Original Article

Comparison of oral malodor and oral microbiome in smokers and non-smokers

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ABSTRACT

Objective: The aim of this study was to investigate the influence of smoking on both the oral malodor and oral microbiome in smokers compared with a control group of non-smokers.
Methods: The study population consisted of 37 patients with complete data for oral malodor, periodontal condition, and oral health behavior. The number of bacteria was determined by real-time PCR analysis.
Results: Levels of hydrogen sulfide in smokers (n=9) were significantly higher than those from non-smokers (n=28). The mean numbers of total bacteria, *Fusobacterium nucleatum* and *Campylobacter rectus* recovered in saliva were significantly higher in smokers. In addition, a multiple linear regression analysis showed that smoking influenced oral microbiome. Bacteria in tongue coatings from 21 patients with no tongue cleaning habit were also investigated. The detection rates of *F. nucleatum* and *C. rectus* per total bacteria in smokers were 3.03% and 0.60%, respectively, this correspond to approximately 5 fold the rates detected in non-smokers. The number of *F. nucleatum* and *C. rectus* also showed positive correlation coefficients with all volatile sulfur compounds (VSC) values.
Conclusions: The results of this study suggest that smoking promotes colonization of periodontopathogenic bacteria in tongue coatings and influences oral malodor by increasing the amount of VSC.

Key words: Smoking, Oral microbiome, Periodontopathogenic bacteria, Oral malodor, Tongue coating

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INTORODUCTION

Several reports suggested that smoking is related to periodontitis as well as to important systemic diseases including coronary heart disease and certain cancers[1]. Dental treatments are also influenced by smoking, for instance, a significant relationship between smoking and the risk of osseointegrated implant failure has been reported[2]. The oral cavity is an environment with close contact with nicotine, one of the most cigarette harmful components of cigarette smoke[3]. The oral cavity is affected by smoking through the action of cigarette toxic compounds on tissue directly or on the bacterial composition indirectly[4].

Some studies evaluated the effect of smoking on the oral microflora and showed greater bacterial diversity during the early days of bacterial colonization in both marginal and subgingival plaque as well as higher numbers of anaerobic bacteria such as F*usobacterium spp*. in smokers than in non-smokers[5,6]. While most reports investigated subgingival or gingival plaque[7-9], few studies examined tongue coating and saliva. It has been demonstrated that the presence of periodontal disease-related bacteria in tongue coating is closely related to halitosis[10,11]. Therefore, it can be hypothesized that smoking cause perturbations of the oral microbial composition and favor the occurrence of halitosis.

The aim of this study was to investigate the influence of smoking on oral malodor and the oral microbiome in smokers in comparison to non-smokers.

METHODS

1. Subjects

From August 2008 until August 2016, all patients who referred to the Clinic for Breath Odor at the Tokushima University Hospital, Japan, were screened for this study. Subjects who measured volatile sulfur compounds (VSC) in mouth air and full-mouth periodontal probing depth (PPD) in addition to collect saliva and tongue coating sample were included. On the other hand, subjects who received antibiotic treatments or having severe systemic diseases were excluded. Thirty seven patients (15 men and 22 women, 51.4±13.9 years) were enrolled for analysis.

2. Evaluation of life style and oral health behavior

The patients first answered a questionnaire regarding “the experience of smoking”, “the number of cigarettes smoked per day”, and “the duration of smoking”. The subjects were divided into two groups: “current smokers” who have been smoking regularly, and “non-smokers” who have never smoked or quit smoking 3 years ago or more. Questions regarding “the use of mouth rinse regularly”, and “the habit of tongue cleaning” were also answered. The participants were divided into two groups based on whether they had the habit of tongue cleaning more than once every two days or not.

3. Clinical measurements

All teeth of all patients were assessed for PPD and bleeding on probing (BOP). The data obtained were used to calculate the percentages of teeth with PPD $\geq$ 4mm and BOP.

