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Application of AgNOR Scores for the Evaluation of Cytological Atypia for Oral Mucosa Exposed to Toombak from a Dental Teaching Hospital, of Sudan

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Authors' contributions

This research was conducted in collaboration between all authors. All authors involved in the study design, protocol writing, results interpretation and final manuscript draft, reading and approval. Authors HIA, RMS and MAY managed the field data collection. Authors HIA, AMW and AAB preformed the laboratory techniques and statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Oral cancer is one of the highly prevalent cancers worldwide and a leading cause of mortality in certain regions like South-Central Asia. Oral cancer in Sudan constitutes a serious health problem, squamous cell carcinoma (SCC) is the most prevalent type of oral malignancy. The aim of this study was to assess cellular proliferative activity of clinically healthy oral mucosal epithelial cells of toombak dippers by means of AgNOR counts and cytological atypia using H&E and Pap stain, to

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find out the best staining results of the three stains, and to find out the most affected age group, frequency of dipping toombak per day, and the effect of snuffing duration in cellular proliferative activity. Smears were collected from normal-appearing mouth floor mucosa and tongue from 100 patients of toombak dippers, 50 non-tobacco users for negative controls and 10 cases were toombak users and they had oral cancer for positive control. AgNORs were counted in the first 50 well-fixed, nucleated squamous cells via microscope. The laboratory analysis showed that; decreasing the number of the normal cases associated with increasing to snuffing duration and to frequency of dipping per day; that the normal cytological appearance was 25 cases of snuffing duration of 2-5 years, 20 cases of snuffing duration of 6-10 years, and 4 cases of 11-15 years of snuffing. Increasing in the cases of inflammation associated with increasing to snuffing duration and to frequency of dipping per day; that the inflamed samples was 3 cases of snuffing duration of 2-5 years, 15 cases of 6-10 years of snuffing, 16 cases in 11-15 years, in 16-20 years were 8 cases, and 3 cases in above 20 years of snuffing. The cases of a typical cells appears associated in cells appears associated with increasing to snuffing duration after 11-15 years of snuffing and to increasing in frequency of dipping per day. The AgNor number like the cytological diagnosis of H&E and Pap stain that the normal count found in short snuffing duration and the abnormal count appears with the increasing in snuffing duration after 11-15 years of snuffing, as general alteration in oral mucosa appears after 5 years and more than 5 times/day, the difference was statistically significant (P value 0.01), and associated with increasing in frequency of dipping per day. In conclusion oral exfoliative cytology using Pap and H&E stain is useful in evaluation of epithelial a typia that is frequently encountered with pre-malignant and early malignant oral lesions. The results in AgNor count like the cytological diagnosis in H&E and Pap stain, the intensive exposure to toombak is a significant factor of increasing cellular a typia. The study recommended that there is a need to standardize the cytological proliferative marker methods to provide rapid, inexpensive, simple and applicable screening test for subjects who have been identified as being at high risk for developing oral cancer, therefore the micro nuclei frequency and AgNOR counts recommended to being used with Pap and H&E stain.

Keywords: Oral cancer diagnosis; AgNOR; H&E; PAP; Sudan.

1. INTRODUCTION

Oral mucosa is a specialized type of tissues that lines the mouth. This tissue is designed to provide protection for the body from infection and debris, and it is capable of producing secretions such as mucus, in addition to absorbing materials introduced into the mouth. The trait of absorption is used to apply certain types of medications, such as oral vaccines [1].

The color of the oral mucosa can vary, depending on the skin color of the body. In some people, it is a pale pink, while others have dark pink to brown tissue. Extremely pale mucosa can be a sign that someone is anemic, while patchy or dark ones can be signs of a medical problem.

A classic problem that develops with the oral mucosa is leukoplakia, in which white plaques of material appear in the mouth. Also so many changes and lesions can appear in the oral mucosa that may or may not change the mucosal color [1].

Social habits of tobacco use and alcohol consumption have been shown to produce a variety of oral mucosal changes [2].

