

Original Article

Exfoliative Cytology of Oral Mucosa among Smokers, Opium Addicts and Non-smokers: A Cytomorphometric Study

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Abstract

Background: The present study was conducted to evaluate keratinization as well as nuclear and cytoplasmic changes of oral epithelial cells among smokers, opium addicts and non-smokers through exfoliative cytology technique.

Methods: Smears of buccal mucosa and mouth floor were collected from 300 males (100 smokers, 100 opium addicts and 100 non-smokers). The nucleus and cytoplasm sizes were determined using image analysis software. Data was analyzed with Mann-Whitney test and Student's *t*-test on SPSS version 13 statistical software. Statistical significance was defined as $P < 0.05$.

Results: The results revealed statistically significant differences in cellular and nuclear size and the nuclear/cytoplasmic ratio between smokers, opium addicts and non-smokers in different age groups. The mean size of the nucleus compared to that of cytoplasm was significantly higher in smokers and opium addicts compared to non-smokers after correction for age.

Conclusion: The results of this study indicate different rates of epithelial cell keratinization in oral cavity among smokers, opium addicts and non-smokers. Also, our results suggest a possible relationship between the number of cigarettes per day, daily opium consumption and an increase in the rate of cellular proliferation of oral mucosal cells. The present study indicated a decrease in cellular diameter as well as an increase in nuclear diameter and nuclear/cytoplasmic ratio in smears taken from both smokers and opium addicts compared to non-smokers.

Key words: Cytology, exfoliative, nuclear, opium, smoking

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Introduction

Oral cancer constitutes the fourth most common cancer among males and the sixth most common cancer in females and is among the most common cancers in many developing countries.¹ Smoking is a common etiologic factor for oral cancers.² Also, opium addiction might play a role in the development of oral cavity cancer.³ Despite a decrease in the incidence of head and neck cancers in some communities, the incidence of oral cancers has not fallen in recent years, one reason of which is the increased use of cigarettes and tobacco in those communities.³ In some apparently healthy smokers, changes are observed in the frequency of epithelial cell proliferation,⁴⁻⁷ the size of nucleus and the size of nucleus in relation to cytoplasm. In addition, an increase is observed in the number of keratinized cells.⁸

Despite the implementation of cancer prevention programs in some countries and the fact that the oral cavity is an accessible

area (so the patients can easily examine their oral cavities), the majority of oral cancers are diagnosed at advanced stages, resulting in poor prognosis and survival rate among patients.^{9,10} In addition, the morbidity and mortality rates of oral cancer have risen despite advances in therapeutic techniques, leading to increased treatment costs and complications.¹ Hence, the early diagnosis of oral cavity cancers is critical in successful treatment of patients.²

Exfoliative cytology technique is a non-invasive method for initial and early diagnosis of cancers as an adjunct to clinical examination. A review of literature shows that this technique has been in use since the 1960s and 70s as a cancer evaluation and diagnostic technique with acceptable sensitivity and specificity.¹⁰

Cytology can be used as a diagnostic technique in detecting early changes of diseases even in the absence of clinical manifestations. This technique has some advantages, including easy and fast implementation, adequate diagnostic value, non-invasiveness, low cost and reproducibility.²

The literature shows controversial results with regard to the effects of smoking on oral cavity.^{5,8} The present study was undertaken to evaluate the potential precancerous changes of oral cavity among tobacco smokers and opium addicts.

Materials and Methods

The present cross-sectional descriptive/analytical study was completed by including patients referred to the Faculty of Dentistry, Kerman University of Medical Sciences, and to clinics all

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over the city of Kerman based on a simple random sampling method. This study was approved by the ethics committee of Kerman University of Medical Sciences (No.K.91.23). The patients and accompanying individuals were divided into three groups of smokers, inhalant opium addicts and non-smokers based on their answers to a questionnaire. One hundred subjects were placed in the smokers group, 100 subjects in the opium addiction group and 100 others in the non-smokers group. The criterion for including opium addicts in this study was using at least 4 grams of opium per day during three or more of the last six years. Also, opium addicts were selected based on DSM-IV-TR criteria (Diagnostic and Statistical Manual of Mental Disorders 4th Edition, Text Revision).^{11,12}

