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Utility of keratinization and Mean NOR as Neoplastic Proliferative predictors of oral mucosal alterations related to carcinogenic exposure

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Abstract:

Objective: *The aim of the present study was to assess the cytomorphometric proliferative alterations of oral mucosa which might be induced by toombak dipping.* ***Methodology****: Two hundred volunteers (100 were toombak users (cases) and 100 were non-tobacco users (controls)) living in the city of Al-Ubayyid, western Sudan were studied by cytological methods using scrapes of oral mucosa cells.* ***Results****: Out of the 100 cases, 47 (47%) individuals were demonstrated with low keratinization and the remaining 53 (53%) persons were demonstrated with excessive keratinization. Abnormal mean Nucleolar Organizer Regions (NORs) count was identified among 26 (26%) of the cases, hence, no abnormal mean NOR count was identified among controls.* ***Conclusion:*** *Toombak dipping is a major factor for occurrence of keratinization of the oral mucosa, as well as, increased mean NOR values.*

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Key words: Cytomorphometric, Sudan, NOR, toombak, keratinization.

INTRODUCTION

The incidence rates of oral cancer are 3.7% for men and 2.6% for women in the Sudan. Several lifestyle risk factors for the development of oral cancer are familiar, including tobacco products, alcohol, infections, dietary factors, chemical irritants and frank carcinogens. Prevalence of oral cancer is 3.2% in Sudan and the disease is mainly attributed to N-nitrosamine rich oral snuff consumption[1].

In 2012, 21% of the global population aged 15 and above smoked tobacco. Men smoked at five times the rate of women; the average rates were 36% and 7% respectively [2]. Present and future generations must be urgently protected from the devastating health, social, environmental and economic consequences of tobacco consumption and exposure to tobacco smoke. Governments use the tobacco control measures in the WHO Framework Convention on Tobacco Control (WHO FCTC) to reduce the prevalence of tobacco use and exposure to tobacco smoke. By implementing these measures, governments reduce the heavy burden of disease and death that is attributable to tobacco use or exposure [3].

In the Sudan, snuff, locally known as Toombak, was introduced approximately 400 years ago. It is always processed into a loose moist form and its use is widespread in the country. Tobacco used for manufacture of the Toombak is of the species *Nicotiana rustica* and the fermented ground powder is mixed with an aqueous solution of sodium bicarbonate.

Toombak dippers develop a clinically and histologically characteristic lesion at the site of dipping. The risk for cancer of the oral cavity among Toombak users was high (RR 7.3-73.0-

fold) [4]. The use of Toombak plays a significant role in etiology of oral squamous cell carcinomas (OSCCs), with the tobacco specific nitrosamines present in Toombak possibly acting as principal carcinogens [5-8].Therefore, the objective of the this study was to assess the cytomorphometric proliferative alterations of oral mucosa which might be induced by toombak dipping.

MATERIALS AND METHODS

This is a prospective case control study, carried out among toombak users in the city of Al-Ubayyid, Western Sudan. Two hundred apparently healthy volunteers were selected for this study. One hundred were toombak users (ascertained as cases) and the remaining hundred were non-tobacco users (ascertained as controls). All toombak dippers were chosen from persons who have dipped toombak at least for the last five years. Patients with clinically apparent oral mucosal lesions, and previous benign or malignant lesions were excluded from this study. All cytological smears were collected by the same examiner from the mucosa of the mouth from the dip site (in toombak dippers). Participants were asked to rinse their mouth with saline solution for a minute before collection of the samples. The specimen collection site was dried by a smooth wipe so as to avoid silver staining of the mucoid material of saliva during application of AgNOR method to the slides. The material was collected by a smooth brush after brushing the floor of the mouth and tongue or dip-site two times, and rinsing and cleaning the brush each time in a saline solution. This was done so as to collect cells from the inner layers of the oral mucosa. The material collected was smeared on two slides and immediately fixed in 95% ethyl alcohol for 15 minutes.