VSC, which are bacteria-derived substances responsible for oral malodor, were measured by gas chromatography in accordance with the modified protocol described by Hinode[12]. VSC in samples were measured with a gas chromatograph GC-8APF (Shimadzu, Kyoto, Japan) equipped with a flame photometric detector and a β, β-ODPN 25% Chromosorb W-HP60/80 column (3.1 m x 3.2 mm, Shimadzu). The chromatograph used an auto-injection system equipped with a 6-port-value, a Teflon sample loop, and a Teflon column. Each subject closed their mouth for 60 seconds prior to collect and analyze a 10-ml sample of air using the above system. In this study, the total amount of hydrogen sulphide (H_2S), methyl mercaptan (CH_3SH) and dimethyl sulphide [$(CH_3)_2S$] was defined as “total VSC”. VSC measurements were performed at least one hour after any oral activities such as eating, drinking and oral hygiene procedures during the morning. In

addition, participants were prohibited to drink alcohol for one day before examination and to smoke on that day".

4. Determination of bacteria

Following the oral malodor analysis, unstimulated saliva was collected in a 50-ml sterile tube from each subject. Saliva samples were vortexed, dispensed into vials (200 µl) and kept at -80°C until used for the real-time polymerase chain reaction (PCR) analysis. Tongue coating samples were collected using sterile 4mm-wide plastic spatula (Asone Co., Osaka, Japan) by swabbing the tongue dorsum 5 times from back to front (approx. 1 -cm-long swabbing motions). Samples were suspended in one ml of phosphate-buffered saline (PBS), vortexed, dispensed into vials (200 µl) and kept at -80°C.

All samples (saliva, tongue coating) were analyzed for the number of total bacteria as well as three periodontopathogenic bacteria (*Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Campylobacter rectus*). The number of bacteria in saliva and tongue coating samples was determined by real-time PCR as previously reported by Amou et al. [10]. MiniOpticon system (Bio-Rad Laboratories, Hercules, CA, USA) with SYBR Green I dye was used for the real-time PCR analysis. Samples were thawed and centrifuged at 10,000 g for 10 min at 4°C. After elimination supernatant 200 µl of InstaGene Matrix (Bio-Rad Laboratories) were added to each pellet samples. The mixture was incubated at 56°C for 30 min, vortexed for 30 s, incubated at 100°C for 8 min, and then stored at -20°C until used for the real-time PCR analysis. Prior to analysis, the mixtures were thawed and centrifuged at 10,000 g for 10 min at 4°C. The supernatant of the samples were used for DNA template. DNA template (2 µl) were added to PCR reaction mixture (18 µl) made of 10 µl of SsoFastTM EvaGreen® Supermix (Bio-Rad Laboratories), 0.04 µl of 100 µM primer (Forward, Reverse) and 7.92 µl of diethylpyrocarbonate-treated water. The liquid mixtures were heat-treated as follows: Conditions for PCR reaction include an initial denaturation step (3 min at 95°C), followed by denaturation (5s at 95°C), annealing (10s at 60°C) and extension (10s at 60°C). The number of cycles for total bacteria, *P. gingivalis*, *F. nucleatum* and *C. rectus* were 40, 45, 38 and 38, respectively. Standard curve for individual bacterial species and base sequence of each primer were followed by the method of Yokoyama et al.[13].

5. Statistical analysis

Data were analyzed using the software IBM SPSS Statistics ver.20 (SPSS Japan Inc. Tokyo). Differences with regard to the number of bacteria or the VSC values between the two groups regarding smoking were assessed using the Mann-Whitney U test. Spearman's rank correlation coefficient and multiple linear regression analysis were used to investigate the factors affecting the number of bacteria and oral malodor. Logarithmic value of the number of bacteria and the ratio of the number of periodontopathogenic bacteria per total bacteria were used for

Table 1 Characteristics of smoker and non-smoker groups in this study.

