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Oral Epithelium Changes Associated with Chronic Toombak Dipping

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ABSTRACT

Background: Oral cancer (OC) is a significant global health issue linked to modifiable risk factors, including tobacco consumption. Consequently, investigating the effects of long-term toombak use on oral epithelium is vital. **Methodology:** The study conducted in El-Obeid City, Sudan, involved male toombak users and included 120 buccal smears: 30 from a control group and 90 from long-term users. Data was collected using a structured Arabic questionnaire on demographics and toombak use patterns. Buccal smears were obtained after oral wash, then fixed in 95% ethanol, dried, and stained with Papanicolaou (PAP) stain for cytological analysis. **Results:** This study examined 120 male volunteers, aged 15 to 73 years, with a mean age of 33 years. Nuclear degenerative alterations revealed karyolysis in 89% of cases and were absent in controls; karyorrhexis was noted in 5.6% of patients versus 3.3% of controls. Multinucleation occurred in 4.4% of cases, with no instances observed in the controls. Cytoplasmic vacuolization was observed in 89% of the cases, with no instances in the controls. Keratinization was observed in 7.8% of the cases and 6.7% of the controls. Cytological evidence of fungal infection was detected in 5 patients, comprising 80% cases and 20% controls. Cytological evidence of bacterial infection was observed in 8 patients (88% of cases and 12% of controls). **Conclusion:** Toombak users exhibit a significantly higher incidence of degenerative cellular changes, particularly karyolysis (indicating severe nuclear degeneration) and cytoplasmic vacuolization (indicating cytoplasmic damage). Additionally, there are corroborative changes such as multinucleation. Cytological evidence linked to infection in both cases and some controls suggests that microbial factors may contribute to tissue damage.

Keywords: Oral, cytology, toombak, pyknosis, karyolysis, keratinization

Introduction

Worldwide, an estimated 177,757 deaths and 377,713 new cases of oral cancer (OC) occur each year, making it a significant global health problem [1]. The disease's rising prevalence in East Africa has made it a pressing public health concern [2]. The incidence of oral cancer is rising in Sudan. Oral squamous cell carcinoma (OSCC) is rising, along with other types of oral cancer. The buccal mucosa, tongue, and lip are the predominant locations [3]. OSCC comprises most oral malignancies worldwide and is closely associated with modifiable risk factors such as tobacco and alcohol use [4].

Tobacco and alcohol consumption are leading risk factors for cancers of the head and neck, although their effects vary depending on the specific type of cancer, the sex of the patient, age group, and regional demographics. While occupational exposure to carcinogenic substances is not a predominant factor, it remains an important consideration in the comprehensive assessment of risk for these malignancies [5].

A systematic review confirms a link between non-smoking tobacco use and an elevated risk of oral cancer. It analyzed five studies on smokeless and chewing tobacco, alongside 25 studies on various types of smokeless tobacco, mainly betel quid and supari. Among these, 21 studies showed a significant positive correlation with oral cancer (OR: 0.67–149.5), while seven studies did not find a significant correlation [6]. And about changes of Tobacco in nucleus & cytoplasm, Cigarette smoking significantly elevates cellular proliferation, as indicated by the increased Argyrophilic nucleolar organizer regions (AgNORs) counts in smokers. The nucleolar organizer regions (NORs) can be identified in the nuclei as brown or black dots with the silver colloidal staining technique in formalin-fixed paraffin sections and in cytology smears. The combined use of Pap staining and AgNORs counting proved effective at detecting cellular proliferation before clinical symptoms appeared in smokers [7].

The mean number of micronuclei, mean micronuclei per cell, frequency of cells showing micronuclei, and nuclear area were significantly increased in tobacco users compared to controls, especially in combined tobacco users. The nuclear-cytoplasmic ratio was increased, and the cytoplasmic area was decreased in tobacco users compared to controls [8]. Therefore, this study aimed to assess the oral epithelium changes associated with chronic toombak dipping among Sudanese people in Western Sudan.