One slide was stained according to the Papanicolaou (Pap)staining method and the other was stained according to AgNOR staining method.

Cytological Interpretation (Pap method): The presence of two or more of the following features indicated the presence of epithelial atypia: nuclear enlargement associated with increased nuclear cytoptasmic ratio, hyperchromatism, chromatin clumping with moderately prominent nucleolation, irregular nuclear borders, bi or multinuclation, increased keratinisation, scantiness of the cytoplasm and variations in size and or shape of the cells and nuclei. For each of these features, three possible grades were provided. The first grade allows for the fact that the characteristic may be completely absent (i.e. "none") or present in its normal form (i.e. "normal"). The other two grades (slight or marked) allow for different degrees of severity (mild, moderate or severe). The examiner had been trained in the calibration of the scoring by screening of normal and abnormal cytological smears in comparison with an atlas containing various abnormalities of the different characteristics [9].

AgNOR interpretation: All quality control measures were adopted during specimen collection and processing. All stained smears were examined by light microscope for the AgNOR quantitation. The NOR mean counts measured by counting the number of silver-stained dots per 20 nucleus for every smear, then the number obtained divided by 20.

Data analysis: The data was analyzed by SPSS (statistic package of social science) computer program. Pearson Chi square test was used with 95% confidence level and P value less than 0.05 was considered statistically significant.

Ethical consent: Each participant was asked to sign an ethical consent form before taking of the specimen and filling of the questionnaire.

RESULTS

In this analytical case control study, cytological changes were assessed among 200 individuals (100 Toombak users and 100 non-tobacco users, their ages ranging from 11 to 70 years, with a mean age of 26 years. The mean age for the cases was (27.5 ±11 years), hence, the mean age for the controls was (24 ±10 years), as it is shown in Table1. Seventy percent of the study subjects were below 30 years.

Table 2, showing the distribution of the study population by presence of keratinization. All of the cases were identified with some degree of keratinization, and, only one control subject was identified with keratinization among control group(Microphotograph 1). Out of the 100 cases, 47 (47%) individuals were demonstrated with low keratinization (Microphotograph 2) and the remaining 53 (53%) persons were demonstrated with excessive keratinization (Microphotographs 3 and 4). Toombak dipping is a major factor for occurrence of keratinization of the oral mucosa and this was found to be statistically significant $P < 0.0001$.

Table 3, showing the duration of toombak use by the degree of keratinization. Higher proportions of low keratinization were found in durations 0-5 and 6-10 representing 15 for each followed by durations 11-15, 16-20 and 20+ constituting 9, 6 and 2 respectively. High frequency of excessive keratinization was detected in duration range 6-10 representing 15 followed by durations 0-5, 20+, 11-15, and 16-20 constituting 12, 10, 8 and 8 respectively. In respect to the total participants in each duration, keratinization was found to increase with the increasing of duration and this was found to be statistically significant $P < 0.004$.

Table 4, showing the distribution of the cases by age and degree of keratinization. In respect to the total number of

participants in each age group, excessive keratinization is relatively increased with increasing of age.

Abnormal mean NOR count was identified among 26 (26%) of the cases, hence, no abnormal mean NOR count was identified among controls (Microphotographs 7, 8 and 9). The mean NOR counts for cases and controls were 2.35 ±1.6 and 1.56 ±0.41 respectively. This different was found to be statistically significant $P < 0.001$, as indicated in Table 5.

The relation between keratinization and NOR finding was shown in Table 6. However, 20 (10%) and 6 (3%) of the specimens showing excessive and low keratinization respectively, were further identified as having abnormal mean NOR count. Keratinization is significant factor for obtaining abnormal mean NOR count ($P < 0.03$).

In regard to the infection, 34, 21 and 9 of the cases were identified with *Actinomyces*, Bacteria and *Monilia* respectively, since, 10, 11 and 3 of controls were demonstrated with *Actinomyces*, Bacteria and *Monilia* in this order as shown in Table 7, and Microphotographs (5 and 6). Cases were more susceptible for infection than controls and this was found to be statistically significant $P < 0.001$.