In the Sudan, the high incidence of oral mucosal changes (dysplasia, hyperkeratosis and other potentially malignant oral mucosal lesions) and equally the high prevalence of these changes have been strongly attributed to the habit of snuff use which is dipped in the mouth and locally known as toombak [2].

Toombak has been used in the Sudan for centuries and its use is widespread. A close relationship between use of toombak and development of oral mucosal changes has been reported. In addition, snuff contains other carcinogens including aliphatic and aromatic hydrocarbons, formaldehyde, ketones, alcohols, phenols, amines, amides, alkaloids, metals, radio element (e.g. polonium-210, uranium-235 and -238) and poly aromatic hydrocarbons (PAHs) [3].

Early diagnosis of symptomatic (scars) and asymptomatic (during routine dental check) is of great importance for oral mucosal changes and

lesions, oral exfoliative cytology, a simple, painless and inexpensive method has become a preferred method for both early diagnosis of the lesion and for establishing quantitative techniques [4].

During the oral exfoliative cytology the presence of two or more features consistent with a typical change in cells which is a sign of malignancy.

Nucleolar organizer regions (NORs) are located in the cell nucleoli during interphase. They are loops of DNA in which ribosomal RNA is encoded . Also changes can occur in the NORs counts that can be detected by the AgNOR test which another method of oral exfoliative cytology [5].

The aim of this study was to assess cellular proliferative activity of clinically healthy oral mucosal epithelial cells of toombak dippers by means of AgNOR counts and cytological atypia using H&E and Pap stain, to find out the best staining results of the three stains, and to find out the most affected age group, frequency of dipping toombak per day, and the effect of snuffing duration in cellular proliferative activity.

2. MATERIALS AND METHODS

This was cross sectional and comparative study for assessment of cytological atypia in epithelial cells of oral mucosa of toombak users, using H&E, PAP stain, and AgNOR. The study was conducted during the period of March 2015 to July 2017.

The Study was carried out in Wad Medani dental teaching hospital in Wad Medani the capital of Gezira state which lies in the western bank of the blue Nile, , Central Sudan.

2.1 The Sample Size Included

The Sample size included the 160 sample; 100 toombak dippers which was the test sample, 50 non-tobacco users for negative controls and 10 cases were toombak users and they had oral cancer for positive control.

2.2 Study Population Included

Study population included the patients who presented to the hospital and who have a history of snuff dipping more than two years of both genders and their ages above 18 years.

2.3 The Study Excluded

The study excluded patients with a history of radiotherapy or chemotherapy for oral or other malignancy, patients with history of smoking or alcohol consumption, patients with a history of systemic diseases, and patients with a history benign or malignant oral lesions (to avoid pias).

2.4 Pre Procedural Evaluation Included

Pre procedural evaluation included pre procedure targeted physical exam for mouth cavity by the dentist, a brief history for the systemic disease and social history and habits and questionnaire filled by the patient which includes; the age of the participant,the duration of snuffing and frequency of dipping toombak per day.

2.5 Sample Collection and Processing

Sample collection and processing; Buccal smear which was done by brush from the dipping site. Participants were asked to rinse their mouth with saline solution for a while before the sample is taken. The materials were collected by a smooth brush after brushing the floor of the mouth at the dipping site two times, then 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 for each specimen was smeared on three slides and immediately fixed in 95% ethyl alcohol for 15 minutes, one slide was stained by H&E, the second slide was stained by PAP stain, and the third slide was stained by Silver nitrate stain for AgNOR.

2.6 Assessment of Slides

Assessment of slides; each specimen had three slides, which was reviewed by a cytopathologist according to criteria of benign and malignant. The results for each stain compared with positive and negative control samples. Assessment of other factors such as the most affected age group, frequency of dipping toombak per day, and the effect of snuffing duration in cellular proliferative activity.

2.7 Statistical Analysis

Statistical analysis for the data was done by SPSS program. The X2 test was used to compare the differences between the results of the diagnosis for the three methods; Agnor, H&E, and PAP stain comparing with negative control

(non toombak users). A $p<0.05$ was considered statistically significant.