Materials and Methods

The study was conducted in El-Obeid city, North Kordofan State, Sudan, involving male participants who use tobacco in the form of toombak dipping. The study comprised 120 volunteers living in the city of EL-Obeid. Of the 120 participants, 90 individuals were toombak dippers (ascertained as case group) and the remaining 30 were non-tobacco users (ascertained as control group). A purposeful questionnaire was designed to obtain participants' identification data. Buccal (dipping site) scraped using a toothbrush was performed, and the obtained cells were smeared on a cleaned

glass slide, then immediately (while it was wet) fixed in 95% ethyl alcohol for 15 minutes, then air-dried and sent to the laboratory for subsequent staining using Papanicolaou (Pap.) stain.

Sample processing: Smears were stained with the Papanicolaou staining procedure. Ethyl alcohol-fixed smears were hydrated in descending concentrations of 95%, 70%, and distilled water for two minutes each. The smears were stained with Harris' hematoxylin for five minutes, rinsed with distilled water, and differentiated in 0.5% aqueous hydrochloric acid for a few seconds to remove any excess stain. To prevent discoloration, they were rinsed promptly with pure water. The smears were blued in alkaline water for a few seconds before being dehydrated with alcoholic concentrations ranging from 70% to 95% for two minutes each. The smears were then treated with Eosin Azure 50 for 4 minutes. To stain the cytoplasm, the samples were treated with Papanicolaou Orange G6 for two minutes, washed with 95% alcohol, and then dehydrated in absolute alcohol. The stains were cleaned with Xylene and put in DPX mount.

Assessment of the Results: Strict quality control methods were implemented to ensure dependability and reproducibility. To evaluate staining quality, smears were inspected using a light microscope at a low power (10X). All smears had good staining quality, with blue nuclei, pink/orange cytoplasm of keratinized squamous cells, and blue/green cytoplasm of non-keratinized squamous epithelial cells. To prevent assessment bias, cytological smears were labeled such that the examiner was unaware of the subjects' case or control groups.

Statistical Analysis

After organizing the collected data on a data sheet and entering it into computer software (SPSS) for analysis, we were able to determine frequencies and cross-tabulations.

Results

This study investigated 120 male participants aged 15 to 73 years with a mean age of 33. Most toombak dippers were aged 31-38 years, followed by 47-54, and 23-30 years, representing 33/90(36.7%), 24/90(26.7%), and 18/90(20%), respectively. Most controls were aged 31-38 & 47-54 years, representing 10/30(33.3%) for each group, as indicated in Table 1, Fig 1.

Table 1: Distribution of the cases and controls by age

Age in years	Controls	Cases	Total
15-22	2	3	5
23-30	5	18	23
31-38	10	33	43
39-46	3	12	15
47-54	10	24	34
Total	30	90	120

