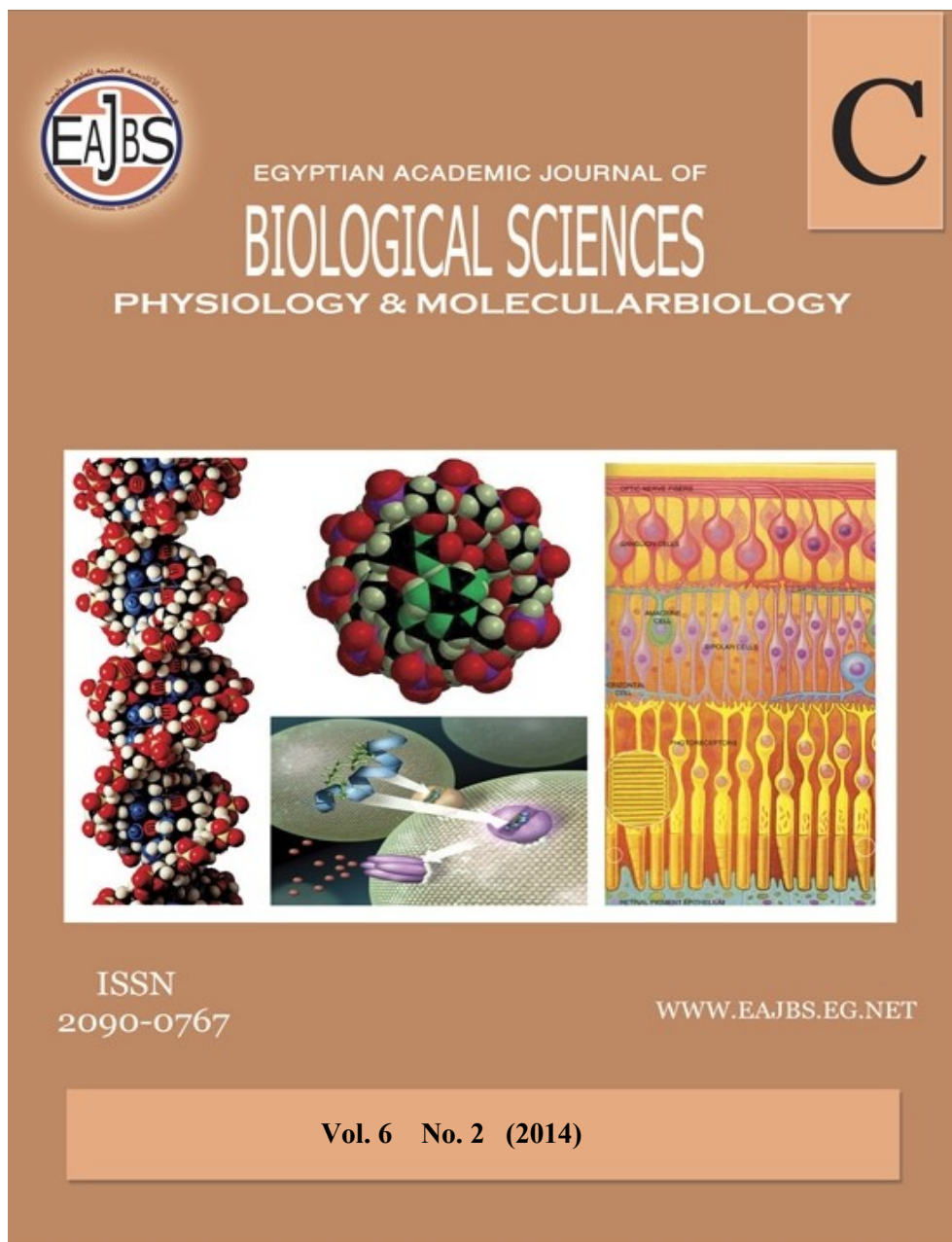


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## Assessment of Oral Cellular proliferative activity among Toombak Dipper in Al-Obeyed City, Sudan

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### ABSTRACT

**Background:** Many factors have been identified as important causative agents that responsible for the development of oral precancerous and cancerous lesions. In Sudan, Toombak have been identified as a major risk factor. Therefore, the aim of this study was to assess the cellular proliferative activity that associated with Toombak use. **Methodology:** In this study 75 apparently healthy individuals were selected for this study, of whom 50 were currently Toombak users (cases) and 25 were non-Toombak users (controls), Pap. Method and Silver technique were used for the staining of oral smears.

