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EFFECT OF TOBACCO CIGARETTES ON RATS' ORAL MICROBIOTA

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УТИЦАЈ ДУВАНСКИХ ЦИГАРЕТА НА ОРАЛНУ МИКРОБИОТУ ПАЦОВА

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ABSTRACT

Objective. The tobacco usage is a risk factor of the variety oral diseases. To date, the effect of this risk factor on the composition of the oral microbial community has been considered by single studies with unclear pathogenic links. One of the possible factors that could be critical is a severe disturbance microbiota of oral cavity under influence of smoking, so the purpose of the present study was to evaluate the microflora of rats exposed to tobacco cigarette smoke.

Methods. We compared the composition of the oral microflora of 20 WAG rats were exposed to smoke of tobacco cigarette and 10 control rats using a culture-based methods targeted at microbial taxonomic and functional profile. Differences between groups were analyzed with nonparametric the Kruskal-Wallis H test. Paired data were analyzed using the Wilcoxon test and Friedman's ANOVA test. Data presented are expressed as mean ± standard deviation (SD) numbers of colony-forming units per ml (CFU/ml). Statistical significance was defined as $p<0.05$.

Results. At the experiment 60th day, comparative analysis of the microbiota showed that microbiological composition varied considerably between the two groups. In the rats influenced by cigarette smoke, exposure time-related reductions in commensal microflora were noted.

At the end of the experiment, the commensal bacteria were markedly reduced. In contrast, the opportunistic bacterial diversity was increased and represented by Gram-negative microorganisms as the major phyla.

Conclusion. We conclude that alterations in the composition, diversity, and function of the oral microbiome occur in association with tobacco smoke components and exposition term. The established risk factors for oral dysbiosis, especially after long-term exposure may contribute to disease development of the oral cavity.

Key words: *tobacco products; mouth; microbiota; rats; Gram-negative bacteria*

САЖЕТАК

Циљ. Употреба дувана је фактор ризика за разне оралне болести. До данас је утицај овог фактора ризика на састав оралне микробне флоре разматран у појединачним студијама са нејасним патогеним везама. Један од могућих критичних фактора јесте изражено оштећење микробиоте усне дупље под утицајем пушења, па је сврха овог истраживања била да се процени микрофлора пацова изложених диму дуванске цигарете.

Методе. Упоредили смо састав оралне микрофлоре 20 WAG *пацова који су били изложени диму дуванске цигарете и 10 контролних пацова користећи методе засноване на култури усмерене на таксономски и функционални профил микроба. Разлике између група анализиране су непараметријским Краскал–Волисовим „*H*“ тестом. Упарени подаци анализирани су помоћу Вилкоксон теста и Фридмановог* ANOVA *теста. Приказани подаци изражени су као средња вредност ± стандардна девијација* SD *бројеви јединица које формирају колоније по ml (*CFU/mL*). Статистичка значајност је дефинисана као* $p<0,05$.

Резултати. Шездесетог дана експеримента, упоредна анализа микробиоте показала је да микробиолошки састав значајно варира између две групе. Код пацова под утицајем дима цигарета примећено је смањење комензалне микрофлоре у зависности од времена излагања. На крају експеримента, комензалне бактерије значајно су смањене. Насупрот томе, опортунистичка бактеријска разноликост повећана је и представљена је грам-негативним микроорганизмима као главним типом.

Закључак. Закључујемо да се промене у саставу, разноврсности и функцији оралног микробиома јављају у вези са компонентама дуванског дима и термином експозиције. Утврђени фактори ризика за оралну дисбиозу, посебно након дуготрајне изложености, могу допринети развоју болести усне дупље.

Кључне речи: *дувански производи; уста; микробиота; пацови; грам-негативне бактерије.*

INTRODUCTION

Cigarettes' attractive design and their accessibility makes cigarette smoking is the preferably choice among globally young population. Tobacco cigarette smoking poses a bigger threat to the youth in Ukraine. According to the 2017 Global Adult Tobacco Survey report, among adults in Ukraine, 22.8% (8.2 million) were current tobacco smokers and 60.4% of them initiated daily smoking before age 18 years and the first smoking experience was in the age group of 12–13 years. This state in cigarette uses highlight the importance of targeting smoking research efforts at health of young adults (1, 2).