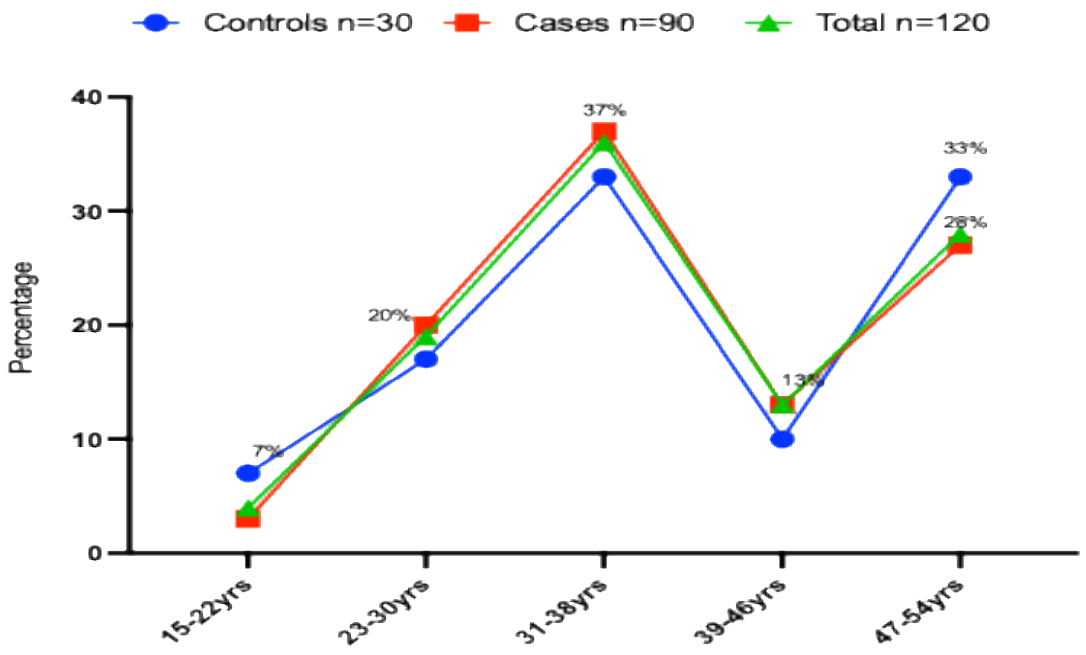

Figure 1: Illustrates the distribution of the study subjects by age

Regarding nuclear degenerative changes, karyolysis was observed in 80/90(89%) of the cases and none of the controls; karryohexis was observed in 5/90(5.6%) cases compared to 1/30(3.3%) of controls. Multinucleation was seen in 4/90(4.4%) of cases and none of the controls.

Regarding cytoplasmic changes, cytoplasmic vacuolization was indicated in 80/90(89%) of the cases and none of the controls. Keratinization was detected in 7/90(7.8%) of the cases and 2/30(6.7%) of the controls, as shown in Table 2, Fig 2. Cytological evidence of fungal infection was observed in 5 subjects, of whom 4/5(80%) were cases and 1/5(20%) was a control. Cytological evidence of Bacterial infection was seen in 8 subjects, of whom 7/8(88%) were cases and 1/8(12%) was a control.

Table 2: Distribution of the study subjects by cellular proliferative changes

Variable	Control	Cases	Total
Nuclear changes			
Normal	29	1	30
Karryohexis	1	5	6
Karyolysis	0	80	80
Multinucleation	0	4	4
Total	30	90	120
Cytoplasmic Details			
Normal	28	2	30
Cytoplasmic vacuolization	0	80	80
Cytoplasmic Inclusion	0	1	1
Keratinization	2	7	9
No infection	28	79	107

