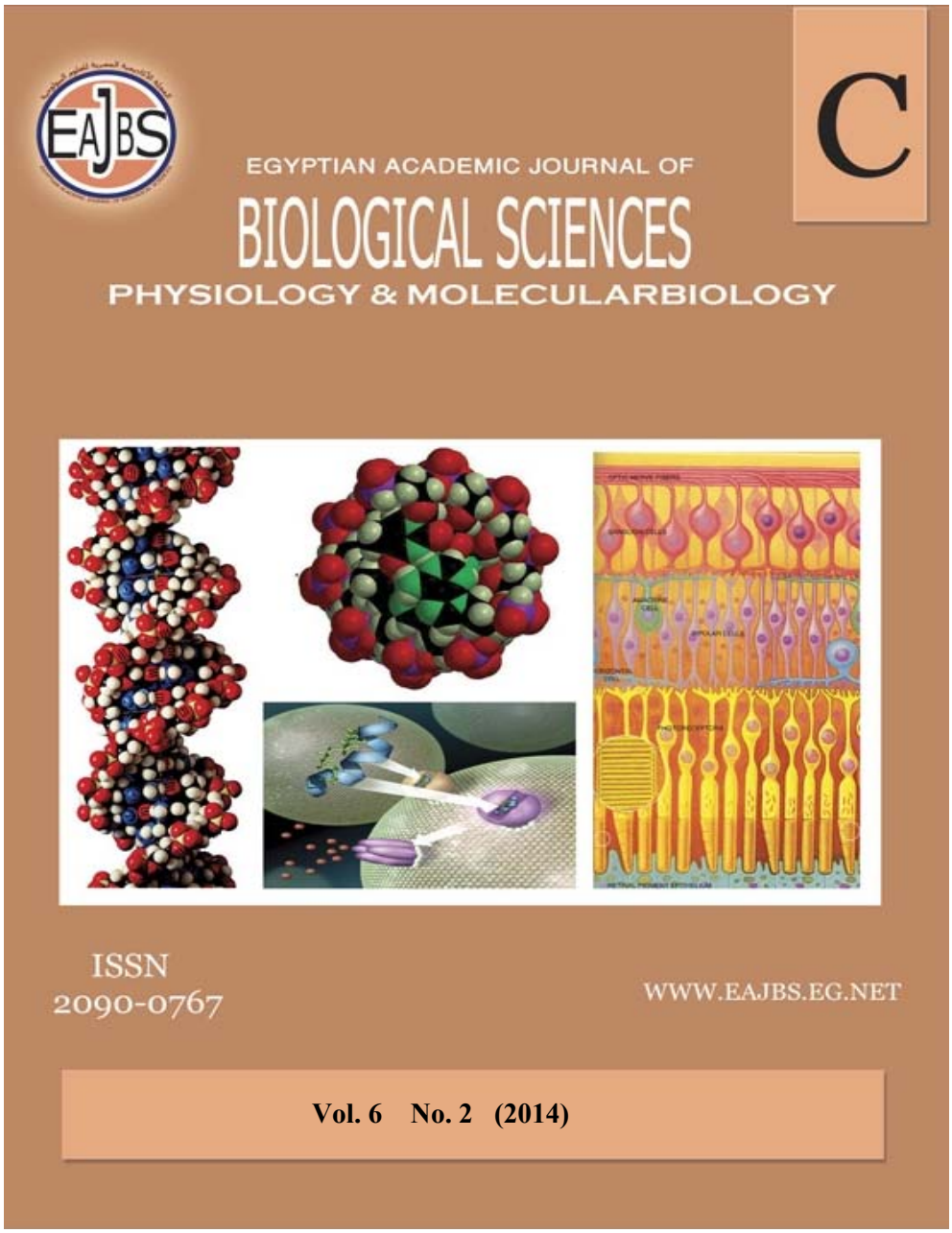


Egyptian Academic Journal of Biological Sciences is the official English language journal of the Egyptian Society for Biological Sciences, Department of Entomology, Faculty of Sciences Ain Shams University .

Physiology & molecular biology journal is one of the series issued twice by the Egyptian Academic Journal of Biological Sciences, and is devoted to publication of original papers that elucidateimportant biological, chemical, or physical mechanisms of broad physiological significance.

www.eajbs.eg.net

Egypt. Acad. J. Biolog. Sci., 6(2): 107 - 113 (2014)

Egyptian Academic Journal of Biological Sciences
C. Physiology & Molecular Biology

ISSN 2090-0767
www.eajbs.eg.net

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

**Awad Eljeed Abugooda Alobaid[1], Ibrahim Bakhit Yousef Elemam[1], Mohammed Abed Algader Alsheikh[1], Abdalhafeez Osman Mahmud[2], Hussain Gadelkarim Ahmed[2]**

1- Department of Histopathology and Cytology, Medical Laboratory Sciences, Al-yarmouk College, Khartoum, Sudan.