Parameter		Smokers (N=9)		Non-smokers (N=28)		*p*-value#
		Mean	S.D.	Mean	S.D.	
Age		49.8	18.1	51.9	12.6	0.972
Number of teeth		22.7	7.9	24.5	6.2	0.697
%PPD≥4mm	(%)	35.2	28.3	29.3	27.3	0.513
%BOP	(%)	29.6	20.4	43.3	29.2	0.190
Salivary flow rate	(ml/min)	0.50	0.29	0.37	0.22	0.107
Gas Chromatograph	H_2S (ppb)	127.9	137.6	56.1	97.9	0.038*
	CH_3SH (ppb)	35.0	47.9	69.2	290.2	0.265
	Total VSC (ppb)	151.5	150.9	128.7	313.7	0.083

PPD：probing pocket depth, BOP: bleeding on probing, S.D.: standard deviation
#: Mann-Whitney U test, *p<0.05, N=37

Table 2 Oral bacteria found in saliva in the smoker and non-smoker groups.

Items		Smoker (N=9)		Non-smoker (N=28)		*p*-value[#]
		Mean	S.D.	Mean	S.D.	
Total bacteria	log [cells/ml]	7.53	0.64	6.68	1.02	0.026*
P. gingivalis	log [cells/ml]	2.42	2.75	1.37	2.22	0.357
F. nucleatum	log [cells/ml]	5.88	1.15	3.82	1.71	0.002**
C. rectus	log [cells/ml]	5.76	1.28	4.36	1.16	0.018*

#: Mann-Whitney U test, *p<0.05, **p<0.01, N=37

Table 3 Relationship between the number of oral bacteria in saliva and the parameters related to oral environment.

Parameter	Total bacteria	*P. gingivalis*	*F. nucleatum*	*C. rectus*	PPD≥4mm	Salivary flow rate	Mouth rinse	Smoking habit
Total bacteria	1							
P. gingivalis	0.463**	1						
F. nucleatum	0.660**	0.524**	1					
C. rectus	0.751**	0.422**	0.781**	1				
PPD≥4mm	0.130	0.297	0.199	0.227	1			
Salivary flow rate	0.144	-0.113	0.060	0.111	-0.138	1		
Mouth rinse	0.046	0.061	-0.035	0.041	0.159	0.010	1	
Smoking habit	0.372*	0.184	0.508**	0.395*	0.079	0.158	0.048	1

Spearman's rank correlation coefficient, *p<0.05, **p<0.01, N=37

analysis. In the analysis about the factor affect the number of bacteria, we divided into two groups in the presence or absence of periodontal pocket (PPD ≥ 4mm or PPD < 4mm).

6. Ethics

The Ethics Committee of Tokushima University Hospital approved the study (protocol approval number 218-2). The method and objectives of this study were explained to the participants, who provided written informed consent prior to their participation in the study.

RESULTS

1. Study population

The study population consisted in 9 current smokers (6 men and 3 women) and 28 non-smokers (9 man and 19 woman), including some past smokers. There were no statistically significant differences with regard to age, number of teeth and periodontal between the two groups of current smokers and non-smokers (Table 1). The proportion of “the use of mouth rinse regularly” and“the habit of tongue cleaning” in smokers and in non-smokers were 55.6% (5/9) and 50.0% (14/28), and 22.2% (2/9) and 50.0% (14/28), respectively. There was no significant difference of both factors between smokers and non-smokers.

2. Influence of smoking on halitosis

The H_2S level in current smokers was higher than that in non-smokers (p<0.05) as shown in Table 1. Moreover, total VSC in current smokers was higher than that in non-smokers, although there were no significant differences (p=0.083).

3. Influence of smoking on bacteria found in saliva

There were no significant differences in the unstimulated salivary flow rate between current smokers and non-smokers (Table 1). As reported in Table 2, the number of total bacteria, *F. nucleatum* and *C. rectus* in saliva of current smokers were significantly higher in comparison to the levels found in non-smokers. To get some insights about the factors that affect the number of bacteria in saliva, the relationship between oral bacteria and the parameters affecting the oral environment was investigated. There were significant positive correlations between "Smoking habit" and the number of bacteria with regard to total bacteria, *F. nucleatum* or *C. rectus*, as reported in Table 3. In addition to consider and evaluate the influence of the other factors, multiple linear regression analysis was conducted as dependent factors which involve "Total bacteria", " *F. nucleatum*" and "*C. rectus*" and as independent factors that affect oral environment (Table 4). As a result, high correlation

Table 4 Multiple linear regression analysis of the factors influencing the number of oral bacteria.