**Results:** Cytological atypia was identified in 10 cases and 4 controls. The mean count NORs count was 4.6 in cases and 2.2 in controls.

**Conclusion:** Toombak is a major risk for occurrence of cellular proliferative activity features that may progress in to oral precancerous or cancerous lesions.

### INTRODUCTION

The incidence rates of oral cancer are 3.7% for men and 2.6% for women in the Sudan (GLOBOCAN, 2008). 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 (GLOBOCAN, 2008). There are mainly 4 smokeless tobacco products: loose leaf or chewing tobacco, snuff, plug tobacco and twist or roll tobacco (IARC, 1985 Cullen *et al.* 1986). The oral use of snuff in North America and Western Europe is causally associated with an increased risk for cancer of the oral cavity and pharynx, and other pre-neoplastic changes such as leukoplakia (IARC, 1985 Cullen *et al.* 1986).

In Sudan, oral snuff, known locally as Toombak, is home-made from finely ground leaves of *Nicotianarustica*, a tobacco species with a particularly high content of nicotine and minor alkaloids.

This tobacco is mixed with Natron or Atron (sodium bicarbonate) (about 4:1), then water is added to the mixture, and after a period of about 2 hours or longer the mixture, called Toombak "saffa" (Idris *et al.* 1995). Natron or atron (sodium bicarbonate ( $\text{Na}_2\text{H}(\text{CO}_3)2.2\text{H}_2\text{O}$ )). Atron, opposed to lime in other parts of the world, is probably added to toombak for its alkaline effects. It has been shown that at high pH (11.0 - 11.8) nicotine is completely protonated and its rate of absorption is increased (Brunnemann and Hoffmann, 1974; Idris *et al.* 1991). Atron probably quickens absorption of nicotine from toombak to the central nervous system (Brunnemann *et al.* 1985). N-nitrosamines: the study by Idris, *etal.* (1991). Have analyzed the Tobacco Specific Nitroamine (TSNA) levels in Toombak and found unusually high levels of these TSNAs compared to the reported levels in any snuff (Tso 1972; Idris *et al.* 1991; Idris *et al.* 1992). Epidemiological evidence suggests that Toombak is a risk factor for cancer of the oral cavity and possibly of the esophagus in the Sudan (Idris *et al.* 1995). Several studies from Sudan have proved that Toombak use is a major risk factor that responsible of high frequencies of potential malignant oral lesions and oral cancers and in particular OSCCs in the Sudan. Most of tumors were observed at the site of dip application (lower lip). Oral cancer seems to be gender-specific, as the majority of cases were males (Ahmed *et al.* 2003; Ahmed and Mahgoob, 2007, Ahmed and Babiker, 2009, Ahmed *et al.* 2011; Edris *et al.* 2001).

Therefore, the aim of this study was to assess the cellular proliferative activity which is possible induced by the habit of Toombak dipping to enable early prediction carcinogenesis process using exfoliative cytology as an easy noninvasive diagnostic and screening procedure.

## MATERIALS AND METHODS

In this study, oral scrapes were obtained from 75 apparently healthy volunteers from the Al-Obeyed city in

Western Sudan. Of the 75 individuals, 50 were Toombak dippers ascertained as cases and the remaining 25 were non-Toombak dippers ascertained as controls. All study subjects were male, as Toombak dipping considered as social stigma among females in Sudan. All cytology smears were collected from the oral mucosa (the Toombak dip site).

