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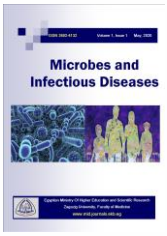

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Original article

The value of PAP and AgNOR techniques in identification of bacterial infections and cytomorphological changes in buccal cavity of Sudanese hookah users

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ABSTRACT

Background: Oral cancer is considered as a major health problem in most part of the world. Many factors have been identified as important causative agents responsible for the development of oral cancerous and precancerous lesions. In Sudan, smoking of tobacco has been identified as major cause. **Aim:** The study aimed to evaluate the value of PAP (Papanicolaou stain) and Silver stained Nucleolar Organizer Regions (AgNOR) techniques in identification of bacterial infections and changes in cells of buccal cavity among Sudanese hookah users. **Methods:** In the current study hundred apparently healthy people were included, Pap and AgNORs stains was used for the staining of buccal smears. **Results:** A total of hundred samples of buccal smears were included in this study. The age of participant ranged from 20 - 70 years. Thirty three samples (33%) had bacterial infection, one sample (1%) had Actinomyces infection, and 66 samples (66%) showed normal cells. Twelve samples (12%) had acute inflammation, 30 samples (30%) had chronic inflammation, and 58 samples showed normal cells. Samples stained with Pap stain, seven smears (7%) had inflammatory changes and 93 samples (93%) were negative. *p* value, and standard deviation, mean AgNORs showed 1.920_+4.50 in cases and 0.682_+1.420 in control. **Conclusions:** Analysis of AgNORs and Pap stain suggest that, use of hookah influences prolifrative activity in cells and also play a role in transmission of different types of microorganisms due to smoking it in the form of groups.

Introduction

Hookah smoking or in cultural traditions called (narghile or waterpipe), is a way of tobacco smoking in which air is passed over heated charcoal, which transfers its thermal energy to the tobacco located in the head of the hookah underneath the charcoal, producing smoke. The smoke composed of both charcoal burning products and compounds released from the heated tobacco, are entrained down the stem of the water pipe and bubbles through water by action of puffing on the water pipe hose before being inhaled by the smoker. The attraction for this mode of smoking tobacco is driven by variety of available tobacco flavors, the absence of visible side-stream smoke, the social aspect of smoking in group [1]. Numerous and toxic pollutants have been previously identified in mainstream hookah smoke including; nicotine, nitrosamines, aromatic amines, polycyclic aromatic hydrocarbons, volatile carbnyl compounds, benzene, carbon monoxide, nitric oxide and heavy metals. In addition, hookah tobacco smoking has

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been associated with negative impacts on the respiratory and cardiovascular systems, periodontal diseases, low birth weight, cancers and high risk for infection due to sharing the mouthpiece [1].

Tobacco smoke and use of hookah are the most important etiologic factors for oral cancers and risk factors for dysplastic lesions. Some studies have reported that the tobacco smoke from hookah contains toxic agents such as carbon monoxide, heavy metals, and carcinogenic chemical agents [2].

Hookah smoke contains polycyclic aromatic hydrocarbons at a 20-fold concentration and heavy polycyclic aromatic hydrocarbons at a 50-fold concentration compared to cigarette smoke. In addition, the amount of carbon monoxide produced by hookah has been reported to be 5 times higher than that of cigarette. In addition, a study showed that during 45 minutes of hookah use, smoke is produced 40 times higher than that with cigarette smoking, which increases several folds the potential to induce disease [3].

In addition, lung cancers and periodontal and respiratory diseases have been reported at a higher rate in hookah users compared to normal individuals. Many studies have confirmed the relationship between cigarette smoke and oral cancers. Several review studies and reports have confirmed an increase in the risk of oral cancers due to the use of hookah. However, no strong relationship was reported between these two factors [4,5].