Dependent factor	Independent factor	Correlation coefficient	*p*-value	R^2
Total bacteria	PPD≥4mm	0.053	0.764	0.162
	Salivary flow rate	0.234	0.183	
	Mouth rinse	0.063	0.725	
	Smoking habit	0.398	0.023*	
F. nucleatum	PPD≥4mm	0.179	0.311	0.286
	Salivary flow rate	0.109	0.541	
	Mouth rinse	-0.104	0.559	
	Smoking habit	0.525	0.002**	
C. rectus	PPD≥4mm	0.328	0.058	0.323
	Salivary flow rate	0.090	0.611	
	Mouth rinse	0.069	0.700	
	Smoking habit	0.467	0.004**	

The age and gender were adjusted, *p<0.05, **p<0.01, N=37

Table 5 Oral bacteria found in tongue coating in the smoker and non-smoker groups.

Items		Smoker（N=7）		Non-smoker（N=14）		*p*-value#
		Mean	S.D.	Mean	S.D.	
Total bacteria	log [cells/ml]	7.27	1.09	7.59	0.68	0.296
P. gingivalis	log [cells/ml]	2.23	2.05	2.42	2.05	0.794
F. nucleatum	log [cells/ml]	5.45	0.97	5.08	0.79	0.412
C. rectus	log [cells/ml]	4.78	0.88	4.50	0.85	0.709
%*Pg*		0.03%	0.04%	0.05%	0.08%	0.682
%*Fn*		3.03%	3.78%	0.54%	0.47%	0.014*
%*Cr*		0.60%	0.53%	0.11%	0.09%	0.044*

#: Mann-Whitney U test, *p<0.05, N=21

Table 6 Relationship between the number of oral bacteria in tongue coating and the values of oral malodor.

The measurement by gas chromatograph		Total bacteria	*P. gingivalis*	*F. nucleatum*	*C. rectus*
H_2S	(ppb)	0.273	-0.069	0.508*	0.483*
CH_3SH	(ppb)	0.455*	0.327	0.703**	0.647**
Total VSC	(ppb)	0.427	0.217	0.726**	0.641**

Spearman's rank correlation coefficient, *p<0.05, **p<0.01, N=21

coefficients of the item “Smoking habit” in total bacteria, *F. nucleatum* and *C. rectus* were observed as 0.398, 0.525 and 0.467, respectively. However, no correlations between each number of bacteria and the presence of PPD ≥ 4mm was observed. There was no significant correlation between the items “smoking” and “number of P. gingivalis” (data not shown).

4. Smoking influences to both the bacteria in tongue coating and halitosis

It has been reported that the tongue cleaning habit affects biofilm of tongue coating[10]. The 21 subjects (10 men and 11 women, 52.4±13.9 year) who do not have tongue cleaning habit were selected to compare the number of bacteria in tongue coating in current smokers and non-smokers (Table 5). The levels of *F. nucleatum* and *C. rectus* per total bacteria (% *Fn* and % *Cr*) in current smokers were 3.03% and 0.60%, respectively, and they were 5.6 fold and 5.4 fold significantly higher than those in non-smokers respectively ($p<0.05$). When the number of each bacteria and oral malodor value were compared, there were significant positive correlations between *F. nucleatum* or *C. rectus* and H_2S, CH_3SH or total VSC observed (Table 6). In addition, there was a significant relationship between total bacteria and levels of CH_3SH ($p<0.05$).

DISCUSSION

There is evidence for a higher level of periodontopathogenic bacteria in smokers[5-9], although, several studies reported that both smokers and non-smokers exhibit similar subgingival microflora[14,15]. This study showed that smoking related with the total number of bacteria as well as certain periodontopathogenic bacteria in the oral cavity. Moreover, it suggested that smoking also influence tongue coating-associated microorganisms.