2- Department of Pathology, College of Medicine, University of Hail, KSA.

Email: Hussaingad1972@yahoo.com, he.ahmed@uoh.edu.sa

**ARTICLE INFO**

Article History
Received: 25/9/2014
Accepted:10/11/2014

***Keywords*:**
Toombak
Cytological atypia
Oral cancer
Al-Obeyed

**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 ($Na_2H$ $(CO_3)2.2H_2O$). 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 Nittrose Amine (*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 Heamatoxlyin 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 mentees 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 |

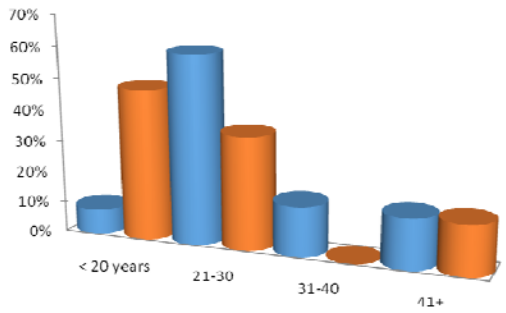


Fig.1: Description of the study population by age

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

| | Cases | | controls | | |
|---|---|---|---|---|---|
| Category | Yes | No | yes | no | p-value |
| Inflammation | 1 | 49 | 1 | 24 | |
| Infection | 2 | 48 | 1 | 24 | 0.001 |
| 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 | |
| High | 50 | 14 | 64 | 0.0001 |
| 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.

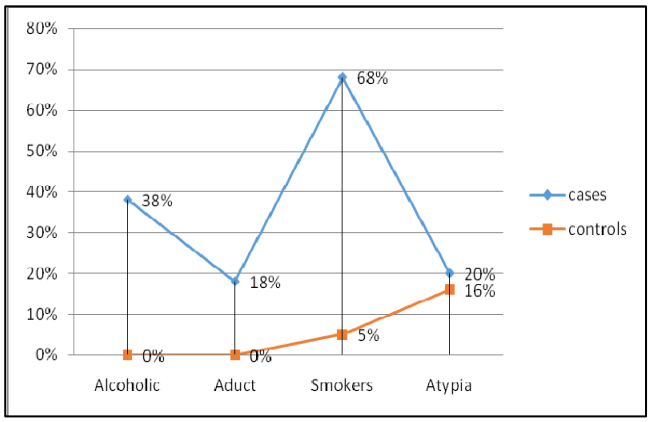


Fig. 2: Description of the study population by habits and cytological atypia

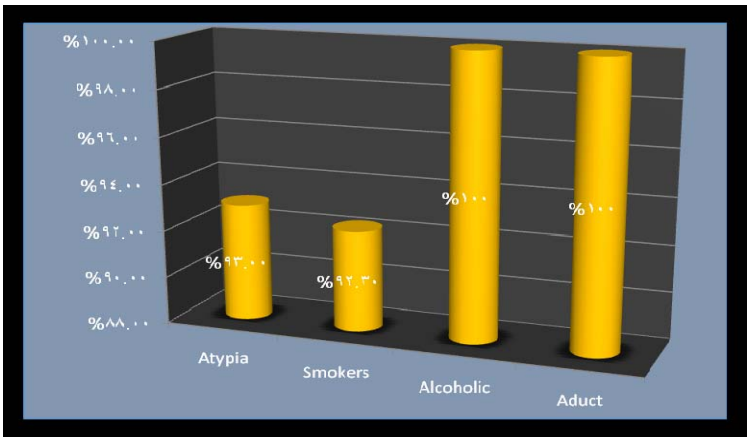


Fig. 3: Description of the cases by abnormal mean *NORs* count and diagnosis and habits.

## 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 (indictor of oral precancerous change).

## REFERENCES

Ahmed HG, Babiker AE. (2009). Assessment of cytological atypia, AgNOR and nuclear area in epithelial

cells of normal oral mucosa exposed to toombak and smoking. Rare. Tumors, 1(1):e18.

Ahmed HG, Idris AM, Ibrahim SO. (2003). Study of oral epithelial atypia among Sudanese tobacco users by exfoliative cytology. Anticancer Res., 23(2C):1943-9.

Ahmed HG, Mahgoob RM. (2007). Impact of Toombak dipping in the etiology of oral cancer: gender-exclusive hazard in the Sudan. J Cancer Res There. 3(2):127-30.

Ahmed HG, Omer AS, AbdAlgaffar SA. (2011).Cytological study of exfoliative buccal mucosal cells of Qat chewers in Yemen. Diagn Cytopathol., 39(11):796-800.

Boldy, DA, Crocker, J, Ayres, JG. (1989). Application of the AgNOR method to cell imprints of lymphoid tissue. J. Payhol, 157(1): 75- 79.

Brunnemann KD, Genoble L, Hoffmann D. (1985). N-Nitrosamines in chewing tobacco: an international comparison. J. Agric. Food Chem., 33:1178-81.