Women were not included in the study due to cellular changes during menstruation, after menopause and also due to the possibility of pregnancy and other hormonal changes.¹³ In addition, men drinking alcoholic drinks or using any drugs affecting the oral epithelium were excluded from the study. The inclusion criterion for smokers and opium addicts was use of cigarettes or opium during the last six months. Non-smokers were defined by no use of cigarette or any addictive material and smoke-producing substances during the preceding year.^{2,14} The aim of the study was explained to each subject and, if consenting to participate, a questionnaire was filled. In addition, the subjects were reassured that the data in the questionnaires would remain confidential and only be used for statistical analysis. All participants in the study consented to the procedure.

In order to determine the effect of the number of cigarettes smoked on the percentages of red, blue and yellow cells, the subjects were divided into 4 groups: A= less than 5, B = 5–10, C = 10–15 and D = more than 15 cigarettes per day. Also, in order to determine the effect of opium dose (grams) on the percentages of red, blue and yellow cells, the subjects were divided into 4 groups: A = less than 5 grams, B = 5–7 grams, C = 7–10 grams and D = more than 10 grams.

Before taking the oral samples, the subject was asked to rinse the oral cavity with normal saline solution for two minutes. Then, a tongue depressor was used to collect samples from two areas of the oral cavity, mouth floor and the buccal mucosa, after these areas were dried with an air syringe. The tongue depressor was scraped on the mucosa with a moderate force, followed by immediate smearing on a microscope slide, which was fixated with a spray of 90% alcohol. Sampling was carried out from 9 to 11 a.m. on a dental chair in a tranquil environment and under adequate illumination. Subsequently, the samples were submitted to the pathology laboratory and stained using Papanicolaou technique. In the laboratory, 20 unfolded epithelial cells with clear outline were counted on each slide and classified, based on staining, into yellow (keratinized), red (partially keratinized) and blue (non-keratinized)^{13,15} groups under a light microscope (Nikon, Japan) at 40× magnification (Figure 1).

Oral epithelial cells were morphologically classified into three types based on the size and the color of cell components. Each of these cellular types represents cells from different layers of oral epithelium at different stages of keratinization. The most keratinized yellow cells are present in the superficial layer of the oral epithelium, the intermediately keratinized large red cells are present in the intermediate layer and finally the non-keratinized small blue cells are present in the basal layer of the epithelium.¹⁶

Twenty cells per slide were analyzed for Cytoplasm Area (CA), Nuclear Area (NA), and Nucleo-Cytoplasmic (N/C) ratio using

a specific software (SCION image for window v.4.0.3.2, Scion Corporation, Frederick, MD, USA). The sampling was performed in a stepwise manner, moving the slide from the left upper corner to right and then down to avoid measuring the same cell twice. For measurement, the software was calibrated and scale setting was changed from square pixels to micrometers squared (μm^2). We followed the instructions given in the manual of the software for measuring the cell sizes. The nucleus and the cell outline were traced using digital cursor on the screen and the software automatically calculated the cell size and the NA (Figure 2). The N/C ratio was calculated manually.¹⁶

All samples were evaluated by two observers who were blind to the particulars of the study; if they did not agree on their readings, a third observer evaluated the sample. The results were analyzed with Mann-Whitney test and Student's t-test on SPSS version 13 statistical software.

Results

In the present study, 300 individuals divided equally in smokers, opium addicts and non-smokers, were examined. The age range of smokers was 25–68 years with a mean of 43.4 ± 7.5 years; the age range of non-smokers was 25–40 years with a mean of 30.2 ± 2.5 years and the age range of opium addicts was 30–60 years with a mean of 45.3 ± 3.7 years.

The smokers used 4–20 cigarettes per day (Mean \pm SD = 14.2 ± 3.5) and had the habit for 4–40 years (Mean \pm SD = 25.5 ± 8.7). Also, the addicts group used 4–12 gram opium per day (Mean \pm SD = 6.1 ± 0.5) and had the habit for 6–30 years (Mean \pm SD = 10.2 ± 5.1). The subjects were divided into three groups of under 30, 30–50 and over 50 years of age. There was a significant relationship between age and the mean percentage of yellow and red cells in smokers and opium addicts. This study showed that smokers and opium addicts had a higher mean of yellow and red cells in their mouth floor compared to non-smokers. In addition, there was a significant relationship between the sampling area (the oral cavity floor) and increased number of yellow and red cells (Table 1).