In regard to the inflammation, 39 and 17 of the cases were identified with few and numerous polymorphs respectively, since, 14 and 1 of the controls were demonstrated with few and numerous polymorphs in this order, as shown in Table 8. Cases were more susceptible for inducing inflammatory process than controls and this was found to be statistically significant $P < 0.001$.

Out of the 26 individuals with abnormal mean NOR count, 1 (0.5%), 6 (3%), 11 (5.5%), 6 (3%) and 2 (1%) of the study subjects were identified among age range 20+, 21-30, 31-40, 41-50 and 50+ respectively, as indicated in Table 9.

Out of the 26 individuals with abnormal mean NOR count, 2 (2%), 3 (3%), 3 (3%), 5 (5%) and 13 (13%) of the cases

were identified among dipping frequency ranges 0-5, 6-10, 11-15, 16-20 and 20+ respectively, as indicated in Table 10.

Out of the 26 individuals with abnormal mean NOR count, 0 (0%), 3 (3%), 6 (6%), 7 (7%) and 10 (10%) of the cases were identified among dipping duration ranges 0-5, 6-10, 11-15, 16-20 and 20+ respectively, as indicated in Table 11.

Table 1. Distribution of study population by age

Age group	Frequency	Valid per cent	Cumulative Per cent
< 20 years	82	41	41.0
21-30	58	29	70.0
31-40	43	21	91.5
41-50	13	6	98.0
51+	4	2	100.0
Total	200	100	

Table (2): Distribution of study population by keratinization.

Keratinization	Cases	controls	Total
No	0	99	99
low	47	1	48
excessive	53	0	53
Total	100	100	200

P< 0.0001

Table (3): Relation between duration of Toombak use and keratinization:

Duration range	Degree of keratinization		
	Low	Excessive	Total
0-5 years	15	12	27
6-10	15	15	30
11-15	9	8	17
16-20	6	8	14
20+	2	10	12
Total	44	53	100

P< 0.004

Table (4): Distribution of Cases by age groups and degree of keratinization

Duration group	Degree of keratinization		
	Low	Excessive	Total
<20	23 %	16%	39
21-30	12	15	27
31-40	9	14	23
41-50	3	6	9
50+	0	2	2
Total	47	53	100

Table (5): Description of study population by mean NOR counts:

Cases\Controls	Mean NOR count			Total
	Normal	Abnormal	Mean	
cases	74	26	2.35	100
controls	100	0	1.56	100
Total	174	26	3.91	200

P< 0.0001

Tale (6): Relation between keratinization and mean NOR counts:

keratinization	NOR comment		Total
	Normal	Abnormal	
Low	41	6	47
Excessive	33	20	53
Total	74	26	100

P< 0.04

Table (7): Distribution of Infections among study population:

Micro organism	Cases\Controls		Total
	Cases	Controls	
No	36	76	112
Actinomyces	34	10	44
Monilia	9	3	12
Bacteria	21	11	32
Total	100	100	200

P< 0.001

Table (8): Distribution of Polymorphs among study population:

polymorphs		Cases\Controls		Total
		Cases	Controls	
	NO	44	85	129
	Few	39	14	53
	numerous	17	1	18
Total		100	100	200

P< 0.001

Table (9): Ages proportion with NOR mean counts:

age gp		NOR comment		Total
		normal	abnormal	
	< 20 years	81	1	82
	21-30	52	6	58
	31-40	32	11	43
	41-50	7	6	13
	51+	2	2	4
Total		174	26	200

P< 0.0001

Table (10): Dipping frequencies proportion of Cases with NOR mean counts:

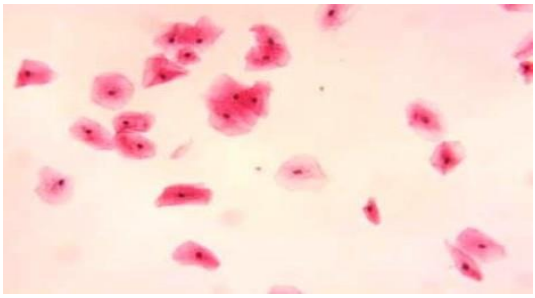
Frequency group		NOR comment		Total
		normal	abnormal	
	0-5	12	2	14
	6-10	10	3	13
	11-15	13	3	16
	16-20	16	5	21
	20+	23	13	36
Total		74	26	100

P< 0.0001

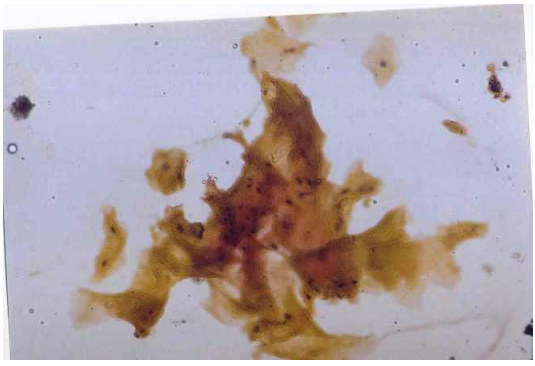
Table (11): Dipping duration proportion with NOR mean counts:

Duration group		NOR comment		Total
		normal	abnormal	
	0-5	27	0	27
	6-10	27	3	30
	11-15	11	6	17
	16-20	7	7	14
	20+	2	10	12
Total		74	26	100

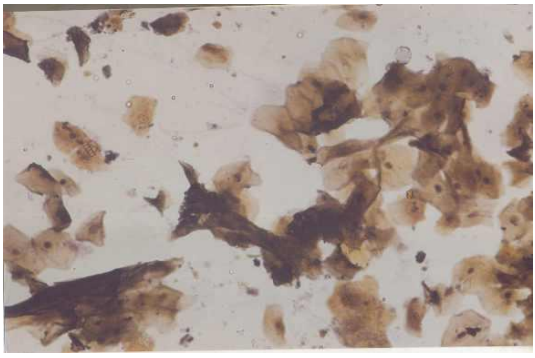
P< 0.0001



Microphotograph 1: Normal Buccal Mucosa from Non-tobacco user (Pap stain 200 X)

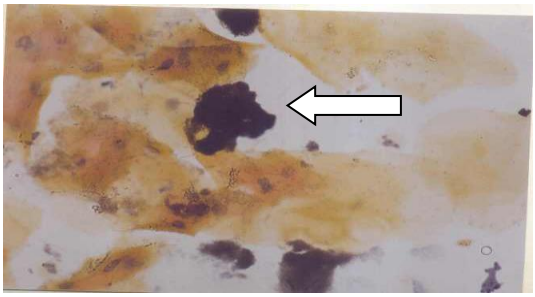


Microphotograph 2: Buccal Smear from dipping area showing low keratinization. Anucleated cells appeared in the field (Pap stain 200 X).

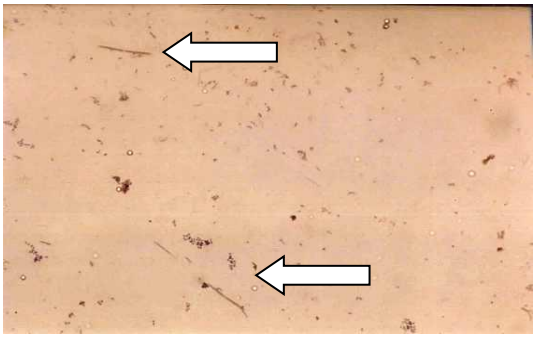


Microphotograph 3: Buccal Smear from dipping area showing excessive keratinization. Anucleated cells appeared in the field (Pap stain 200 X).