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 staining method (Paiva *et al.* 2004). And the other was stained according to AgNOR staining method described by Pluton *et al.* (1986).

### Papanicolaou staining method:

Ethyl alcohol fixed smears were hydrated in descending concentrations of 95% alcohol through 70% alcohol to distilled water, for two minutes in each stage. The smears were then treated with Harris Heamatoxylin for five minutes to stain the nuclei, rinsed in distilled water and differentiated in 0.5% aqueous hydrochloric acid for a few seconds to remove the excess stain. This was immediately followed by rinsing in distilled water to stop the action of discoloration. Then the smears were blued in alkaline water for seven minutes and dehydrated in ascending alcoholic concentrations from 70% to 95% with two changes of 95% alcohol for two minutes each. The smears were next treated with Eosin Azure 50 for four minutes. For cytoplasmic staining, smears were treated with Papanicolaou Orange G6 for two minutes, rinsed in 95% alcohol and then dehydrated in absolute alcohol. The smears were cleared in Xylene and mounted in DPX (Distrene Polystyrene Xylene).

### AgNOR staining method:

The 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 were treated with silver stain to detect AgNORs as

follow: Smears were rehydrated by passing through descending graded alcohol to water, excess water were shaken off and slides were placed horizontally in humidified staining container. Smears were covered by silver solution, and were placed in a dark place for 45 minutes. Slides were then washed in three changes of water. Smears were immersed in 5% Sodium thiosulphate for 10 minutes followed by washing in running tap water. In the last step smears were dehydrated, cleared in xylene and mounted in *DPX* and were ready for examination. *AgNOR* sites appear as intranuclear black dots in a pale yellow background. Two investigators, blind to the study groups, analyzed the silver stained cells under light microscope (Olympus *BX1*, Japan) at 40x magnification.

**Data analysis:**

All results were analyzed by Statistical Package for the Social Sciences [SPSS] version 16.0. The means were obtained and chi-square and other variables odd ratio were calculated for comparison and presented in form of figures and tables and t.test analyze

the numerical *NOR* in test and control. *P* value <0.05 was considered significant.

**Ethical consent:**

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, University of Alyarmouk.

**RESULTS**

In this prospective study 75 apparently healthy volunteers were studied, their ages ranging from 16 to 61 years with a mean age of 28 years. Most of study subjects were among age group 21-30 years representing 39 followed by age group < 20 years as indicated in Table 1. The age distribution is relatively similar among cases and controls with exception of 21-30 for cases and age range < 20 years for controls as indicated in Fig.1.

Table 1: Distribution of age by cases and controls

Age	Cases	controls	Total
<20	4	12	16
21-30	30	9	39
31-40	8	0	8
40+	8	4	12
Total	50	25	75

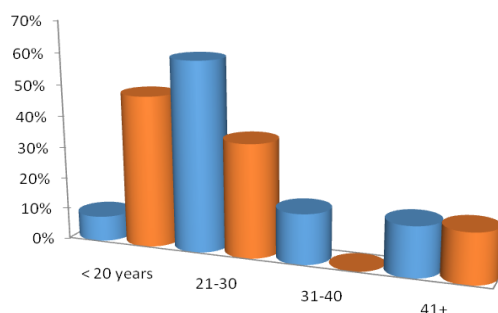


Fig.1: Description of the study population by age

Table 2 showing the distribution of the cytological diagnosis by cases and controls.

Cytological atypia was identified among 10 cases and 4 controls and could not

be identified in 40 and 21 of the cases and controls, respectively. Inflammatory infiltrate and evidence of infection were found in one and two of the cases

respectively as shown in Table2 (see photos 1, 2). The mean NORs counts for cases were found to be 4.6 compared to only 2.2 in control group. Moreover, all of the cases

were found with high mean NORs count compared to 14/25(56%) of the controls, as seen in Table 3 (see photo3).