Brunnemann KD, Hoffmann D. (1974). The pH of tobacco smoke. Food Cosmet Toxicol., 12(1):115-124.

Crocker, J, Egan, MJ. (1988). Correlation between NOR sizes and numbers in Hodgkin's lymphoma. J Pathol, 156(2): 1794 - 1801.

Cullen JW, Blot W, Henning field J, Boyd G, Mecklenburg R, Massey MM. (1986). Health consequences of using smokeless tobacco: summary of the Advisory Committee's report to the Surgeon General. Public Health Rep., 101(4):355-73.

Derenzini, M, Trere, D, Pession, A, Montanaro, L, Sirri, V, Ochis, RL. (1998). Nucleolar function and size in cancer cells. Am J Pathol, 152(5): 1291- 1297.

Edris AM, Ahmed HG, Mohammed EA. (2011). Accuracy of oral exfoliative cytology in Sudanese patients undergoing oral biopsy. RSBO. 8(3):255-260.

Idris AM, Ahmed HM, and Malik MO. (1995). Toombak dipping and cancer of the oral cavity in the Sudan: a case-control study.Int J Cancer., 63(4):477-480.

Idris AM, Ahmed HM, Mukhtar BI, Gadir AF, el-Beshir EI. (1995). Descriptive epidemiology of oral neoplasms in Sudan 1970-1985 and the role of toombak. Int J Cancer., 61(2):155-168.

Idris AM, Nair J, Friesen M, Ohshima H, Brouet I, Faustman EM, Bartsch H. (1992). Carcinogenic tobacco-specific nitrosamines are present at unusually high levels in the saliva of oral snuff users in Sudan. Carcinogenesis. 13(6):1001-5.

Idris AM, Nair J, Ohshima H, Friesen M, Brouet I, Faustman EM, Bartsch H. (1991). Unusually high levels of carcinogenic tobacco-specific nitrosamines in Sudan snuff (toombak). Carcinogenesis;12(6):1115-8.

Idris, AM, Warnakulasuria, KA, Hoffmann, D, (1994). Toombak: a major risk factor for cancer of the oral cavity in Sudan. Prev M, 23(6): 832-9.

International Agency for research on cancer: GLOBOCAN (2008). Available from: http://globocan.iarc.fr/factsheets/populations/factsheet.asp?uno=900#MEN

Mehrotra R, Yadav S. (2006). Oral squamous cell carcinoma: Etiology, pathogenesis and prognostic value of genomic alterations. Ind J of Can, 43(2); 60-66.

Paiva RL, Sant'Ana Filho M, (2004). AgNOR quantification in cells of normal oral mucosa exposed to smoking and alcohol. A cytopathologic study. Anal Quant CytolHistol, 26:17580.

Ploton D, Menager M, Jeannesson P. (1986). Improvement in the staining and in the visualization of the argyrophilic proteins of the nucleolar organizer region at the optical level. Histochem J., 18:514.

Tobacco habits other than smoking. (1985). IARC Monogr EvalCarcinog Risk Chem Hum., 37: 37-136.

Tso TC. (1972). Physiology and Biochemistry of Tobacco Plants. Stroudsburg, PA: Dowden, Hutchinson and Ross. PP. 393.

## ARABIC SUMMARY

**تقيم النشاط الخلوي الفموي لدى مستخدمي التمباك في مدينة الابيض بالسودان**

**عوض الجيد أبوجودة العبيد[١] – إبراهيم بخيت يوسف الأمين[١] – محمد عبدالقادر الشيخ[١] – عبدالحفيظ عثمان[٢] حسين جادالكريم أحمد[٢]**

١- قسم علم الامراض، المختبرات الطبية ، كلية اليرموك ، السودان

٢- قسم علم الامراض، كلية الطب ، جامعة حائل، المملكة العربية السعودية.

**الخلفية**: تم تحديدالعديد من العوامل المسببة المهمة والمسؤول عن تطور الاورام السرطانية. في السودان يعتب التمباك من اكثر المخاطر لسرطان الفم. لذلك تهدف هذه الدراسة الي تقيم النشاط الخلوي لمستخدمي التمباك.

**المنهجية** :في هذه الدراسة تم اختيار ٧٥ أفراد اصحاء لهذه الدراسة، منهم ٥٠ كانوا مستخدمي تمباك في الوقت (عينة دراسة) الحاضر المستخدمين ٢٥ كانوا غيرمستخدمين (عينة ضابطة). أستخدمت طريقتي باب والسلفر لصبغ المسحات الخلوية.

**النتائج**: تم تحديد اللانمطية الخلوية في١٠ حالات و ٤ ضوابط . كان متوسط العدد للنور ٤.٦و ٢.٢ في الحالات وفي الضوابط.

**الاستنتاج**: التمباك هوخطر رئيسي لحدوث ملامح النشاط الخلوي التكاثري الذي يمكن ان يتحول لحالات قبل سرطانية أو سرطانية في الفم.