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Tobacco cigarettes belong to combustible tobacco products. This popular consumption of combustible cigarettes practically is related with tobacco smoke associated diseases and deaths (3, 4). Currently evidence suggests that tobacco smoke components play an influential role in disrupting human normal microbial communities. The oral microbiota refers to the complex and dynamic microbial ecosystem, including bacteria, fungi, viruses, archaea and protozoa. The oral bacterial species are introduced by following phyla: Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes, Fusobacteria, Saccharibacteria, Synergistetes, Gracilibacteria, Chlamydia, Chloroflexi, Tenericutes and Chlorobi. The fungal species are presented by Candida, Cladosporium, Aureobasidium, Saccharomyces, Aspergillus, Fusarium and Cryptococcus. The Candida species are the most frequent among others fungal species. Archaea belongs to a minor part of the oral microbiota (5, 6). The known species of Archaea are Thermoplasmatales, Methanobrevibacter, Methanobacterium, Methanosarcina, and Methanosphaera. Archaea are methanogens and numbers of them are increased in patients with periodontitis (7).

Normal bacterial biofilm benefits the host by preventing the excessive growth of harmful microorganisms. Various studies revealed that certain genera of bacteria had been found in the oral microbiota of both humans and rats. The healthy oral cavity is mostly associated with the Gram-positive bacteria, whereas the Gram-negative bacteria and filamentous forms predominated when the gingiva is inflamed (8, 9). Indeed, disturbance of the oral microbial equilibrium is found in patients with dental caries, periodontitis, cancers arising in the oral cavity and oropharynx (10, 11). The disease-associated microorganisms possess an elevated virulence potential by high metabolic functions. A pathogenic community that includes Streptococcus mutans, Candida albicans, Bifidobacterium dentium and Lactobacillus spp. have been recognized as pathogens that are associated with caries. Periodontal disease is caused by mainly Gram-negative species. Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, and Bacteroides forsythus have been identified in patients with periodontal disease (12). Specific oral bacterial community compositions have been found in several systemic diseases, such as bacterial endocarditis, coronary heart disease, aspiration pneumonia gastrointestinal cancer, even immune diseases (12, 13).

The significant associations between periodontitis and infective endocarditis have been elucidated. There are multiple rodent models have demonstrated that oral bacteria and even dental extraction show histologic evidence of endocarditis (14, 15). Staphylococcus aureus, Streptococcus viridans and Enterococcus spp. are the most common pathogens identified in endocarditis patients. Coronary heart disease is a fatal systemic disease that has been associated with dental diseases and atherosclerotic plaques are commonly infected with gram-negative periodontal pathogens such as Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis (13).

The harmful effects of tobacco cigarette smoke on the microbial community to be associated etiologically with a variety of systemic and local diseases (16-18). Even though the oral microflora of smokers is clearly different to that of nonsmokers, changes caused by cigarette smoke on the members of microbiome continue to be studied (19-21). The vulnerability of oral microbiome provokes our interest in microbiome alterations in smoking youths.

The purpose of the present study was to evaluate the microflora of rats exposed to tobacco cigarette smoke.

MATERIALS AND METHODS

Experimental Design

Experimental animals

The experimental design was performed according to the method of a previously reported by Koichi Tomoda and colleagues (22). In the 14-week study, fifteen male and fifteen female WAG rats at 10 weeks of age, weighing up to-109 g were chosen. They were kept in standard laboratory conditions in the Vivarium of Kharkiv National Medical University (KhNMU). The average temperature was 21 ± 2^{o}C while the relative humidity was 50-70%. WAG rats were received standard rodent chow and drinking water ad libitum. All procedures were carried out under the control of our committee in accordance with The Guidelines for Animal Experiments in KhNMU and Guiding Principles for the Care and Use of Laboratory Animals approved by The European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. The Commission on Ethics and Bioethics of KhNMU at its fist meeting on 15/01/2020 approved that this experiment complied with the bioethical requirements of the EU Directive 2010/63/EU on the protection of animals.