Studying the oral microbial flora introduced streptococci as the main group of early colonizers in the oral biofilm with a fundamental role in the development of oral diseases [6]. Studying the subgingival microbiology assessment has identified *Streptococcus (S.) mutans* as the main cause of dental decay; *S. sanguis* also plays a role in the oral plaque [7]. The risk of respiratory infections also increases in smokers, possibly due to provision of an environment for bacterial colonization [8], especially in sterile sites, such as trachea and altered epithelial secretion and inflammation [9].

Few studies show the smoking effect on nuclear organization region of buccal mucosal cells and this effect is determined by numerical determination of nuclear organizer regions of buccal mucosa [10].

Oral cancer is one of the six most common cancers worldwide, and globally more than 50% of head and neck cancers occur in developing countries, remarkably in Africa. Over all 200,000 cases of head and neck cancers occur each year in Africa, among which 80,000 are oral cancer. Epidemiological and clinical studies suggest a causative role of tobacco use in the evolution of oral potentially malignant and malignant disorders [11].

Oral cancer is one of the most important types of cancer that threaten human life; it refers to cancer that develops in any portion that poses the oral cavity. The remarkable cause of oral cancer is tobacco and human papilloma virus. Researchers considered the techniques improving the diagnostic criteria of oral cancer are scarce and scanty, this study beside other researches run to participate in filling this gap.

The study aimed to evaluate the value of Papanicolaou stain (PAP) and silver stained nucleolar organizer regions (AgNOR) techniques in identification of bacterial infections and changes in cells of buccal cavity of Sudanese hookah users.

Materials and Methods

This was a prospective case control study, included samples collected from oral cavity hookah users in Khartoum state as test group and non smokers control group. The studied samples were Sudanese hookah users in Khartoum State.

Sample size

Hundred volunteers obtained from them buccal smears, which were collected by using tongue depressor used to scarab cells from oral mucosa. Questionnaire to obtain essential data was provided for each respondent.

Data analysis

Data was analyzed by using SPSS software.

Method

A.Papanicolaou staining technique

It is polychromatic stain that uses multiple dyes to give different colors according to various components of the cells. It's the most important stain utilized in the practice of cytopathology [12].

Smears were fixed with 95% ethanol for 15 minute then rinsed in tap water, then we added harris Hematoxylin 1-3 minutes then rinse in tap water, then dipped in 95% ethanol, then add eosin azure 2.5 minutes, then dip in 95%ethanol 2 changes, then add 100%ethanol for 1minute, clear in 2 changes of xylene 2 minutes for each, then will mounting with DPX [13].

B. Nuclear organizer regions (Ag-NOR):

The air dried smears were stained according to the AgNOR staining method. Working solution was freshly prepared by mixing one volume of 2% gelatin in 1% formic acid solution and two volumes of 50% aqueous silver nitrate solution. All smears incubated with this silver solution for 30 minutes at room temperature in a dark area and they were protected in the dark until each slide analyzed [14]. Two cytopathologists examined and interpretated the silver-stained cells under light microscope (Olympus BX-51, Japan) at 10x & 40x magnification. All smears screened horizontally from left to right and AgNORs counted in the nuclei of the first 50 non-overlapping, inner layers, nucleated epithelial cells. Superficial cells with pyknotic nuclei not counted. The AgNOR count made adopting the method described by **Crocker et al.** [15]. Silver stained nucleolar organizer regions, which are visible as black-dark brown dots located within the nuclei of the cells, was counted; overlapped black dots counted as one structure [13].

Statistics

The mean and standard deviation (mAgNOR ± SD) of AgNOR dots in 100 tumor nuclei were estimated. A similar thing was done for the 50 tumor nuclei. The data collected were statistically analyzed to generate Pearson correlation coefficient (*p*-value) using Statistical Package for Social Sciences (IBM SPSS Statistics 21) to compare the values observed in each tumor grade and the values observed for counting 50 and 100 tumor nuclei [16].

Ethical consideration

Each participant was asked to sign a written ethical consent form during the interview, before the specimen was taken. The informed ethical consent form was designed and approved by the ethical committee of the Faculty of Medical Laboratory Research Board, National University-Sudan.