Table 2: Describes the frequency of infection, inflammation and cellular change

Category	Cases		controls		p-value
	Yes	No	yes	no	
Inflammation	1	49	1	24	0.001
Infection	2	48	1	24	
Cell atypia	10	40	4	21	

Table 3: Distribution of the study population by mean NORs count

NOR	Cases	Controls	Total	p-value
normal	0	11	11	0.0001
High	50	14	64	
Total	50	25	75	

Fig. 2 Showing description of the study population by habits and cytological atypia. The proportions of all categories were high among cases compared to controls, including smoking, alcoholic, atypia and adduct drug use constituting 68%, 38%, 20% and 18%, in this order as shown in Fig. 2 In regard to the

relation between high mean NORs count (abnormal) and diagnosis and habits among cases. Alcoholic, adduct drug, cytological atypia and smoking, were found to represent, 100%, 100%, 93%, and 92.3%, respectively, as shown in Fig. 3.

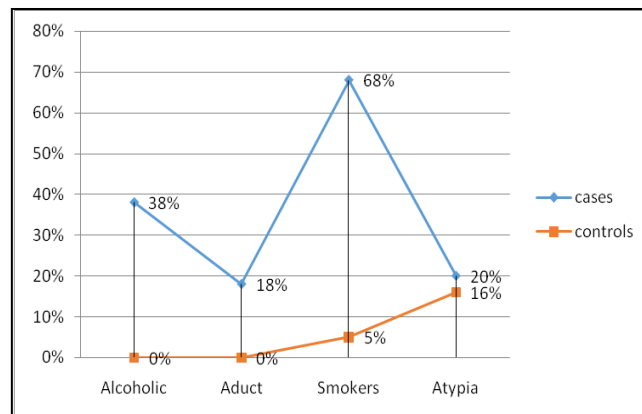


Fig. 2: Description of the study population by habits and cytological atypia

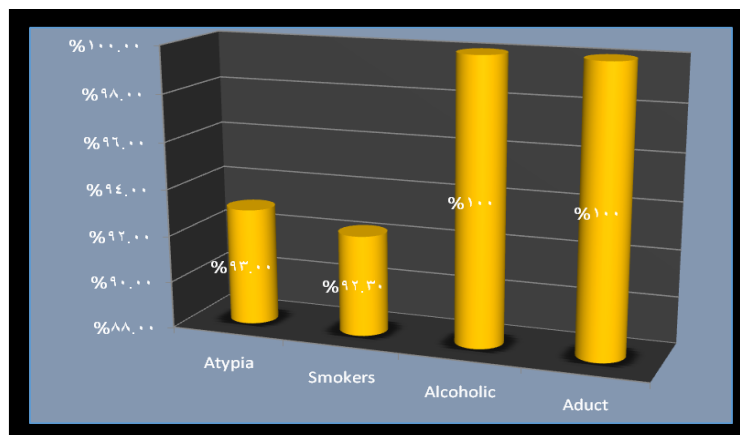


Fig. 3: Description of the cases by abnormal mean NORs count and diagnosis and habits.

## DISCUSSION

More than 95% of the carcinomas of the oral cavity are of squamous cell type; in nature they constitute a major health problem in developing countries, representing a leading cause of death. The survival index continues to be small (50%), as compared to the progress in diagnosis and treatment of the other malignant tumors (Mehrotra and Yadav 2006). In the Sudan, oral cancer is one of the major health problems, due to the habit of Toombak using (Idris *et al.* 1995). These facts explain the findings of the current study, particularly, the significant ( $P < 0.0001$ ) variation in the mean NORs count (4.6 among cases compared to 2.2 in controls). NORs have been shown to be the site of *rDNA* which are transcribed to *rRNA*. They can routinely highlight by virtue of argyrophilia of their associated proteins (Derenzini *et al.* 1998). It has been reported that, particularly human somatic cells could contain 10 demonstrable NORs in nuclei, but many resting cells contain only one particle (Crocker and Egan 1988). Due to increased proliferative activity of neoplastic cells, higher number of NOR particles in cancerous cells might be expected (Boldy *et al.* 1989). This in addition to the presence of a reasonable of cytological atypia among cases compared to controls. These findings incriminate the role of Toombak use as a risk for development of cellular proliferative activity which may progress to oral cancer. In the Sudan, snuff, locally known as Toombak, it is wide-spread in the country. The results of this study agreed with the study by (Ahmed and Mahgoub, 2007), who found that, the risk among Toombak user was high. Furthermore, study by (Idris *et al.* 1994), revealed that Toombak contains at least 100- fold higher concentration of tobacco- specific N-amine *TSNA*. The majority of patients with oral lesions in the present study have prolonged history of Toombak use as well as smoking. It seemed that the effects steadily increase with increasing duration and frequencies of use as shown in the literature, as it well established

