during their growth period.

To evaluate the effect of cigarette and cigar's smoke, three different cigarette brands with variable nicotine and tar concentrations and one cigar type were selected. This selection was carried out according to have the highest difference between their nicotine and tar concentration. The cigarettes' brand and their nicotine and tar concentration are provided in Table 1.

Table 1. The nicotine and tar concentration per cigarette for different cigarette brands (mg)

	Nicotine	Tar
Winston	1	14
Winston Ultralight	0.5	6
Kent (Light)	0.1	1

First liquid culture environment was provided by dissolving 8 gr. Of brass nitrite (Merk-1-05443) in one liter of distilled water and autoclaved under 121$^{\circ C}$ of temperature during 15 minutes. Each strain of *S. mutans* and *S. sanguis* was incubated separately in different liquid culture and a suspension was provided. 14 plates of blood agar were used (totally 28 plates) for culturing of each strain. Then 0.1 ml of bacterial suspension was spread on each blood agar plate. The plates were coded according to the strain of bacterium and the environment of incubation. The plates were transferred to the jars. The lightened candle and also the lightened cigar and cigarettes were collocated in the jars. The jars were incubated at 37˚c in seven different environments, i.e., Atmospheric air, increased CO2, microaerophilic, cigar and three types of cigarette (Kent, Winston and Winston ultralight) smokes for 48 hrs. Candle was used to provide 5-10% of CO2 and lighted cigarettes or cigar in the closed jars were used to produce smokes. In all cases, the cigarettes and cigar burned to about half of their length that was because of the depletion in oxygen contents of the jar.

After 48 hours of incubation all the plates were removed from the incubator and were photographed by using the same photography protocol (same digital camera, constant distance between the camera lens and the plate, shutter speed, diaphragm size and focus center). All the taken photographs were analyzed for measuring the diameter of colonies by using the Adobe Photoshop software as will be described later (Zonuz 2008). In brief, the colonies diameters were analyzed in Photoshop software with a maximum application of digital zoom. Pixels that formed the pictures of colonies were counted. The area of each pixel was 0.1 mm2. Where the distance between colonies was less than two mm, they were excluded because it was not possible to have an accurate measurement in a so concentrated area. The colonies were assumed flat and their areas were obtained and then the diameter of each colony was calculated.

To calculate the real colonies' diameters, when the plate size of the colony diameter was changed at the photographic time, we used the following formula:

$$\textbf{Real Colony Diameter} = \frac{r}{ZP} * ZC \quad (1)$$

Where r and ZP are the real plate diameter (mm) and the zoomed plate diameter (mm) and ZC is the zoomed colony diameter in computer (mm) during the photographic time.

It should be noticed that the convex surface of colonies in our two-dimensional screen was assumed to be flat. The assessment had been done on 420 colonies for each strain (totally 840 colonies) in seven different environments.

The data was analyzed using General Linear Model (GLM) and Post Hoc statistical tests.

Figure 1 presents a view of *S. mutans* and *S. sanguis* colonies in some of the investigated environments.

a) S.mutans Colony near to atmosphere

b) S.mutans Colony near to Winston (ultralight)

c) S.sanguis Colony near to atmosphere

d) S.sanguis Colony near to cigar

Figure 1. Example cases of bacterial colonies in different environments

Results:

The smallest S. *mutans* colony diameter was in atmospheric air environment (0.319 mm) and the largest one was in ultralight Winston environment (0.95 mm). Table 2 shows the s.mutans mean colony diameters in all environments. By using the Post Hoc analysis, the mean colony sizes were compared in different environments. The differences were statistically significant in all cases except in CO2 environment with Winston cigarette and the microaeropillic environment with cigar ($p<0.05$).

Table 2. The *S. mutans* colony diameters in different environments (mm)

Environment	Count	Diameter (Average)	Diameter (Minimum)	Diameter (Maximum)
Atmosphere	60	0.3686	0.319	0.426
CO2	60	0.6706	0.488	0.917
Microaeropillic	60	0.6115	0.53	0.73
Cigar	60	0.6232	0.428	0.775
Winston	60	0.6645	0.55	0.85
Winston(ultralight)	60	0.7613	0.64	0.95
Kent	60	0.7087	0.555	0.822

Table 3 presents mean diameter differences of S. mutans in different environments. The highest mean difference was related to the pair of atmospheric air environment and ultralight Winston cigarette (0.3928 mm).