3. THE ORDINARY HAEMATOXYLIN AND EOSIN (H&E) STAIN

Buccal smear which was done by brush from the dipping site hydrated in descending grades of alcohol concentration, at 95% through 70% to distilled water for 2 minutes in each stage. For staining of the nucleus, the smears treated with Mayer's Haematoxylin for 8 minutes and differentiated by rinsing in acid alcohol for seconds, bluing in running tap water for 8minutes, counterstaining in Eosin for 1 minute, and rinsed in water. The smears dehydrated in 70% alcohol through 95% and 100% alcohol, and then blotted in a filter paper, cleared in xylene and mounted in DPX, after that the smears were ready for microscopic examination.

3.1 Interpretation of the Results

Nucleus; deep blue colour. Cytoplasm and background tissue; pink colour. RBCs; orange colour [6].

4. 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. Then the smears were treated with Harris' hematoxylin 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 of stain. They were then immediately rinsed in distilled water, to stop the action of discoloration. Then the smears were blued in alkaline water for a few seconds and dehydrated in ascending alcoholic concentrations from 70%, through two changes of 95% alcohol for two minutes for each stage. The smears were next treated with Eosin Azure 50 for four minutes. For cytoplasmic staining, they were treated with Papanicolaou Orange G6 for two minutes, rinsed in 95% alcohol and then dehydrated in absolute alcohol. The smears were then cleared in Xylene and mounted in DPX (Distrene Polystyrene Xylene) mount.

4.1 Results Interpretation

The nuclei should appear blue/black. Cytoplasm (non-keratinizing squamous cells) Blue/green. Keratinizing cells pink/orange [6].

Atypia was assessed cytologically by using the presence of two or more of the following features which were consistent with atypia: nuclear enlargement associated with increased nuclear: cytoplasmic ratio, hyperchromatism, chromatin clumping with moderately prominent nucleoli, irregular nuclear membranes and bi- or multi-nucleation, scant cytoplasm, and variation in size and/or shape of the cells and nuclei [7].

5. AgNOR STAIN

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 incubated with this silver solution for 30 minutes at room temperature in a dark medium and then were protected in the dark until each slide was analyzed.