All rats were randomly assigned to two groups. Five male and five female rats of group 1 were intact animals. During the 12-week study period, ten males and ten female rats of group 2 were exposed to tobacco cigarette smoke. Two weeks prior to the first tobacco-smoke exposure, all rats were conditioned to placement and restraint in the Boyarchuck chamber for twenty minutes/day. The fresh airflow was inhaled by all rats, during acclimatization period. All smoke exposure experiment was carried out using «Liggett & Meyers», Blue Label, filter cigarettes (Philip Morris International, USA), which have nicotine and tar contents of 0.5 mg and 6.0 mg per cigarette, respectively.

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The rats of group 2 were exposed to cigarette smoke using the Boyarchuck chamber for twenty minutes/day, for five days a week (Monday to Friday) each week. The cigarette was smoked at a rate of 15 puffs per minute with an inhalation of 2 seconds of smoke mixed with 7 volumes of fresh air, followed by 2 seconds of air in the Boyarchuck chamber. The mixture of air and smoke moved from the chamber to the connected holders, in which the rats were kept separately. The nose and mouth of the rats were exposed to smoke but not the body itself. The rats in the group 1 were exposed to a fresh airflow for twenty minutes in the Boyarchuck chamber.

Sample Collection

The samples of rat oral biofilm were collected using swabs soaked in 0.9% NaCl sterile solution. Procedures were performed four times: at the beginning of the experiment, on the 30th, 60th and 90th days. After collection of samples, the swabs were inserted in test tubes with 0.95 ml of sterilized physiological solution.

Culture and identification of isolates

Serial dilution of the samples with buffer solution, 50-µl portions of the diluents were spread onto Petri dishes with following culture media: Blood agar, Mannitol Salt agar, Eosine Methylene Blue agar, MacConkey agar, Mueller-Hinton agar and Sabouraud dextrose agar. Blood agar, Mannitol Salt agar, Eosine Methylene Blue agar and MacConkey agar were used to detect the bacterial flora. These growth media were cultured at 37^{o}C for 48 hours.

To monitor the growth of fungi, the samples were taken up in Sabouraud dextrose agar and incubated at 37^{o}C for 7 days. The colonies on the plates were counted as colony-forming units per ml (CFU/ml) and Gram-stained. Species and biotypes of the bacteria were identified with Coagulase test, Catalase test, Oxidase test, Urease test, IMVIC test (Indole, Methyl red, Voges-Proskauer and Citrate utilization tests) and germ tube test.

Statistical analysis

The statistical analyses were performed using the STATISTICA software (StatSoftInc., USA, version 7.0). Eight independent parameters were compared using a Kruskal-Wallis test. Wilcoxon matched pairs test was used to compare dependent variables. Friedman test was used to assess whether there are any statistically significant differences between the distributions of three or more paired groups. Data were expressed as the mean values and standard deviation (SD) numbers of CFU/ml. The results were considered statistically significant at $p<0.05$.

RESULTS

The use of culture-based methods enabled to obtain microbial species derived directly from the rat oral cavity before and after exposure to tobacco cigarette smoke. At the beginning of the experiment, Gram-positive aerobic bacteria corresponded to the most commonly encountered in oral microbiota of all rats (Table 1).

Gram-positive and Gram-negative bacteria comprised 91.2% and 8.8% of all isolates, respectively. Gram-positive aerobic bacteria were represented by Bacillus spp., Staphylococcus spp., Streptococcus spp., Enterococcus spp., Corynebacterium spp. At the same time, Enterobacter spp. and Escherichia spp. were the most prevalent genera of Gram-negative bacteria.

On the 30th cigarette smoke exposure day while we were comparing oral microbiome of group 2 with ones of group 1, the respective tendency of disorder in microbiological composition was found. There was a shift toward a decrease the number of commensal microflorae, an increase the abundance of opportunistic microorganisms including Staphylococcus aureus, Enterobacter aerogenes, Escherichia coli and appear some new one of them Moraxella catarrhalis and Candida albicans.

At the experiment 60th day, comparative analysis of the microbiota in group 1 and group 2 showed that microbiological composition varied considerably between the two groups. In group 2 rats influenced by cigarette smoke, exposure time-related reductions in commensal microflora were noted.