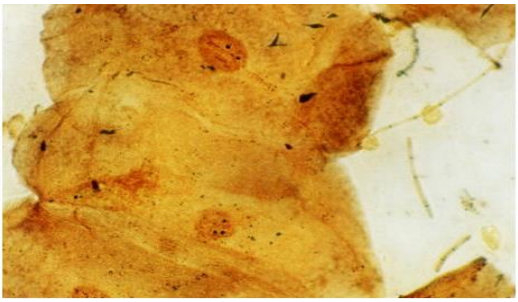
Microphotograph 4: Buccal Smear from dipping area showing excessive keratinization. Anucleated cells appeared in the field (Pap stain 400 X).



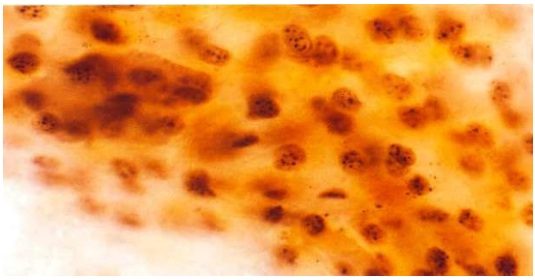
Microphotograph 5: A smear stained by Pap. Stain showed an actinomytes infection with few inflammatory cells (400 X).



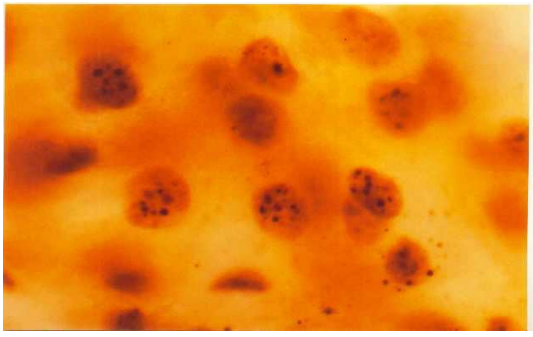
Microphotograph 6: A smear stained by Pap. Stain showed a Monilia infection (400 X).



Microphotograph 7: AgNOR Stained Smear from normal buccal mucosa Showing normal NOR Mean Counts (400 X).



Microphotograph 8: AgNOR Stained Smear from Dipping Area Showing Abnormal NOR Mean Counts ((200 X).



Microphotograph 9: AgNOR Stained Smear from Dipping Area Showing Abnormal NOR Mean Counts (400 X).

DISCUSSION

Squamous cell carcinoma (SCC) of the oral cavity and pharynx is one of the most frequent malignant neoplasms, especially in

the Sudan, where toombak dipping is frequently practiced. Toombak is a serious carcinogen that induces oral cancer due to its main content (TSNAs)[10].

In this study, out of the 100 cases, 47 (47%) individuals were demonstrated with low keratinization and the remaining 53 (53%) persons were demonstrated with excessive keratinization. Since keratinization with anucleation may lead to premalignant lesion called leukoplakia, there is a strong association between cancers of the oral cavity and pharynx and tobacco use.

Use of toombak has been showed to produce a variety of oral mucosal changes such as dysplasia and hyperkeratosis. An association between the severity of mucosal lesions and longer lifetime duration (> 10 years) of toombak use was found, but the severity was not related to the daily frequency of the habit [11]. In the sudan, the high incidence of OSCCs and an equal high prevalence of potentially malignant oral mucosal lesions has been strongly attributed to the habit of toombak use [12].

Duration and frequency of toombak use have a direct proportion with the degree of keratinization. In regard to the age, keratinization is relatively increased with the increasing of age.

Quantitatively significant difference existed in the number of AgNORs between the cases and controls, 26 % of the cases showed abnormal mean AgNORs counts. No abnormality detected among controls. Quantity is strictly proportional to the proliferative activity of the cell and does not necessarily indicate malignancy. It is the qualitative characteristics of AgNOR that help to differentiate hyperplastic, premalignant, and malignant lesions. AgNOR counts have been of a great value in recognizing of various benign and malignant lesions, to establish prognosis and to determine the proliferative activities of the cells [13]. The values for the premalignant lesions were lower than that of inflammatory lesions and carcinomas. The

mean AgNOR count ranged from 1.77 (normal mucosa) to 8.47 (moderately differentiated SCC)[14].