Table 2 presents the number of cigarettes smoked and opium consumed per day, the number of years of cigarette and opium use, and the number of yellow, red and blue cells in the mouth floor and buccal mucosa. There were significant differences in the percentage of yellow and red cells in the oral cavity in terms of the number of cigarettes smoked and opium consumed per day and the number of years of smoking.

Table 3 shows the ratios of nuclear size to cytoplasmic size in the three groups under study in terms of age and intraoral site. As the table shows, in the smokers and opium addicts groups and in the subjects aged 30–50 years and over 50, there was an increase in the means of ratios of nuclear size to cytoplasmic size, with a significant relationship in this respect. As the Table 4 shows, the smokers and opium addicts groups had increased nucleus/cytoplasm ratio which correlated with the location of sampling. In addition, there were significant differences in the number of smoking years and the means of nucleus/cytoplasm ratios between the two groups of fewer than 5 years and 5–15 years and groups of 16–30 and over 30 years.

Discussion

Squamous cell carcinoma is the most common malignant tumor

Table 1. Mean of red-staining, blue-staining and yellow-staining cells in the 3 groups for age groups and intraoral site.

Groups	Age groups	Intraoral site						
		<30	30-50	>50	*P-value	Floor of mouth	Buccal	*P-value
Smoker	Yellow-staining cells	25.9	35.5	45.1	0.001**	43.8	23.3	0.001**
	Red-staining cells	35.4	38.8	42.8	0.004**	41.5	22.1	0.001**
	Blue-staining cells	38.7	25.8	12.1	0.002**	14.7	54.6	0.0001**
Opium addiction	Yellow-staining cells	23.1	40.2	36.7	0.011**	42.7	21.1	0.001**
	Red-staining cells	33.2	21.7	45.1	0.024**	42.2	25.3	0.001**
	Blue-staining cells	38.2	27.1	15.7	0.002**	12.1	53.6	0.001**
Non smoker	Yellow-staining cells	34.27	33.7	33.56	0.125	23.3	24.5	0.12
	Red-staining cells	35.42	34.6	34.21	0.142	22.1	25.4	0.18
	Blue-staining cells	30.31	31.7	32.32	0.144	54.6	50.1	0.14

* Mann-Whitney test, **P value significant.

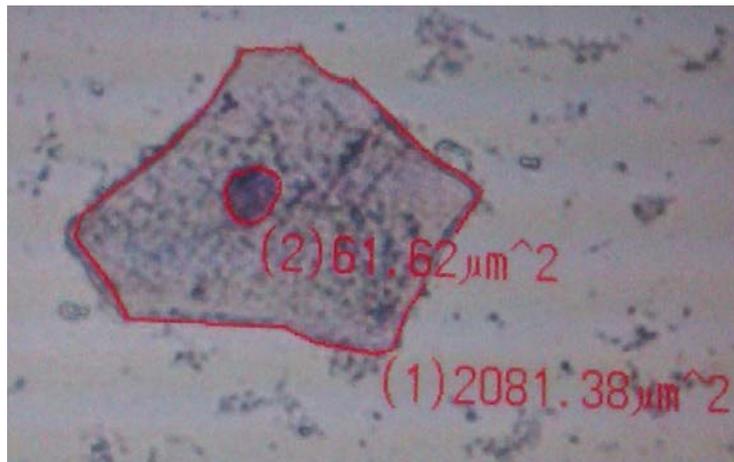


Table 2. Mean of red-staining, blue-staining and yellow-staining cells in the smoker and opium addiction groups for the number of cigarettes and opium consumed (gr) per day and duration (years).