At the end of the experiment, the commensal bacteria were markedly reduced. In contrast, the opportunistic bacterial diversity was increased and represented by Gram-negative microorganisms as the major phyla that were introduced: Enterobacter aerogenes, Enterobacter cloacae, Klebsiella pneumoniae, Moraxella catarrhalis, Candida albicans and Escherichia coli, likely because they took advantage of the empty niches created by the cigarette tobacco smoke exposure. The rat oral microbial equilibrium was disrupted by the exposure to the complex and harmful nature of inhaled tobacco smoke. Moreover, duration of exposure to tobacco cigarette smoke caused deleterious and suppressor effects on the commensal microflora that could trigger the persistent colonization by opportunistic pathogens, such as Klebsiella pneumoniae, Moraxella catarrhalis, Candida albicans and Escherichia coli.

To determine whether oral microbiome composition of group 2 differed according to 30th, 60th and 90th days smoke exposure status, we used Friedman ANOVA Chi-Square test. We further examined whether genus-level microorganisms' relative abundances differed according to duration of cigarettes smoke influence at the beginning, on

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Table 1. Influence of 12 weeks of cigarette smoke exposure on the rat oral microbiota, number of microorganisms (log 10 CFU/ml).

Microbial species	Group 1 (n=10)				Group 2 (n=20)			
	Study period, day							
	0 day	30 day	60 day	90 day	0 day	30 day	60 day	90 day
Bacillus spp.	6.19±0.51	6.10±0.53	6.08±0.51	6.01±0.46	6.10±0.49	5.44±0.36	5.03±0.50	4.84±0.47
					Chi Sqr.= 60.00, p=0.0000			
Kruskal-Wallis test: H=92.25, p=0.0000								
Staphylococcus epidermidis	3.81±0.49	3.71±0.45	3.73±0.41	3.93±0.53	4.59±1.19	4.37±1.26	3.01±0.52	2.42±0.49
					Chi Sqr.= 64.18, p=0.0000			
Kruskal-Wallis test: H=63.86, p=0.0000								
Staphylococcus aureus	1.76±0.46 (n=4)	1.63±0.42 (n=4)	1.62±0.36 (n=4)	1.59±0.42 (n=4)	1.73±0.48 (n=7)	2.08±0.47 (n=9)	2.93±0.48 (n=14)	3.56±0.69 (n=14)
					Chi Sqr.= 51.00, p=0.0001			
Kruskal-Wallis test: H= 90.00, p=0.0000								
Streptococcus viridans	3.83±0.59	3.87±0.64	3.96±0.63	4.00±0.61	4.06±0.54	3.85±0.51	3.00±0.51	2.68±0.47
					Chi Sqr.= 54.47, p=0.0000			
Kruskal-Wallis test: H=57.45, p=0.0000								
Enterococcus faecalis	1.97±0.48	1.96±0.46	2.08±0.47	2.07±0.44	2.04±0.41	2.27±0.39	3.01±0.50	3.42±0.37
					Chi Sqr.= 58.86, p=0.0000			
Kruskal-Wallis test: H=84.58, p=0.0000								
Corynebacterium spp.	1.90±0.45	1.93±0.50	1.97±0.48	2.17±0.31	1.94±0.43	2.04±0.43	2.81±0.42	3.17±0.52
					Chi Sqr.= 56.94, p=0.0000			
Kruskal-Wallis test: H=84.18, p=0.0000								
Enterobacter cloacae	2.12±0.47	2.22±0.39	2.13±0.40	2.06±0.44	2.16±0.39	2.29±0.37	2.60±0.07	3.01±0.47
					Chi Sqr.= 56.76, p=0.0000			
Kruskal-Wallis test: H=81.37, p=0.0000								
Enterobacter aerogenes	2.08±0.48	1.95±0.47	1.97±0.46	2.07±0.49	2.15±0.46	2.34±0.42	2.61±0.22	3.09±0.48
					Chi Sqr.= 59.68, p=0.0000			
Kruskal-Wallis test: H=76.20, p=0.0000								
Klebsiella pneumoniae	0	0	0	0	0	0	2.59±0.08	3.23±0.46
					Wilcoxon matched pairs test: Z=3.92, p=0.0000			
Kruskal-Wallis test: H=116.66, p=0.0000								
Escherichia coli	2.45±0.04	2.37±0.32	2.34±0.44	2.31±0.39	2.33±0.38	2.63±0.07	3.16±0.46	3.65±0.08
					Chi Sqr.= 59.12, p=0.0000			
Kruskal-Wallis test: H=93.23, p =0.0000								
Moraxella catarrhalis	0	0	0	0	0	2.14±0.51 (n=16)	3.63±087 (n=16)	4.68±0.87 (n=16)
					Chi Sqr.= 96.00, p=0.0000			
Kruskal-Wallis test: H=117.13, p=0.0000								
Candida albicans	0	0 [0;0]	0 [0;0]	0 [0;0]	0 [0;0]	2.49±0.06	3.50±0.23	3.62±0.05
					Chi Sqr.= 43.27, p=0.0000			
Kruskal-Wallis test: H=115.57, p=0.0000								