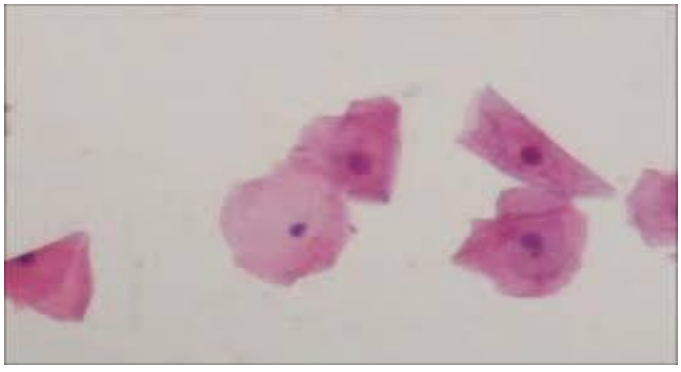

Fig. 1. Normal buccal cells stained with H & E stain

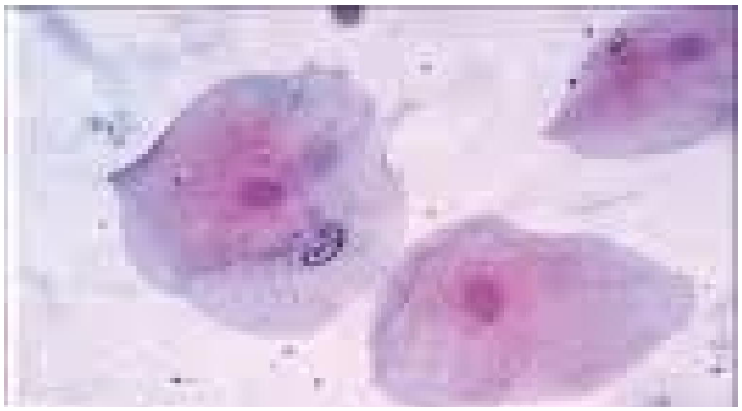

Fig. 2. Normal buccal cells stained with PAP stain

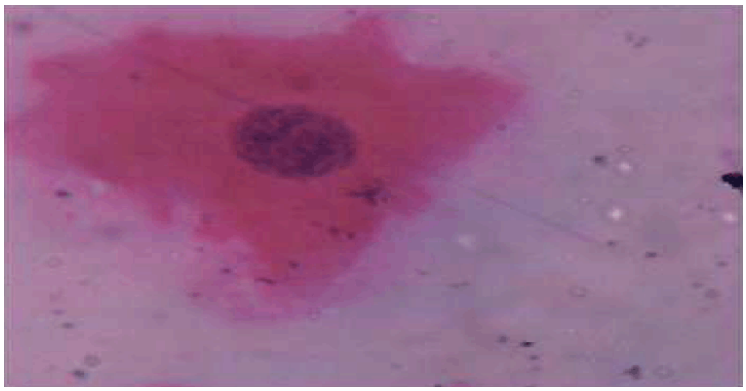

Fig. 3. A typical cell Using PAP stain

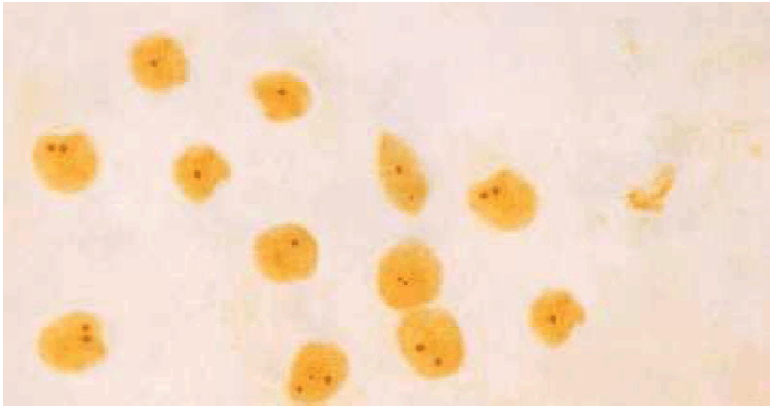

Fig. 4. Buccal cells with normal AgNor count

5.1 Results Interpretation

AgNORs were visible as black-dark brown dots located within the nuclei of the cells [5]. Normal cells AgNORs appeared as discrete black brown dots present within the nucleus, their count 1-3. Abnormal *AgNOR* sites appear as intra nuclear black dots in a pale yellow background, their count more than 4 dots.

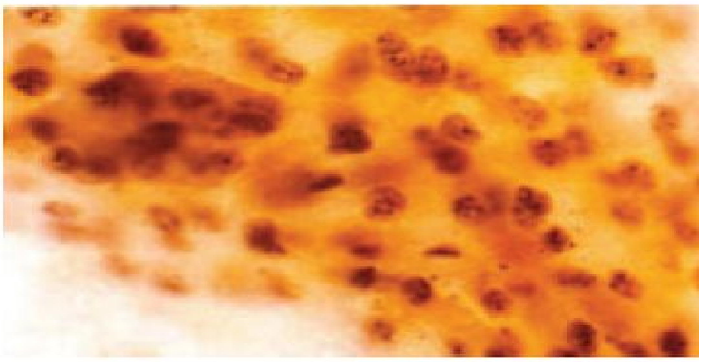

Fig. 5. AgNor; buccal cells from toombak user showing more than 4 dots per nucleus

6. RESULTS

6.1 The Age of the Study Population

Most of the subjects in the present study are older than 50 years old (30%), followed by the subjects aged between 41-50 years (25%), 31-40 years (23%), and 20-30 years (22%) (Fig. 6).