Results

The study was conducted during the period from October 2020 to January 2021. The study aimed to evaluate the value of Pap and AgNOR techniques in identification of bacterial infections and changes in cells of buccal cavity of Sudanese hookah users. A total of hundred samples of buccal smears were included in this study. The age of participant ranged from 20 to 70 years.

Infections

From hundred samples stained with Pap stain included both cases and control 33 samples (33%) had bacterial infection, one sample (1%) had Actinomyces infection, and 66 samples (66%) were negative.

Inflammation

From hundred samples stained with Pap stain twelve samples (12%) diagnosed with acute inflammation, 30 samples (30%) had chronic inflammation, and 58 samples (58%) were negative.

Inflammatory changes

From hundred samples stained with Pap stain, inflammatory changes detected in seven samples (7%), while 93 samples (93%) were negative. Regarding AgNORs technique *p*-value, STD deviation, mean AgNORs showed 1.920_+4.50 in cases and 0.682_+1.420 in control group.

Table 1. Comparing the mean of AgNOR in users and control.

AgNORs	Mean	Std.deviation	*p*.value
Case	4.50	1.920	
Control	1.420	0.682	0.000

Table 2. Frequency of inflammation among studied groups.

p value	Total	Inflammation		Variables
		Normal	Yes	
0.000	50	15	35	Case
	50	43	7	Control
	100	48	42	Total

Table 3. Distribution of study population according to type of inflammation

p value	Total	Inflammation type			Variables
		Normal	Chronic	Acute	
0.000	50	15	24	11	Case
	50	43	6	1	Control
	100	58	30	12	Total

Table 4. Frequency of infection among studied groups.

p value	Total	Infection		Variables
		Normal	Yes	
0.000	50	23	27	Case
	50	43	7	Control
	100	66	34	Total

Table 5. Frequency of study population according to type of infection**.**

p value	Total	Infection type			Variables
		Normal	Bacterial	Actinomyces	
0.000	50	23	26	1	Case
	50	43	7	0	Control
	100	66	33	1	Total

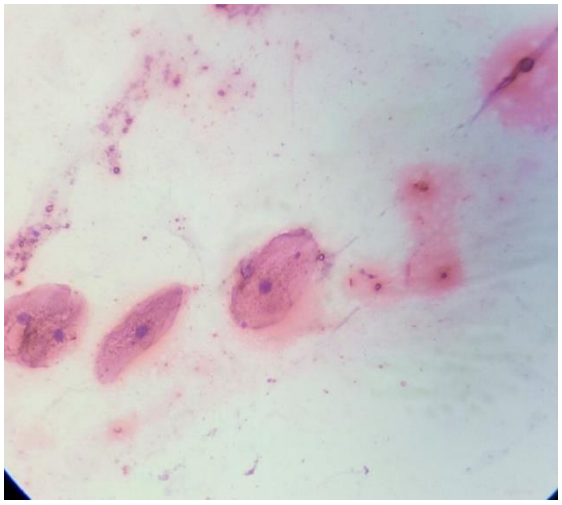
Table 6. Frequency of atypia among studied groups.

p value	Total	Atypia		Variables
		Normal	Abnormal	
0.006	50	43	7	Case
	50	50	0	Control
	100	93	7	Total

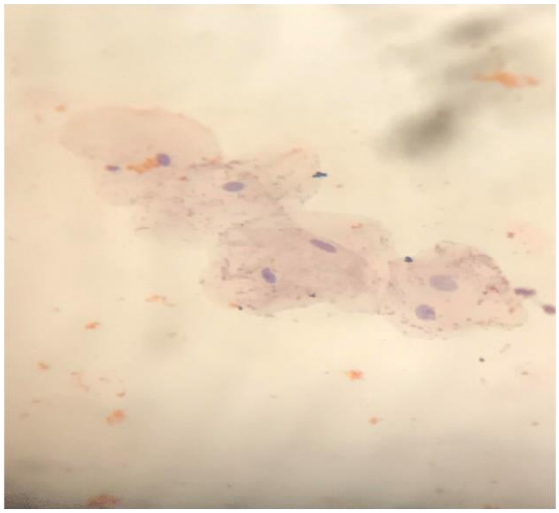
Table 7. Correlation between atypia type and cytological findings.