Keratinization is a significant factor, which causes abnormal mean NOR counts, since, 20 (10%) and 6 (3%) of the specimens showed excessive and low keratinization respectively.

In regard to the infections and inflammatory conditions, cases were more susceptible than controls, and this was found to be statistically significant ($P< 0.001$). Erosions and exposure of oral mucosa to toombak irritation are the major causative factors.

The brush biopsy technique is a critically discussed method for detection of oral pre-cancerous stages and manifest carcinomas. Conventional cytology has a reasonable accuracy measures rendering it suitable for identification of atypical cells in normal, inflamed or benignly hyperproliferative mucosa. The value determined for the restricted sensitivity in conventional brush biopsy cytoanalysis of 79% correlates well with results reported in the literature [15].

On the basis of the present study, it appears that AgNORs - silver stained nucleolar organizer regions - which stain for NOR-associated proteins act as markers of cell proliferation. Their quantity in terms of number of AgNORs per nucleus is strictly a marker of proliferative activity of the cells. The assessment of cellular proliferative activity of clinically healthy oral mucosal epithelial cells of toombak dippers and smokers by means of AgNOR counts and nuclear areas via nuclear morphometry was performed. Smears were collected from normal-appearing mouth floor mucosa and tongue of 75 toombak dippers, 75 smokers and 50 non-tobacco users between the ages of 20 and 70 with a mean age of 36 years. AgNORs were counted in the first 50 well-fixed, nucleated squamous cells and nuclear areas were calculated via microscopic stage micrometer. Cytological atypia was ascertained in 6 tobacco users and could not be ascertained in non-tobacco users.

Statistically mean AgNOR numbers per nucleus in the non-tobacco users (2.45±0.30) was lower than the toombak dippers (3.081±0.39, $p<0.004$), and the smokers (2.715±0.39, $p<0.02$), and mean nuclear areas of epithelial cells of toombak dippers (6.081±0.39, $p<0.009$) and smokers (5.68±10.08, $p<0.01$) was also significantly higher than non-smokers (5.39±9.4). The mean number of nuclei having more than 3 AgNORs was 28%, 19% and 7% in toombak dippers, smokers and non-tobacco users, respectively. These findings support the view that toombak dipping and smoking are severe risk factors for oral mucosal proliferative lesions and exfoliative cytology is valid for screening of oral mucosal lesions [11].

In another study, the presence of nucleolar organizer regions (NORs) compared in normal oral mucosa, dysplasia and microinvasive carcinoma. NOR quantification was performed with an image analyzer after staining by the argyrophilic nucleolar region technique. The morphometric results were statistically different for normal mucosa, dysplasia and microinvasive carcinoma. It was concluded that an increase of NOR activity follows the disease progression and may reflect the degree of cellular proliferation and malignancy [16].

In general, toombak dipping is a male tradition, however, small numbers of females practice this habit in hide, because it's considered as a social stigma. So, all of the participants in study were males (100%) at age zone ranging from 11 to 70 years with a mean 26 years.

In conclusion: Quantitative assessment of AgNORs by cytochemical methods might be useful to predict cellular proliferative activity. Cytochemical methods such as silver Nucleolar regions assessment is highly recommended for the assessment of cellular proliferative activity, simple and cost-effective methods. The success of conventional Pap smear in the evaluation of cytological (morphological) changes in oral mucosa caused by Toombak make it to be a very useful diagnostic tool

especially in oral screening program, however, Toombak use is a major risk factor for oral keratinization. Oral exfoliative cytology can be used to detect epithelial atypia which is frequently encountered in premalignant oral and early malignant lesions. More sophisticated methods such as Immunohistochemistry and in situ hybridization are recommended for precise evaluation of these traditional methods.

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