6.2 The Duration of Snuffing of the Study Population

Most of the subjects in the present study are in duration of snuffing 6-10 years (35%) followed by 2-5years (28%), 11-15years (25%), 16-20 years (9%), and finally 20 years (3%) Table 1.

6.3 Frequency of Dipping per Day

According to frequency of toombak dipping per day, most of the subjects using toombak 2-5 times per day (52%), followed by the subjects using 6-10 times (46%) and 11-15 times (2%) per day (Table 2).

6.4 Comparison between H&E and Pap Stain in the Diagnosis of Samples

H&E and Pap stain showed the same cytological diagnosis that; most of samples were normal 49(49%), the inflamed samples were 45(45%), and atypical cells were in 6 samples (6%) (Table 3).

6.5 The Cytological Diagnosis of Samples Using AgNor Count

According to AgNor count most of samples were normal 94(94%) and just 6 (6%) samples were abnormal (Table 4).

6.6 Comparison of the Snuffing Duration with Cellular Appearance Using H&E and Pap and AgNor

According to the cytologic appearance, normal cases were found to be decreasing while the frequency of dipping increased. The number of the cases with frequency of dipping 2-5, 6-10 and 11-15 times/day was 25, 20 and 4 respectively.

The inflamed samples were found to be increasing with increasing of the frequency of dipping. The number of the cases with frequency of dipping 2-5, 6-10, 11-15,and 16-20 times/day was 3, 15, 16, 8 and 3 respectively.

The cases of cytological a typical cells appear in 11-15 years of snuffing in 5 cases, and only one case in 16-20 years.

When using AgNor count the samples were normal in snuffing duration of 2-5 years and 6-10 years.

The abnormal AgNor count appear in dipping period 11-15 years in 5 cases, and only one case in 16-20 years of dipping (Table 5).

6.7 Comparison of the Dipping Frequency/ Day with Cellular Appearance Using H&E and Pap and AgNor Count

According to the cytologic appearance, normal cases were found to be decreasing while the frequency of dipping increased. The number of the cases with frequency of dipping 2-5, 6-10 and 11-15 times/day was 30, 15 and 4 respectively.

The inflamed samples were found to be increasing with increasing of the frequency of

dipping. The number of the cases with frequency of dipping 2-5, 6-10, and 11-15 times/day was 5, 20 and 30 respectively.

The cases of a typical cells associated with increasing of frequency of dipping/day; the number of the cases with frequency of dipping 6-10 and 11-15 times/day was 2 and 4 respectively.

Table 1. Duration of snuffing among study group

Duration per year	Frequency	Percent
2 -5 yrs	28	28.0
6 - 10 yrs	35	35.0
11 -15 yrs	25	25.0
16 - 20 yrs	9	9.0
above 20 yrs	3	3.0
Total	100	100.0

Table 2. Frequency of dipping per day

Frequency / day	Frequency	Percent
2 -5 times / day	52	52.0
6 -10 times / day	46	46.0
11 - 15 times / day	2	2.0
Total	100	100.0

Table 3. The cytological diagnosis of H&E stain and Pap stain

Cytology diagnosis	H & E	Pap stain
normal	49 (49%)	49 (49%)
Inflammation	45 (45%)	45 (45%)
Cytological a typia	6 (6%)	6 (6%)
Total	100 (100%)	100 (100%)

Table 4. Mean AgNor count among study group

Count	Frequency and percentage
Normal (1-3)	94 (94%)
Abnormal (4 and above)	6 (6%)
Total	100 (100%)

7. DISSICUSION

The duration of snuffing is found to be increasing with the age of the patients.

These results agreed with Hussain Gadelkarim Ahmed [8], he said that increasing in age associated with increasing of snuffers and oral changes in toombak use.