Groups		Number of cigarettes and opium per day				*P-value	Duration (years)				*P-value	
		A	B	C	D		<5	5-15	16-30	>30		
Smoker	Yellow-staining cells	Mouth floor	21.2	25.9	39.8	47.5	0.01**	18.1	22.8	35.4	45.2	0.001**
		Buccal	32.3	31.8	34.5	32.6	0.4	31.5	33.7	33.5	33.8	0.6
	Red-staining cells	Mouth floor	25.3	31.8	42.7	50.5	0.02**	30.1	35.2	44.1	48.1	0.02**
		Buccal	34.4	33.5	32.2	32.7	0.5	32.1	31.1	33.9	34.1	0.5
	Blue-staining cells	Mouth floor	53.5	42.3	17.5	2	0.0002**	51.8	42	20.5	6.7	0.0002**
		Buccal	33.4	34.7	33.3	34.7	0.5	36.4	35.2	32.6	32.1	0.6
Opium addiction	Yellow-staining cells	Mouth floor	20.12	24.2	41.3	50.5	0.01**	20.1	20.8	38.2	48.1	0.001**
		Buccal	30.3	33.5	36.5	35.2	0.5	30.2	31.5	35.7	32.8	0.7
	Red-staining cells	Mouth floor	26.1	32.1	44.5	55.1	0.002**	30.1	35.2	44.1	48.1	0.02**
		Buccal	30.4	32.1	32.5	33.5	0.5	31.5	32.8	33.4	33.5	0.5
	Blue-staining cells	Mouth floor	51.4	43.2	19.5	4	0.0001**	54.1	41.5	22.8	8.9	0.0002**
		Buccal	31.2	31.5	31.3	32.5	0.5	33.2	32.9	33.8	33.1	0.6

* Mann-Whitney test, **P value significant, A = less than 5, B = 5-10, C = 10-15 and D = more than 15. A less than 5gr, B = 5-7 g, C = 7-10 g, and D = more than 10.

of the oral cavity and the sixth most common cancer.¹⁷ One of most frequently reported etiologic factors for this malignancy is the use of tobacco.¹⁸

The Iranian population has one of the highest incidence rates of oral squamous cell carcinoma in the world. The cause of these ex-

traordinary rates has been investigated and it has been noted that the main risk factors include poor diet, genetic susceptibility and opium consumption.³ A significant amount of opium is still consumed in many regions of the world, especially in Asia.¹⁹ and the risk of oral cancer risk in opium addicts is high in Asian people.

Table 3. Cytomorphometric analysis (median) in the 3 groups for age groups and intraoral site.

Variable	Nuclear area(μm^2)				Cytoplasmic area (μm^2)				Nuclear/ Cytoplasmic ratio(μm^2)				
	Smoker	Opium addiction	Non smoker	*P-value	Smoker	Opium addiction	Non smoker	*P- value	Smoker	Opium addiction	Non smoker	*P- value	
Age groups	<30	54.52	53.50	55.58	0.16	1912.14	1914.12	1942.22	0.2	0.028	0.028	0.028	0.072
	30–50	72.70	73.72	63.25	0.007**	2024.21	2022.28	2187.21	0.0001**	0.035	0.036	0.028	0.001**
	>50	77.87	77.55	58.15	0.0001**	2112.19	2125.20	2155.32	0.0001**	0.036	0.036	0.024	0.001**
Intraoral site	Floor of mouth	65.12	66.10	60.80	0.004**	2332.24	2330.28	2180.52	0.001**	0.030	0.028	0.021	0.002**
	Buccal	59.62	58.20	61.35	0.07	2185.44	2188.49	2189.25	0.18	0.027	0.027	0.028	0.08

* Mann-Whitney test, **P value significant

Table 4. Cytomorphometric analysis (median) in the smoker and opium addiction groups for the number of cigarettes and opium consumed per day and duration (years).