Fungal infection	1	4	5
Bacterial infection	1	7	8
Total	30	90	120

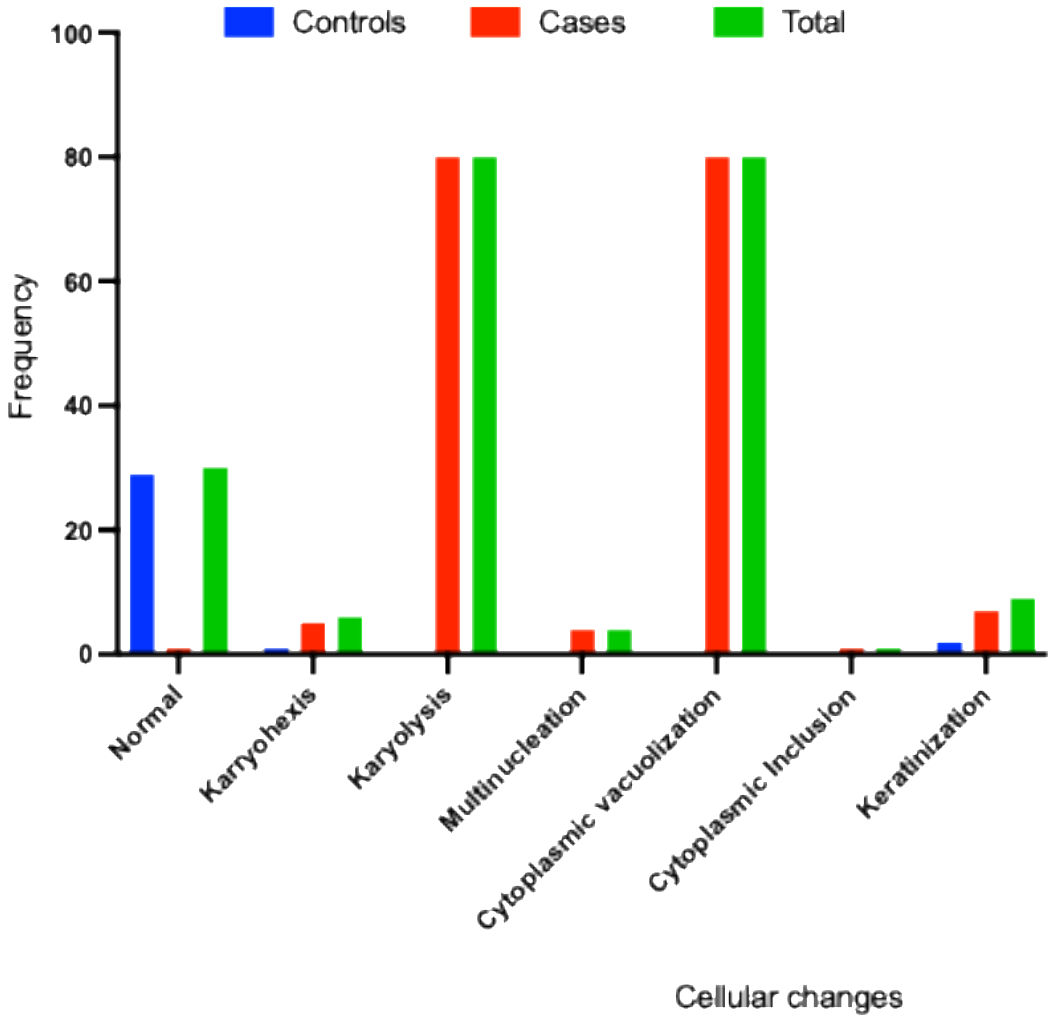

Figure 2: Description of the Nuclear & Cytoplasmic changes among Cases and Controls

Concerning the distribution of nuclear nucleation and nuclear contour between cases and controls, keratosis was absent in 29 out of 30 (97%) controls, and infection was absent in 28 out of 30 (93%) controls. In contrast, among cases, there were 84 out of 90 (93%) with parakeratosis, 4 out of 90 (4%) with fungal infection, and 7 out of 90 (8%) with bacterial infection, as illustrated in Table 3 and Figure 3.

Table 3: Distribution of Nuclear Nucleation and Nuclear Contour in Cases and Controls

Variable	Controls	Cases	Total
Nuclear Nucleation			
Nuclear Nucleation Seen	29	83	112
Nuclear Nucleation Un seen	1	7	8
Total	30	90	120
Nuclear contour			
Regular	14	17	31
Irregular	16	73	89
Total	30	90	120