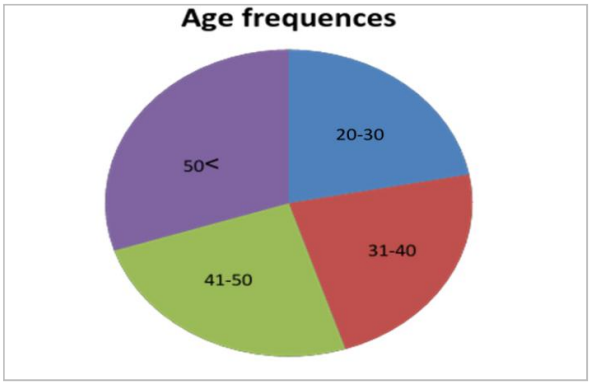

Fig. 6. Age distribution among study group

Table 5. Comparison of the snuffing duration with cellular appearance using H&E and Pap and AgNor count

Duration of snuffing	Cytology appearance			AgNor count	
	Normal smear	Inflammation	Cell a typia	1-3	4 and above
2 - 5 yrs	25 89.3%	3 10.7%	0 0.0%	28 100.0%	0 0.0%
6 -10 yrs	20 57.1%	15 42.9%	0 0.0%	35 100.0%	0 0.0%
11 -15 yrs	4 16.0%	16 64.0%	5 20.0%	20 80.0%	5 20.0%
16 - 20 yrs	0 0.0%	8 88.9%	1 11.1%	8 88.9%	1 11.1%
above 20 yrs	0 0.0%	3 100.0%	0 0.0%	3 100.0%	0 0.0%

Table 6. Comparison of the snuffing frequency/ day with cellular appearance using H&E and Pap and AgNor count

Frequency / day	Cytology appearance			AgNor count	
	Normal smear	Inflammation	Cell a typia	1-3	4 and above
2 -5 times/ day	30	5	0	0	0
6-10 times / day	15	20	2	0	2
11-15 times/ day	4	30	4	0	4

The results of the duration of snuffing of the study population agree with Ghada Radwan [9], she said that the severity of risk depends on duration of contact of the naxious agent with the tissues.

The results of the frequency of dipping per day agree with Anass M and Hussain G [10], they said that the frequency has a role in oral changes, and disagree with Idris AM et al. [23], they said that the severity was not related to frequency on the daily use [11].

H&E and Pap stain had the same cytological diagnosis but Pap stain is more superior to H&E stain. These results agree with Rania M SidAhmed [12], she compared between H&E, Pap and MGG in the diagnosis of urine samples, she found that Pap stain was superior to H&E and MGG stain.

Argyrophilic nuclear organizer region (AgNor) is a silver staining procedure to observe the nucleolar organizer region (Nor) of the nucleous that is suitable for prognostic assessment. Mean Nor count measured by counting the number of silver stained dots per 50 nucleous for every smear, then the number obtained divided by 50, the high mean Nor count was found in (6%) in cases [13].

The results showed that AgNor count like the cytological diagnosis of H&E and Pap stain that the normal count found in short snuffing duration and the abnormal count appears with the increasing of snuffing duration after 11-15 years of snuffing, the difference was statistically significant (*P value* 0.01).

When we compared the snuffing duration and frequency of dipping/day with cellular appearance using H&E, Pap and AgNor count we found that the normal cases were found to be decreasing while the snuffing duration and frequency of dipping increased. The inflamed samples were found to be increasing with increased of the snuffing duration and frequency of dipping, and the overall study population decreased after 16-20 years of snuffing, also with increasing frequency of dipping /day. The cases of a typical cells associated with increasing of snuffing duration after 11-15 years and with increasing of frequency of dipping.

These results agreed with many authors like Derenzini et al. [14], they said that 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.

Sandhya Gulia [15] and Emani sitaramam [16], said that the mean AgNor count in atypia smears showed higher count than normal oral Epithelium and silver nitrate stain has a major role in diagnosis of oral samples.

It has been reported that, particularly human somatic cells could contain demonstrable NORs in nuclei, but many resting cells contain only one particle [5]. Due to increased proliferative activity of neoplastic cells, higher number of NOR particles in cancerous cells might be expected, this in addition to the presence of a reasonable of cytological atypia among cases compared to controls [13].