that, chemical carcinogenesis is a prolonged, processed, and progressed with increasing of exposure, the duration of tobacco exposure seemed to have effects on empowering of the risk of oral cancer (Ahmed and Babiker, 2009). As part of the development of a screening procedure for oral cancer and pre-cancer, exfoliative cytology (*EFC*) was applied to a retrospective cohort to assess the presence and severity of oral epithelial atypia (*ET*) in 300 subjects (100 Toombak dippers; 100 cigarette smokers; 100 non-tobacco users) without prior knowledge of the subjects' tobacco exposure. *ET* was ascertained in 29 subjects and could not be ascertained in the remaining 271. Among the 29 subjects with *ET*, there were 11 (38%) Toombak dippers, 14 (48%) cigarette smokers and 4 (14%) non-tobacco users. Among the 271 subjects without *ET*, there were 89 (33%) Toombak dippers, 86 (32%) cigarette smokers and 96 (35%) non-tobacco users. For the *ET* among Toombak dippers and cigarette smokers, adjusted OR and the 95% *CI* were found to be 3 (0.91-9.7) and 4 (1.2-12.3), respectively (Ahmed *et al.* 2003).

In conclusion: *OSCC* represent great burden, and enormous health problems in the Sudan, particularly among snuff dippers. Fortunately, early stages of cancer cells can be revealed by oral exfoliative cytology. In addition to *Pap*. Smear, *NOR* technique could be, sensitive, and objective adjuvant tool for early identification of neoplastic cells in oral smear.

Furthermore, cytology should be implemented as a routine investigation in dental clinic. Heavy Toombak dippers as well as smoker should undergo periodic screening cytological examination. Use of both *Pap*. Test and *NORAg* techniques are reliable in the screening of oral cellular proliferative activity (indicator of oral precancerous change).

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## ARABIC SUMMARY

تقيم النشاط الخلوي الفموي لدى مستخدمي التبناك في مدينة الابيض بالسودان

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2- قسم علم الامراض, كلية الطب, جامعة حائل, المملكة العربية السعودية.

**الخلفية:** تم تحديد العديد من العوامل المسببة المهمة والمسؤول عن تطور الاورام السرطانية. في السودان يعتبر التبناك من اكثر المخاطر لسرطان الفم. لذلك تهدف هذه الدراسة الي تقييم النشاط الخلوي لمستخدمي التبناك.  
**المنهجية:** في هذه الدراسة تم اختيار 75 أفراد اصحاء لهذه الدراسة، منهم 50 كانوا مستخدمي تبناك في الوقت (عينة دراسة) الحاضر المستخدمين 25 كانوا غيرمستخدمين (عينة ضابطة). أستخدمت طريقتي باب والسلفر لصبغ المسحات الخلوية.  
**النتائج:** تم تحديد اللانمطية الخلوية في 10 حالات و 4 ضوابط. كان متوسط العدد للنور 4.6 و 2.2 في الحالات وفي الضوابط.  
**الاستنتاج:** التبناك هوخطر رئيسي لحدوث ملامح النشاط الخلوي التكاثري الذي يمكن ان يتحول لحالات قبل سرطانية أو سرطانية في الفم.