Table 3. Mean diameter differences of S.mutans colonies in different environments

P-value	A – B	second Environment	First Environment
0.000	0.3021	CO2	Atmosphere
0.000	0.2430	micro aeropillic	Atmosphere
0.000	0.2546	Cigar	Atmosphere
0.000	0.296	Winston	Atmosphere
0.000	0.3928	Winston(ultralight)	Atmosphere
0.000	0.3401	Kent	Atmosphere
0.000	0.0591	micro aeropillic	CO2
0.002	0.0475	Cigar	CO2
0.999	0.0061	Winston	CO2
0.000	0.0907	Winston(ultralight)	CO2
0.034	0.038	Kent	CO2
0.964	0.0117	Cigar	micro aeropillic
0.000	0.053	Winston	micro aeropillic
0.000	0.1498	Winston(ultralight)	micro aeropillic
0.000	0.0972	Kent	micro aeropillic
0.014	0.0413	Winston	Cigar
0.000	0.1381	Winston(ultralight)	Cigar
0.000	0.0855	Kent	Cigar
0.000	0.0968	Winston(ultralight)	Winston
0.007	0.0442	Kent	Winston
0.000	0.0526	Kent	Winston(ultralight)

The smallest *S. sanguis* colony diameter was found in the atmospheric air environment(0.35 mm) and the largest one was observed in the ultralight Winston environment (1.481 mm). Table 4 shows the *S. sanguis* mean colony diameters in different environments.

Table 4. Mean diameters of S. sanguis colonies in different environments (mm)

Environment	Count	Diameter (Average)	Diameter (Minimum)	Diameter (Maximum)
Atmosphere	60	0.5369	0.35	0.649
CO2	60	0.7806	0.677	0.894
micro aeropillic	60	0.8219	0.634	1.06
Cigar	60	1.2749	1.115	1.456
Winston	60	0.7542	0.641	0.868
Winston(ultralight)	60	1.0175	0.681	1.481
Kent	60	0.8369	0.723	1.03

The differences among the means of all groups were significant except in the four cases, i.e.,

Co2 environment compared to Kent cigarette, Co2 environment compared to microaerophillic environment, Co2 environment compared to Winston cigarette and microaerophillic environment in comparison with Kent cigarette.

Table 5 shows mean diameter differences of S. sanguis in different environments. The highest mean value of differences was observed between the atmospheric air environment and cigar's environment (0.7379 mm).

Table 5. Mean diameter differences of S.sanguis colonies in different environments

P-value	A – B	second Environment	First Environment
0.000	0.2436	CO2	Atmosphere
0.000	0.2850	micro aeropillic	Atmosphere
0.000	0.7379	Cigar	Atmosphere
0.000	0.2173	Winston	Atmosphere
0.000	0.4805	Winston(ultralight)	Atmosphere
0.000	0.3000	Kent	Atmosphere
0.531	0.0414	micro aeropillic	CO2
0.000	0.04943	Cigar	CO2
0.908	0.0263	Winston	CO2
0.000	0.2369	Winston(ultralight)	CO2
0.166	0.0563	Kent	CO2
0.000	0.4530	Cigar	micro aeropillic
0.046	0.0677	Winston	micro aeropillic
0.000	0.1955	Winston(ultralight)	micro aeropillic
0.995	0.0150	Kent	micro aeropillic
0.000	0.5206	Winston	Cigar
0.000	0.2574	Winston(ultralight)	Cigar
0.000	0.4380	Kent	Cigar
0.000	0.2632	Winston(ultralight)	Winston
0.000	0.0826	Kent	Winston
0.000	0.1806	Kent	Winston(ultralight)

According to the results, we found that the growth of both bacteria was increased in different environments compared to their growth in the atmospheric air. The ratio of these two increase rates is shown in Figure 2.