De Paula AMB [17], in the part of the development of a screening procedure for oral cancer and precancerous cellular activity, exfoliative cytology was applied a retrospective cohort study to assess the presence and severity of oral epithelial atypia in 200 volunters snuffing toombak and 100 without prior exposure to tobacco. He found that the micronuclei frequencies increase with an increase in the duration and frequency of Toombak use and this was found statistically significant. Atypical changes in results of AgNOR, micronuclei and cytological examination show that cellular proliferation is significantly higher in Toombak users and this might be attributed to the fact that production of a malignant cell requires cell proliferation and DNA activity [17].

Numerous studies have shown that the number of AgNORs are significantly higher in malignant tumors than in physiological reactive and bengin processes, also toombak dippers more susceptible to inflammation [17-19].

Oral cancer is one of the most common cancers worldwide. In Sudan, the use of Toombak plays a significant role in the etiology of squamous cell carcinoma. Specifically, nitrosamines present in Toombak possibly act as principal carcinogen [20].

Ankle and Kale [21], found that smear is useful in evaluation of epithelial atypia that is frequently encountered in pre-malignant and early malignant oral lesions. There is a need to standardize the cytological proliferative marker methods to provide rapid, inexpensive, quantitative, reproducible, technologically simple, and applicable monitoring and screening procedures for subjects who have been identified as being at high risk for developing oral cancer [21]. Ahmed and Mahgoub [22], published that in the Sudan snuff locally known as Toombak, it is wide-spread in the country, the risk among Toombak user was high.

Furthermore, study by Idris AM et al. [23], revealed that Toombak contains at least 100-fold higher concentration of tobacco- specific N-amine TSNA.

Ahmed HG and Babiker AA [24], established that, chemical carcinogenesis is a prolonged process and progressed with increasing of exposure, the duration of tobacco exposure seemed to have effects on increasing the risk of oral cancer.

Ahmad A. AbdulMajeed and Camile S. Farah [25], published that many attempts have been made to identify objective molecular biomarkers to diagnose and prognosticate oral epithelial dysplasia (OED) because histopathological interpretation is subjective and lacks sensitivity. The purpose of their article is to review current knowledge on biomarkers of protein expression for OED by IHC approaches. They used a lot of biomarkers and found that pattern of expression of many proteins decreases during oral carcinogenesis like E.cadherin, Ep-CAM, Catenins, Podoplanin, Syndecan, P 12, P 16, P 21, P 27and P57, on the other hand the pattern of protein expression increases in many proteins like Agnor, PCNA, Ki-67, Cyclins, VEGF, HTER and others.

Hangying Dong et al. [26], said that the potential doubling time (Tpot) has been proposed as a pretreatment estimator of intratreatment tumor proliferative capacity. Ki-67 immunostaining and AgNOR silver staining were examined as possible alternative methods by comparing them with the Tpot in an in vitro system using two different cell lines under varying growth conditions. a strong correlation was found between Ki-67 labeling index and AgNOR scores (r 5 0.927, *P* 5 0.0003). Both Ki-67 labeling index and AgNOR scores had statistically significant inverse correlations with cell doubling time (Td), length of S phase (Ts), and Tpot, as well as a positive correlation with iododeoxyuridine (IdUrd) labeling index.

8. CONCLUSION

Oral exfoliative cytology using Pap and H&E stain is useful in evaluation of epithelial a typia that is frequently encountered in pre-malignant and early malignant oral lesions.

The results of AgNor count like the cytological diagnosis of H&E and Pap stain that the normal count found in short snuffing duration and the abnormal count appears with the increasing of snuffing duration, the difference was statistically significant (*P value* 0.01).

The duration of snuffing is found to be increasing with the age of the patients.

The prolonged exposure to toombak is significant factor in increasing cellular a typia.

ETHICAL APPROVAL

Was taken from the university ethical research board (ERB) and the hospital administration. Each participant was asked to sign a written ethical consent form during the interview, before the specimen was taken. Consents were taken from patients to take samples after agreement of the participant, name of patients were taken but symbolized by numbers instead to guarantee confidentiality.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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