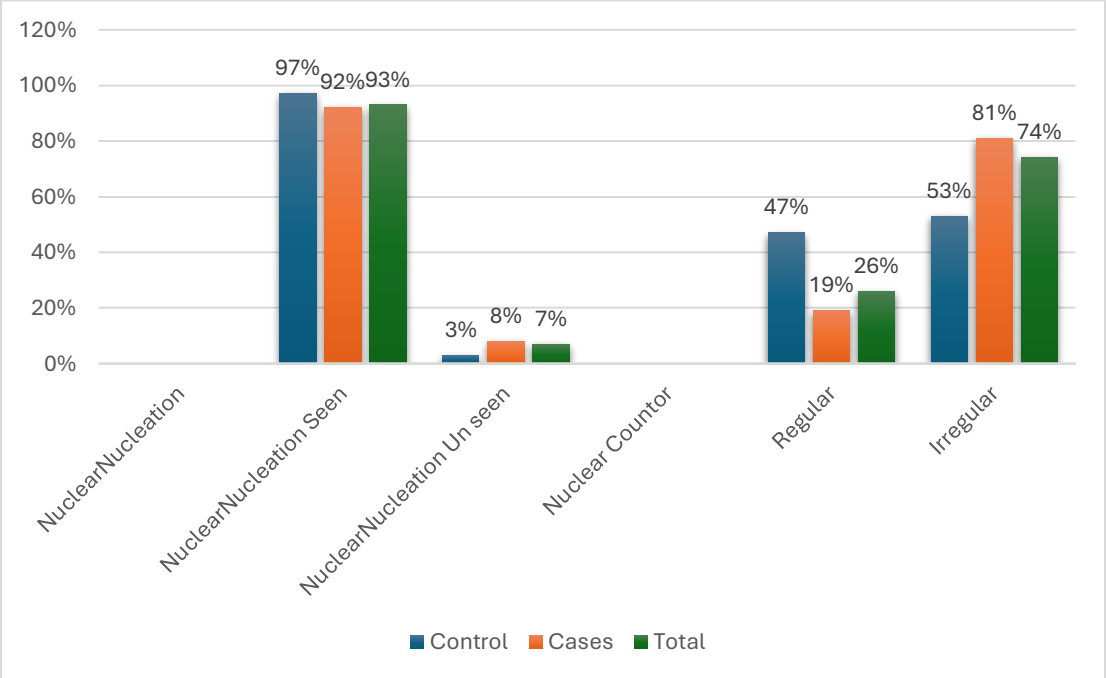

Figure 3: Distribution of Nuclear Nucleation & Nuclear contour among Cases and Controls

Discussion

This study included 120 male volunteers (mean age around 33 years; age range 15–73 years) and compared 90 toombak users (cases) with 30 non-tobacco users (controls). The findings indicate a significant correlation between toombak usage and cellular damage, especially inside the nucleus and cytoplasm. Similar findings have been documented in other studies conducted in Sudan [9-11].

Karyolysis (89% in cases; 0% in controls) was the most significant observation. Karyolysis denotes the disintegration of nuclear material and is often regarded as an indicator of significant cellular injury or irreversible degeneration. The total lack of karyolysis in controls enhances the probability that the degenerative nuclear alteration is associated with tobacco exposure rather than inherent variability. Karyorrhexis occurred in 5.6% of cases compared to 3.3% of controls, indicating its relative rarity in both groups, with only a slight disparity between them.

Karyorrhexis denotes the fragmentation of nuclear material and may arise after cellular degeneration or damage. The reduced gap relative to karyolysis may indicate that the primary mechanism of injury in this dataset favors nuclear dissolution over fragmentation or that karyorrhexis occurs less frequently or is less specific in this setting. Multinucleation (4.4% in cases; 0% in controls) further substantiates a detrimental impact in cases. Multinucleation may be linked to aberrant cell division, nuclear instability, or stress-induced cellular change. Although the frequency is minimal, its absence in controls is significant and aligns with a tobacco-related effect. Tobacco exposure alters oral cellular biology, triggering a loop of inflammation, oxidative stress, and premature cellular senescence. Exfoliated oral cells often display nuclear irregularities, including micronuclei, binucleation, karyolysis, and pyknosis. These act as direct indicators of DNA damage and abnormal cell division [12,13].

The predominant cytoplasmic abnormality seen was cytoplasmic vacuolization, occurring in 89% of cases and 0% of controls. This vacuolization frequently signifies cytoplasmic damage, impaired membrane function, or metabolic strain. The concurrent high frequency of karyolysis and vacuolization (both at 89% in instances) indicates a synchronized pattern of nuclear and cytoplasmic injury, aligning with the consequences of cytotoxic or damaging exposure. Cytoplasmic vacuolization in the oral mucosa is a common cellular response to tobacco consumption, resulting from nicotine and other agents that provoke cellular stress, autophagy, and lysosomal impairment. In exfoliated cytology, these vacuoles appear as transparent voids within the cytoplasm and serve as initial indicators of mucosal tissue damage [14].

Keratinization (7.8% in cases vs. 6.7% in controls) showed only a slight difference. This may imply that keratinization is either less sensitive to toombak exposure in your sample, takes longer to develop, or is influenced by factors other than tobacco alone (e.g., local irritation, epithelial maturation stage, or sampling variability). Tobacco exposure, whether by smoking or smokeless forms, directly modifies the oral mucosa by inducing excessive keratinization as a protective response to thermal, chemical, and mechanical irritants. This disorder frequently presents as leukoplakia or smokeless tobacco keratosis. This protective layer safeguards against minor irritations but may also serve as an early sign of cellular atypia and probable cancer [15].