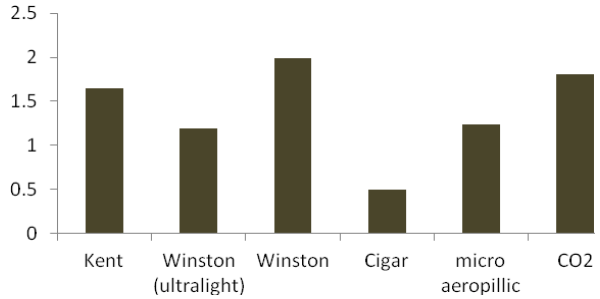

Figure 2. The ratio of increase in S mutans's growth rate to the increase in S sanguis's growth rate (S.mutans/S.sanguis) in different environments

To determine the reciprocal effect of the bacterial type strain and the type of culture's environment on the colony's mean growth, the test of General Linear Model (GLM) was used. The results showed that the colony's growth in different environments has a strong dependency on the type of the bacteria (P=0.000).

Discussion:

The current study indicates the significant difference of *S. mutans* and *S. sanguis* growth in the environments of cigar, cigarettes, CO2 and microaerophillic, comparing with the atmospheric air. This means that these bacterias showed an increased growth under these environments, in comparison with the atmospheric air.

In all cases *S. mutans* showed higher growth compared to *S. sanguis*, except in the vicinity of the cigar's smoke environment.

The proportion of increase in the growth of *S. mutans* compared to *S. sanguis* in cigar environment was 0.5, while this proportion was between 1.2 and 1.9 in other environments.

There are a few studies which have assessed the effect of cigarette's smoke on S. sanguis's growth. Zonuz et al evaluated the effect of cigarette's smoke on the growth of *S. mutans* and *S. sanguis* (Zonuz 2008). However; in their investigation the effects of low O2 pressure (microaerophilic) and cigar smoke were not studied.

Some of the previous investigations indicated a stimulated growth of *S. mutans* in the cigarette smoke environment but some others showed a decrease in the *S. mutans*'s growth. Vaananen et al. reported that the growth of cariogenic bacteria in passive smokers are higher than their growth in non-smokers (Väänänen 1994). Heintze and Sakki reported independently that the number of *S. mutans* and lactobacilli are higher in smokers compared to non-smokers (Heintze 1984, Sakki 1996). Based on the Bagg's report (1999), it can be concluded that the cigarette smoke has more inhibitory effect on the growth of gram positive cocci than gram negative bacilli.

Most of the mentioned studies have been performed clinically (in vivo) and a wide range of variables may have interfered to achieve a certain conclusion. Cigarette type, quantity of nicotine and tar, and the cigarette consuming time can be found among these variables.

Our study showed that the rate of *S. mutans* and S.Sanguis growths in cigar and different cigarette environments is variable. The highest growth of *S. mutans* occurred in the ultralight Winston cigarette smoke environment, while *S. sanguis* was shown to have highest growth in the cigar smoke environment (Tables 2 and 4).

In Johnson and Keene's investigation, they found that the nicotine's effect on the growth of *S. mutans* depends on its dose. They concluded that *S. mutans*'s growth is inhibited in high nicotine concentrations, increased in intermediate concentrations and decreased in low concentrations (Keene 1999). Nevertheless, in 2008, Zonuz et al. found that there is a steady augmentation in the increase of *S. mutans* and *S. sanguis* growth as the nicotine content of the cigarettes increases (Zonuz 2008).

There is also another similar study conducted by Cogo et al. (2008) that couldn't support the relation established by Johnson and Keene. They concluded that nicotine didn't have any effect on the bacterial growth (Zonuz 2008).

There are a number of differences between Johnson's study and the current one. The differences are about the effect of nicotine dose on *S. mutans*'s growth. In Johnson's study, just the pure nicotine's effect was evaluated, whereas in our investigation, the whole effect of cigar and cigarette smokes was examined. The mentioned smokes compared to the pure nicotine have much more ingredients that could affect the bacterial growth. It is interesting that when *S. mutans* was near to the Kent's cigarette smoke (which has less nicotine), more bacterial growth compared to Winston and less bacterial growth compared to the case of near Winston Ultralight was observed.

In this study, in order to evaluate cigar and cigarette's effect, all environments were installed in jars, and the cigar and cigarette were then added to them. The combustion of cigar and cigarette necessarily consumes O2 and thus, the Co2 will be produced. According to the existing studies, many viridances of streptococci, can survive better in anaerobic or microaerophilic condition compared to the air atmospheric condition. Also, more than 135 varieties of Stereptococci, which are called Capnophillic show a better growth near to 5-10% of Co2. S. mutans and S. sanguis are included in this group (Pulliam 1980). In other words, it seems that in addition to cigarette smoke's ingredients, the two factors of low O2 and high Co2 have influence on bacterial growth. In order to assess these two factors, we evaluated these environments, separately.