p value	Total	Atypia types					Variables
		Normal	Prenuclear hallow	Hyperkeratosis	Coarse chromatin	Vacuolated cytoplasm	
	50	43	1	3	2	1	Case
	50	50	0	0	0	0	Control
	100	93	1	3	2	1	Total

Photograp 1. Buccal smear. Slide showed bacterial cells in background.PAP stain. X 40



Photograph 2. Buccal smear. Slide showed nucleus polymorhic.PAP stain. X40



Photograph 3. Buccal smear. Slide showed abnormal count.AgNOR stain. X 40.

Discussion

The prevalence of smoking and tobacco use is increasing in the world [17,18]. Tobacco smoking, increases the risk of all-cause mortality, and is responsible for 90% of causes of cancer, cardiovascular diseases, ischemic heart disease, chronic obstructive pulmonary disease, and stroke [19, 20].

In Sudan, oral cancer is one of the most important reasons for life threatening due to trending of tobacco smoking in various types and ways [21]. In this study, we targeted hundred samples from hookah users in Khartoum state within age group ranged between 20 to 70 years. Hundred samples stained with Pap stain 50 cases and 50 control. The other 100 samples stained with AgNORs the same as done in Pap stained smears. Result showed that mean AgNORs 4.50, Std deviation 1.920 in cases and mean 1.420, Std deviation0.682 in control and *p* value 0.000. Our study is agreed with many studies inside and outside Sudan, such as a study of **Kadivar and Attar** in Iran [22]. Also **Hashemipour et al .** [23], **Ahmed el al.** in Sudan [12] and **Mhaske et al.** [24].

Sharma and Saxena reported that AgNOR appears to be useful technique in distinguishing between normal mucosa, mucosa with and without lesions exposed to carcinogens, such as tobacco and frank oral carcinoma [25]. **Jajodia et al.** mentioned that liquid based cytology and conventional cytology are complementary techniques for cytological screening and combining them with AgNOR can increase the diagnostic yield. With objective criteria for assessment, cytology can be an indispensable tool for screening oral lesions in a resource-limited set-up, especially in high-incidence regions [26].

In Pap smears frequency of inflammation showed out of hundred samples 42 samples diagnosed with inflammation, twelve samples have acute inflammation and 30 chronic inflammation. The result of the present study is somewhat close to the result of **Sohair et al**. [27]. Pap stain is the preferred method in field studies for scoring and detecting abnormalities of cells in buccal mucosa [27]. In our study were found this agent in 1% Actinomyces which is easily identified on cytological smears. The results varied from study to study.

Huttunen et al. mentioned that smoking can damage nearly all organs of the human body and is one of the main risk factors for respiratory infection and infectious diseases in other systems, in a dose-dependent manner [28].

Jiang et al. reported that smoking can substantially increase the incidence and the mortality of infections in a clear dose dependent manner. In addition, smoking leads to a poor prognosis. The recurrence rate and uncured rate of infection in smokers are higher than in nonsmokers. Smoking cessation can reduce damage owing to smoking and reduce the risk of infection [29].

Conclusion

Analysis of AgNORs and Pap staining techniques revealed that smoking of hookah influences proliferative activity in cells. Hookah play a role in transmission of different types of microorganisms.

Author contributions

MAE and AAI: Study design, conduct of the study, collection, and interpretation of data, manuscript writing, and
revision.

EMA and AAI: Study design and manuscript writing and revision.

SS: Collection and interpretation of data, manuscript

writing, and revision
MAE and EMA: Statistical analysis, interpretation of data, manuscript writing, and revision.
All authors have read and approved the final manuscript.

Competing interests: The authors declare that they have no conflict of interest.

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