Groups	Variable	Number of cigarettes and opium per day					Duration (years)				
		A	B	C	D	*P- value	<5	5–15	16–30	>30	*P-value
Smoker	Nuclear area (NA)	68.56	65.36	70.56	79.78		62.58	69.06	75.89	78.98	
	Cytoplasmic area (CA)	2214.22	2145.52	1901.22	1958.19		2116.32	2221.21	1922.20	1943.25	
	Nuclear/ Cytoplasmic ratio(N/C)	0.030	0.030	0.040	0.040	*0.001*	0.029	0.030	0.040	0.046	0.002**
Opium addiction	Nuclear area (NA)	67.65	64.38	72.6	81.80		60.45	71.10	78.80	80.90	
	Cytoplasmic area (CA)	2215.32	2045.52	1900.20	1948.12	*0.001*	2102.30	2220.20	1912.10	1932.12	
	Nuclear/ Cytoplasmic ratio(N/C)	0.030	0.031	0.038	0.041		0.028	0.032	0.041	0.042	*0.001*

*A = less than 5, B = 5–10, C = 10–15 and D = more than 15. A less than 5gr, B = 5–7 g, C = 7–10 g, and D = more than 10, Mann-Whitney test, **P value significant

According to official figures, the prevalence of opium addiction in Iran is estimated at 2–2.8%.²⁰

Lyons and Yazdi studied the relationship between the frequency of consumption of a selected number of foods and oral cancer risk in a case-control study of 105 Italian cases. A significant and strong direct correlation was found between oral cancer and tobacco products such as cigars, opium, and smokeless tobacco.^{21,22}

In early stages, oral cancer sometimes demonstrates slow growth and may not be noticed by the patient. Diagnosis of the underlying pathology is an important step in management of any disease. In this context, exfoliative cytology can be used because it is simple, fast, inexpensive, and non-invasive and carries little risk. Currently, use of exfoliative cytology has increased as an adjunct to screening of precancerous lesions and malignancies of the oral cavity.²³

In the present study, exfoliative cytology technique was used to evaluate cellular changes in the buccal mucosa and the mouth floor of smokers and opium addicts in comparison with a control group. The results showed that the percentage of yellow and red cells increases with age in smokers and opium addicts. A study by Zimmermann and Zimmerman¹³ showed that the keratinized cells in the hard plate, gingival and the mouth floor of senile patients and smokers are more numerous compared to young patients and non-smokers. It appears that both smoking and aging influence keratinization. Of course, it should be noted that aging undoubtedly increases the number of years of smoking and this has a cumulative effect on keratinization. Smokers and opium addicts exhibited higher means of yellow and red cells in their mouth floor and lower means of blue cells compared to non-smokers, which is in consistent with the results of the study by Zimmermann and

Zimmermann.¹³

Khandelwal and Solomon²⁴ showed a significant increase in the cellular area of keratinocytes in mucosa of oral squamous cell carcinoma of patients. Also, results from a study by Kausar et al.²⁵ demonstrated that sada gura, consumed as a single agent or in combination with betel quid, leads to a significant induction of cytogenetic damage in the buccal epithelial cells of habitués.

Studies have shown that smoking is associated with increased keratinization in all mucous membranes of the oral cavity and even less keratinized areas of the oral cavity are at a risk of deleterious effects of smoking.^{26,27,28,29} However, there are significant differences in this respect between high risk areas, such as the mouth floor, the ventral aspect of the tongue and lateral border of the tongue, and other oral cavity regions which are somehow considered keratinized mucosa, such as gingival and the hard plated.²⁶

The results of the present study showed a significant relationship between the percentage of yellow and red cells in the mouth floor and the number of cigarettes smoked and amount of opium used as well as the duration of use. Studies have shown that some changes are observed in smokers with apparently healthy oral mucosa, which include an increased number of keratinized cells⁸ and changes in the nucleus: cytoplasm ratio.^{9,18} The cellular keratinization functions to protect the cell against noxious agents such as the heat of cigarette smoke and chemical agents such as hydrocarbons originating from tobacco.^{4,30} Zimmermann and Zimmerman,¹³ Kapczynski,²⁹ and Ogden et al²⁷ acknowledge the presence of cell alterations related to the number of cigarettes smoked per day and Silva²⁸ mentions that the number of cigarettes smoked must be considered as a factor.