The results of this study indicate that microbial participation exists within the study population and may lead to cellular damage. Infection and inflammation can induce oxidative stress, tissue damage, and degenerative cellular patterns, potentially elucidating the increased prevalence of severe cytoplasmic and nuclear alterations among toombak users. Nonetheless, there is a clarity issue about interpretation: while “80% were cases and 20% were controls” pertains to only 5 subjects, it likely represents approximately 4 cases and 1 control (similarly, 88%/12% of 8 subjects equates to about 7 cases and 1 control). Incorporating the precise counts of cases and controls (e.g., “fungal: 4 cases/1 control; bacterial: 7 cases/1 control”) with percentages would enhance the paper, enabling readers to verify the denominators and mitigate confusion.

Strengths

The study comprised toombak users (cases) and non-tobacco users (controls), facilitating the evaluation of cytological

changes in response to tobacco exposure. The utilization of various cytological indications, including nuclear degeneration (such as karyolysis, karyorrhexis, and multinucleation) and cytoplasmic alterations (such as vacuolization and keratinization), offers a comprehensive assessment of cellular harm instead of depending on a solitary marker. The presence of significant degenerative alterations, particularly karyolysis and cytoplasmic vacuolization, in a substantial proportion of cases and their absence in controls enhances the clarity of the most distinguishing findings. Assessing fungal and bacterial evidence helps clarify probable factors contributing to cellular damage.

Limitations

Cross-sectional design (incapable of establishing causality): Since exposure and cytological data are evaluated concurrently, the study can uncover relationships but cannot conclusively determine that toombak induces the observed cytological harm. *Restricted control of confounding variables*: Factors that are recognized to affect oral/epithelial cytology, such as oral hygiene, age, concurrent oral lesions, nutritional status, alcohol consumption, smoking type/duration, chronic inflammation, and infection risks associated with hygiene, may obscure the association between toombak use and cytological abnormalities. *Limited control group*: A mere 30 controls were incorporated. Although the case–control comparison was robust for certain characteristics, an expanded control sample could enhance the precision of estimates for rarer discoveries (e.g., multinucleation, karyorrhexis). *Clarity in reporting infection percentages*: The documented percentages of fungal and bacterial infections are 80% in cases and 20% in controls for 5 participants, and 88% in cases and 12% in controls for 8 subjects. These likely represent approximate ratios (e.g., ~4 cases per 1 control; ~7 cases per 1 control), although the manuscript must specifically disclose the precise number of cases and controls in each infection group to eliminate any ambiguity. *Lack of microbiological validation*: The "cytological evidence" of fungal or bacterial infection may not accurately represent the actual infection status. In the absence of confirmation testing (such as culture, smear microscopy with confirmed staining, or PCR when applicable), misclassification may occur. *Singular methodology and possible observer variability*: Cytological interpretation may differ among observers or among slides. Indicating whether slides underwent double reading or blinding and presenting inter-observer agreement, if obtainable, would enhance dependability. *Generalizability:* The participants were exclusively male and sourced from a specific environment; thus, the findings may not apply to females or to groups with varying exposure patterns or healthcare accessibility.

In conclusion, the findings indicate that Toombak users exhibit a significantly higher incidence of degenerative cellular changes, particularly karyolysis (indicating severe nuclear degeneration) and cytoplasmic vacuolization (indicating cytoplasmic damage). Additionally, there

are corroborative changes such as multinucleation. Cytological evidence linked to infection in both cases and some controls suggests that microbial factors may contribute to tissue damage. However, the primary distinguishing feature between the groups is the presence of severe degenerative cytology, which is absent in the controls.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' Contributions

Khalifa MMH: conceptualization, writing-original draft, and writing-review and editing. Mohammed MMM: conceptualization, data collection, and analysis. Alobaid AEA: writing-review, editing, and critical revision. Ismail HAA: conceptualization, writing, review, and data collection. Regal HYA: conceptualization, writing, review, editing, and administration. Ahmed HG: conceptualization, editing, and supervision.

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Conflict Of Interest

The authors declare no conflict of interest.

Ethical Approval

The study received ethical approval from the Institutional Research Review Board of the Prof Medical Research Consultancy Center on 5 January 2025. Approval no. HREC 0001/MRCC.1/26.

Statement on Ethics:

The participants' personal information was maintained in strict confidentiality. The data collected from participants were exclusively shared in an anonymous format with the research team. Before enrolment, each participant needed to provide consent for participation in this study. The results corresponded with the updated Helsinki Declaration concerning the ethical implications of research involving human subjects.

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