Data are expressed as Log_{10} CFU the mean ± S.D. per 1 ml sample.* $p < 0.05$: significant relative to the group 1 (control group) by Kruskal-Wallis test

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Table 2 Biochemical characterization of Moraxella catarrhalis

Characteristic	Moraxella catarrhalis
Gram reaction	Negative
Colony morphology	Round, convex, greyish-white
Cell morphology	Small cocci (less 1.0 μm)
Hemolysis	No
Oxidase	Yes
Acid from glucose, fructose, maltose or saccharose	No
Reduction of nitrate/nitrite	Yes
Lipase	positive
Ornithine decarboxylase	Negative
Urease	Negative
Alkaline phosphatase	Negative
Galactosidase	Negative
Indole	Negative
Penicillin resistance	No

Figure 1. Moraxella catarrhalis growing on blood agar.

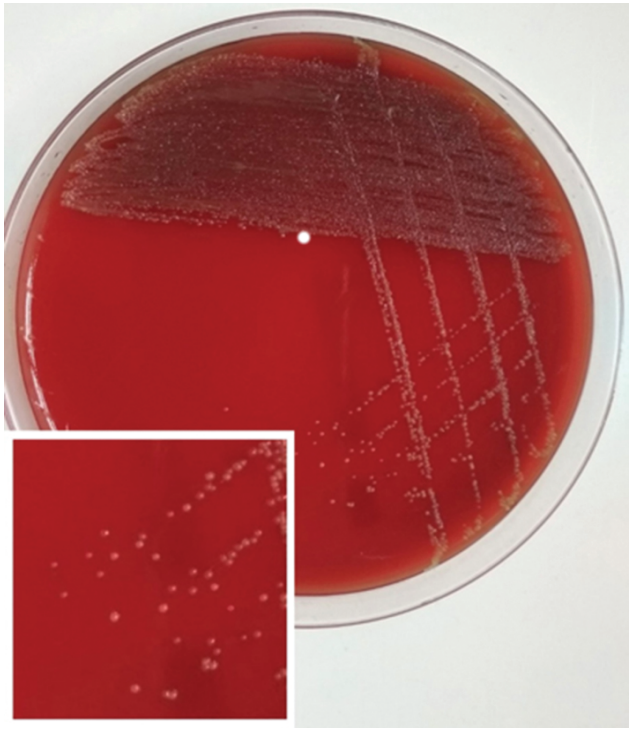
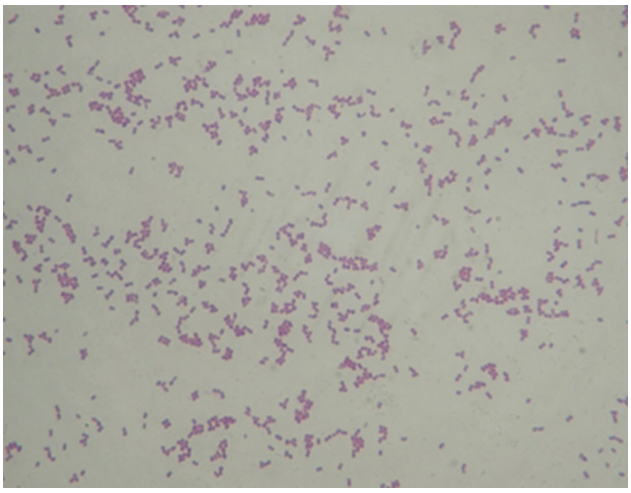