It has been reported that the microflora on the tongue and in the saliva are similar[16]. In this study, current smokers showed higher numbers in total bacteria, *F. nucleatum* and *C. rectus* in saliva compared with non-smokers. In addition, % *F. nucleatum* and % *C. rectus* were also significantly higher in tongue coating of current smokers. *F. nucleatum* and *C. rectus* are strictly anaerobic bacteria associated with periodontal disease[17]. Obligate anaerobes are found in high proportions in subgingival plaque since they are protected from toxic oxygen metabolites[18]. However, Hanioka et al. reported that the periodontal pocket oxygen tension in smokers is lower than in non-smokers regardless of the pocket depth[19]. In this study, there were no significant difference in %PPD ≥ 4mm between current smokers and non-smokers. While the oxygen tension in the air is generally about 21%, oxygen in the space over the tongue ranged between 12 to 14% and in periodontal pocket is about 1 to 2%[16]. Smoking might allow the development of an environment of low oxygen tension on the tongue dorsum as well as shallow periodontal pockets[19]. Therefore, smoking changes the oral environment, which is better adapted for colonization by anaerobic bacteria. This suggests that *F. nucleatum* and *C. rectus* are likely to better survive in the oral environment of smokers.

Host factors such as age and periodontal condition may influence the number of periodontopathogenic bacteria in the oral cavity. In this study, we found no significant relationship between age and PPD and the number of periodontal disease-related bacteria as determined by a multiple linear regression analysis. These results suggest that smoking is an important parameter modulating the composition of the oral microflora. Kilian et al. have reported that the complex equilibrium between resident species in the oral cavity is responsible for the maintenance of a healthy state[20]. They also showed that smoking causes perturbations of the oral microbiome (dysbiosis)[20]. Our study confirms data of dysbiosis associated with smoking.

F. nucleatum is known to play a key role in biofilm formation through its ability to bridge early and late colonizers[21]. The fact that smoking increases the proportion of *F. nucleatum* may contribute to the higher susceptibility of smokers to periodontal disease.

In agreement with the results of our study, Kubota et al. also reported that the prevalence of *C. rectus* was higher in smokers than in non-smokers[9]. It is thought that smoking in pregnant women may be a high risk factor for the development of periodontal disease, since C. rectus has been associated with chronic periodontal disease and that growth of this bacterium is promoted by the presence of female hormones such as estradiol whose concentration increases during pregnancy[22]. There were no significant difference regarding P. gingivalis

between current smokers and non-smokers in accordance with the study of Gomes et al.[23].

Our study brought evidence that smoking modulate oral malodor. Cigarette smoke contains various components such as acetaldehyde, benzene and ammonia which cause "cigarette smell"[24, 25]. Because of that, it is often difficult to diagnose oral malodor in smokers by the organoleptic test. Therefore, VSC values were measured by gas chromatography for our analysis. Bornstein et al. reported that smoking was associated with higher VSC values[26]. In this study, H_2S levels in current smokers were higher than in non-smokers. Periodontal disease-related bacteria used in this study are VSC produced bacteria[27-29]. By investigating the relationship between the number of bacteria in tongue coating and oral malodor, we found a significant positive correlation between VSC values and total number of bacteria in tongue coating, in agreement with the study of Washio et al.[30]. In addition, there were also positive correlations between VSC values and the numbers of *F. nucleatum* and *C. rectus*. Some reports mentioned that bacteria found in the tongue coating increase VSC values strongly compared to those found in saliva[10] or in the subgingival sulcus[31]. Therefore, it is suggested that the high ratio of periodontopathogenic bacteria in tongue coating is associated with oral malodor. Consequently, smoking may be related with the development of oral malodor by increasing the proportion of VSC-producing bacteria.

This study contributed to the evidence of smoking influence regarding oral microbiome and oral malodor, and results may be useful to conduct evidence based-guidance for smoking cessation in dental clinic. The major limitation of this pilot study relates to the small number of current smokers. In order to clarify the effects of smoking for the oral environment, it is necessary to design a longitudinal study monitoring the oral microbiome and the VSC values after smoking cessation.

CONCLUSION

Our results suggested that smoking affects the oral environment and promote colonization of periodontopathogenic bacteria in tongue coatings. In addition, it suggested that smoking influence oral malodor by increasing the amount of volatile sulfur compounds.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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