However, we cannot relate the increased growth, to only decrease in O2 and increase in Co2 pressure. If it was so, the bacterial growth in microaerophilic condition, next to co2, next to cigar and different cigarette's environments, should have been the same. However, checking tables 3 and 5, it can be seen that the mean diameter difference of S. mutans and S. sanguis colonies in most environments of cigar and cigarettes, comparing with co2 and microaerophilic environment is significant.

In the current investigation, compared to the study of Zonuz et al., the range of S. mutans and specially S. sanguis growth augmentation was greater in cigarette and Co2 environments. Among the causes of this difference, various nutritive materials available in different environments are mentionable. In spite of using blood agar in both studies, the nutritive materials used in their environments might have been different.

The low level of PH in culture environment decreases the bacterial growth. Thus, the difference of PH, is likely to be one of the factors that affect the differential bacterial growth observed in our culture environment and Zonuz's environment. Among the variables that cause the higher growth of *S. mutans* (compared to S. sanguis) in cigarette, Co2 and microaerophilic environments, we can mention the lower oxidation _ reduction potential of S. mutans compared to that of S. sanguis. Therefore, in microaerophilic environment, it shows higher growth compared to S. sanguis (Marcotte 1998, Heng 2006, Rustemeier 2002). However, to clarify this subject completely, further investigations are needed.

According to the obtained results, increase in S. mutans's growth was low only in cigar environment compared to S. sanguis, and this range of decreased growth is likely to be a result of different ingredients, for example, more sugar is contained in cigar (compared to cigarette) in order to give a better taste and smell. Fulker et al. (1987) observed that the tobacco's extract lacking sucrose in culture environment of BHI and BSS had an augmenting influence over the growth of S. mutans and S. sanguis and S. Salivarius, whereas in the sucrose-containing one only S. sanguis increased. observed From the combustion of sugar, some compositions like acetaldehyde, Formaldehyde, etc will be released (Shenkin 2004, Keene 1999).

Nils Homan et al's study (2000) showed that the high level cigar consumption will increase the saliva's acetaldehyde which will increase s.salivarius, s. viridance Corheane bacterium and yeast. Whereas S. mutans' growth in both smokers and non-smokers was equal (Homan 2000). Thus, the acetaldehyde resulted from sugar combustion can detain the S. mutans's growth by the help of other ingredients included in cigar's smoke. It seems that this may be only one possible contributing factor which differently affects the growth of S. mutans (compared to S. sanguis) next to cigar's smoke.

Conclusions:

In the Co2 environment, both *S. mutans* and *S. sanguis* had an increase in their growth: (S. MUTANS: 81.95 % and S. sanguis: 45.37 %). Both bacteria showed an increase in their growth in microaerophillic environment, though the difference between them was not very significant (S.mutans: 62.92 % and S. sanguis: 52.08 %). In cigar's environment, the growth rates of *S. mutans* and S. sanguis were 69.07 % and 137.43 %, respectively. Contrary to the other environments, in this one, S. sanguis showed a higher growth compared to *S. mutans*.

In cigarette's environment, Winston had 80.3% of *S. mutans*'s growth and 40.47% of S. sanguis's growth.

Ultralight Winston had 106.56% of *S. mutans*'s growth and 89.49% of S. sanguis's growth.

Kent had 92.26% of *S. mutans*'s growth and 56.87% of S. sanguis's growth.

So we can conclude that cigarette's smoke may increase the risk of dental caries, and cigar's smoke may decrease it.

Suggestions:

1- An in vivo investigation should be done to determine if the effect of cigar and cigarette smoke over the growth of these two bacteria is the same in mouth environment or not.

2- A study should be done to determine the effect of each cigar and cigarette's ingredients independently, over these two bacteria.

3- Another study should be done to determine if there is a relation between the pressure, level of O2 and Co2 of the air breathed by smokers and non-smokers, and the level of cariogenic beacteria.

4- The level of plate's acidity, before and after the installation in different environments, should be evaluated.

5- A study should be done to evaluate the temperature's effect on *S. mutans* and S. sanguis's growth, as one of the effective factors over the growth of these two bacteria when cigar and cigarette are used. These items were recognized as effective factors over the growth of S. sanguis and *S. mutans*.

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The authors do not have any competing interest.

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