Figure 2. Morphology of the rat oral Moraxella catarrhalis isolate. Single colonies from cultures grown on agar were Gram stained. The image magnification is ×100.



the 30th, 60th and 90th days. To analyze the influence of tobacco cigarette smoking on the oral microflora, we identified microorganism's communities that differed significantly in rats of group 2 exposed by tobacco smoke during 60 days and 90 days compared to rats of group 1 (Table 1). The oral S. aureus carriage was significantly higher among rats of group 2 (70%) than rats of group 1 (40%). Moreover, the number S. aureus colonies was in twice time higher in group 2 than in group 1. Our study revealed raise S. aureus population in group 2 depending on time of smoke exposure. While the number rate was stable in S. aureus isolates from group 1 rats (4/10; 40%), the highest number of S. aureus colonies was observed in rats of group 2 on the 90th day of experiment (Table 1). Bacteria of the genus Moraxella were isolated from sixteen out of twenty rats of group 2 (Figure 1). They were biochemically identified as Moraxella catarrhalis (Table 2).

After exposure to tobacco smoke the majority rats of group 2 were colonized by M. catarrhalis and suffered from epistaxis. Moraxella catarrhalis is Gram-negative, aerobic, asaccharolytic small 0.6-1.0 μm cocci occur predominantly in pairs or short chains (Figure 2). We note that the higher number of Moraxella catarrhalis colonies in group 2 at 90th day of experiment than ones at 60th day, therefore, the stronger associations found between number of Moraxella catarrhalis colonies in group 2 and duration of smoking. There were direct relationships between

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number of Moraxella catarrhalis colonies in rats were exposed to tobacco cigarette smoke and cumulative exposure to tobacco cigarettes.

DISCUSSION

The effect of the cigarette smoke on oral microbiota composition is particularly relevant for young smokers as local and general health indicator. Since the oral microbiota influences many aspects of health, it has become the subject of our study. In this study, we have been evaluated the effects tobacco cigarette smoke on the oral microflora four times with interval 30 days. The use of culture-based methods enabled to obtain microbial species derived directly from the rat oral cavity before and after exposure to tobacco cigarette smoke. At the end of experiment, oral microbiota of group 1 was largely richer in Gram-positive aerobic bacteria, whereas Gram-negative bacteria were subdominant members of microflora of the group 2 and Gram-positive aerobic bacteria were scarce. At the genus level, group 2 oral microbiota are comparatively enriched in Enterobacter spp., Escherichia spp., Klebsiella spp., Moraxella spp., Candida spp. and depleted in Bacillus spp. Many of the detected opportunistic microorganisms are capable of causing respiratory system and gastrointestinal tract infections (23-25). This equilibrium was disrupted by the exposure to tobacco smoke. The complex and harmful nature of inhaled tobacco smoke altered rat oral microbial homeostasis. Tobacco smoke could influence on oral microorganism composition directly or indirectly through cell damage, change qualities of saliva, oxygen deprivation or other potential mechanisms that leads to disturbance of oral biofilm formation (21, 26).

Moreover, duration of exposure to tobacco cigarette smoke caused deleterious effects on the commensal microflora that could trigger the persistent colonization by opportunistic pathogens, such as Klebsiella pneumoniae, Moraxella catarrhalis, Candida albicans and Escherichia coli. These changes were determined on the 60-th day of experiment and significantly elevated on the 90-th day after the influence by tobacco smoke on the rats. Cigarette smoke disrupted the oral microbiota homeostasis, due to a significant downregulation of commensals involved in the formation of biofilm on mucosa which led to a decrease of oral trans epithelial resistance and contribute to adverse effects on oral epithelial cells (27).