Sampaio, et al.,¹⁰ showed that the number of cigarettes smoked

and the duration of smoking influence staining of the oral epithelial cells and all the subjects who smoked more than 20 cigarettes per day for more than 15 years exhibited more yellow cells, which is consistent with other studies.^{13,31} Drouilly³² did not observe significant differences in the types of epithelial cells obtained in smears of the mouth floor in smokers or non-smokers. On the other hand, other studies yielded different results. These studies reported an increase in the number of buccal cell and intermediate cells in the mucosa of mouth floor in smokers, which is consistent with the results of other studies.^{30,33,4,34,35}

The differences in the results of these studies might be attributed to differences in cytologic sampling techniques, characteristics of the sample used, as well as the quantity and duration of the smoking habit. In studies in which deep sampling techniques has been used, a higher number of cells were collected from deeper layers and therefore, more keratinized cells were found. In addition, the age of the subjects is a contributing factor. Furthermore, the subject's mouth should be rinsed with normal saline solution before sampling and ignoring this step results in staining of food debris and exfoliated epithelia cells.

In the present study, smokers and opium addicts aged 30–50 years and over 50, showed an increase in the nucleus/cytoplasm ratio, which was attributed to the site of sampling, i.e., the floor of the mouth. In addition, there was a significant relationship between the number of cigarettes smoked per day and duration of smoking and the nucleus/cytoplasm ratio. A decrease in cellular diameter and an increase in nuclear size are two important morphologic changes which are attributed to precancerous and cancerous changes. During the transition from the normal tissue to precancerous and cancerous lesions, some cellular changes take place at the molecular level, which can be determined.³⁶

Cowpe and Longmore³⁷ showed that exfoliative cytology can determine malignant changes through estimating the nucleus/cytoplasm ratio, which is consistent with the results of other studies in this respect.^{38,39,40} Ramaesh, et al.,⁴¹ reported the highest cellular diameter in the healthy mucosa and the lowest cellular diameter in the dysplastic lesions and oral SCC, which is consistent with a study by Einstein and Sivapathasundharam on tobacco users in southern India.¹⁵ In this study, a decrease in cellular diameter in smokers and opium addicts might indicate a precancerous change.

Franklin and Smite reported that increased nucleus/cytoplasm ratio might be due to changes in the size of the nucleus relative to the size of the cytoplasm and is possibly a reflection of significant changes in the cell at the morphologic level.⁴²

The results of the present study showed that smoking and opium use leads to cellular changes in the mucosa. Although the mucosa appears normal, there were significant differences in cellular diameters between smokers, opium addicts and non-smokers, consistent with the results of a study by Hande and Chaudhary.⁴³

Woyceichoski, et al.⁴⁴ evaluated the effect of cocaine in crack on the oral mucosa and showed that this addictive agent increases the diameter of the nucleus and decreases the size of cells, thus increasing their ratio compared to healthy individuals. In addition, they reported a significant relationship between the duration and extent of crack use and cellular changes.

Studies by Sellappa, et al.,⁴⁵ Joshi, et al.,⁴⁶ Mondal, et al.,⁴⁷ Suhas, et al.,⁴⁸ and Stich, et al.,⁴⁹ showed that the areca nut, tobacco and gasp cause some changes in the number and size of nucleus and cytotoxicity and genetic damage in the buccal mucosa cells. Besides, the research by de M Thiele et al. revealed that using

crack cocaine results in increasing the rate of proliferation in buccal mucosa cells.⁵⁰

A study by Kadivar and Attar⁵¹ on 25 healthy people, 25 smokers and 25 opium addicts showed that the maximum rate of cellular proliferation occurs in buccal mucosa of opium addicts and the minimum rate in healthy ones. Also, they found that cigarette smoking and in particular, opium increase the rate of cellular proliferation in buccal mucosa.

Finally, one of the limitations of this study was to exclude brushing, dentist visits, DMFT, etc. It is recommended that these factors should be accounted for in future works.

Conclusion

The results of this study indicate different rates of keratinizing epithelial cells in oral cavity of smokers, opium addicts and non-smokers. Also, these results suggest a possible relationship between the number of cigarettes per day and opium consumed per day and increased rate of cellular proliferation in oral mucosal cells. The present study showed a decrease in cellular diameter, an increase in nuclear diameter and an increase in the nuclear/cytoplasmic ratio in the smears of all smokers and opium addicts compared to non-smokers. Also, exfoliative cytology can be used as a diagnostic and preventive tool in oral premalignant lesions.

No Conflict of Interest

Acknowledgments

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