Our study shows the long-term exposure to tobacco smoke disturbs the dynamic balance between protective oral microflora and pathological microorganisms. Oral microbiota dysbiosis is closely associated with periodontal disease (12). In addition, some classes of bacteria such as Acinetobacter, Clostridium, Klebsiella, and Pseudomonas aeruginosa were revealed in cigarettes made in the European Union (28). Therefore, another mechanism that could lead to disorder of oral bacteria profiles in group 2 could be due to exposure to bacteria in cigarettes, leading to bacterial acquisition and colonization.

Notably, the analysis of the microbiological composition in group 2, depending on the duration of smoking, showed a statistically significant increase in the number of opportunistic representatives. Importantly, a significant percentage (80.0%) of rats in group 2 at 90th experimental day had high level of opportunistic members, including Moraxella catarrhalis, Candida albicans, Klebsiella pneumoniae and Escherichia coli suggesting that tobacco smoke interferes with oral microbiota homeostasis. Furthermore, manifestation of rhinitis associated with the top disrupted respiratory tract in group 2 included several oral microbiota disorders that are characterized by structurally and numerically abnormal microbial members. This together with the observed up-regulation of respiratory system function in group 2 is suggestive of occurrence of oral dysbiosis and dysfunction of the respiratory system in ones.

In our survey of microbial flora that colonize the rat oral cavity, performed at the Kharkiv National Medical University, organisms of the Moraxella genus were isolated from rats with epistaxis, or “bloody nose syndrome.” Overall, our results are confirmed by other data provide evidence that M. catarrhalis induces a complex proinflammatory mechanisms and may also contribute to the pathogenesis of inflammation respiratory and oral tissues response caused by tobacco smoke toxicants (29-31). Cigarette smoke consists of a complex of toxic compounds including polycyclic aromatic hydrocarbons, extremely toxic aldehydes, volatile organic compounds, nicotine, heavy metals which induce various disorders in humans and animals (32-35).

They do adverse effects synergistically on the composition of the oral microbiota. They increase oxidative stress and reactive oxygen species induces elevate levels of proinflammatory cytokines, and chemokines that lead to infiltration different tissues by neutrophils. In addition, neutrophils reaching the gastrointestinal epithelium regulate the it`s inflammatory responses through release of tissue-damaging compounds, which cause disturbance of oral microbiota (36, 37).

Exposure to cigarette smoke elevates the gastro-intestinal tract pH, which possibly benefits some bacteria, enabling them to thrive and cause gastro-intestinal microbiota dysbiosis. At the same time, toxic chemicals in cigarette smoke may induce oral microbiota alterations via antimicrobial activity and disorder of regulation of the oral composition (38). The effect of heavy metal on the oral dysbiosis as part of the gastrointestinal tract microbiota depends on various factors, including heavy metal species and toxicity, dose effect, chronic or acute exposure, and absorption pattern.

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Interestingly, different types of dysbiosis are likely related to various diseases, including inside and outside the intestine.

There are limitations of our study. With respect to the diversity of the oral microbiota interaction, a limitation of the current study design was culture-based analytical technologies, and we acknowledge that a 16S rRNA gene sequencing-based analysis would have yielded superior results. Nevertheless, our data provides evidence that exposure of tobacco cigarette smoke was a main contributor to the observed smoking duration-associated disturbances in microbiota composition. Future studies should investigate the content of the oral microbiome post-smoking cessation.

In conclusion, the long-term co-effect of complex chemical mixture cigarette smoke perturbs balance of rat oral microbiota through various mechanisms. Cigarette smoke dramatically induced colonization oral cavity by opportunistic microorganisms in a time-dependent manner, and these effects paralleled increased diversity of them. During experiment, group 1 rats were primarily colonized with Gram-positive aerobic bacteria and maintained these species. In contrast, group 2 rats were colonized with opportunistic microbes: Enterobacter aerogenes, Klebsiella pneumoniae, Candida albicans, Staphylococcus aureus, Escherichia coli, and Moraxella catarrhalis. The most common colonizers of the rat oral cavity at the 60th and 90th days are gram-negative bacteria, including Enterobacter aerogenes, Klebsiella pneumoniae, Escherichia coli, and Moraxella catarrhalis.

CONFLICT OF INTEREST

The authors declare that they have no competing interests. There is no financial relationship between other people or organizations that may have improperly influenced our work, thus avoiding the possibility of biasing the study.

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