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Research Article
Evaluation of Exfoliated Buccal Mucosal Cells from Snuff Dealers: A Quantitative Cytomorphometric Analysis

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Abstract

Background and Objective: Smokeless tobacco (snuff) use has been associated with many oral disease processes but there is a paucity of scientific information on its effects on dealers who process and package the substance for sales. The present study, therefore, investigated cytomorphometric changes in the exfoliated epithelial cells from the oral mucosa of snuff dealers. **Materials and Methods:** Study groups comprised 40 individuals (20 snuff dealers and 20 controls). Oral cytology smears were obtained using a wooden spatula and smeared on a microscope slide. Smears were fixed in 95% alcohol and stained with Giemsa stain for microscopical examination. Twenty cells were assessed for each of the participants and the cytoplasmic diameter (CD) and nuclear diameter (ND) were measured using AmScope Software. The ratios of nuclear diameter to cytoplasmic diameter (N/CR) were thereafter determined. The effect of age, sex, length of working experience (years of occupational exposure), snuff use, cigarette smoking and alcohol drinking habit on the parameters were also determined. **Results:** No significant difference was observed in CD, ND and N/CR of snuff dealers when compared to control ($p>0.05$). However, variations were only observed based on length of working experience and alcohol drinking habit ($p<0.05$). **Conclusion:** Data obtained from the present study has revealed variations in cytomorphometric parameters of exfoliated buccal cells of snuff dealers which is associated with years of occupational exposure and alcohol drinking habits.

Key words: Cytomorphometry, buccal mucosal cells, cytoplasmic diameter, nuclear diameter, nuclear/cytoplasmic ratio, oral leukoplakia, mouth sores

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Tobacco is mainly of two types, smoked and smokeless tobacco. Smokeless tobacco usually comes in two forms: Snuff and chewing tobacco. Snuff (also known as 'dip') is made from pulverized tobacco leaves and is usually inhaled into the nasal cavity to enhance absorption[1]. Tobacco use is proven to be a potential health threat and a major cause of morbidity and mortality in the world having been associated with oral cancer, oral leukoplakia and cancer of some selected organs[2]. It is well-known to contain carcinogens such as various nitrosamines, nitrosonornicotine and other substances[3]. About 5 million people are believed to die yearly from tobacco-related causes and an estimated 7 million people in developing countries will die by 2030 by World Health Organization's Projection[4,5].

Categorically, tobacco consumption can either be by smoking or smokeless tobacco. Smokeless tobacco refers to the use of tobacco by other means such as via oral or nasal route, other than smoking. There is increased use of smokeless tobacco worldwide, especially among vulnerable individuals (children and elderly) who are easily affected. Consequently, diverse buccal mucosal cells changes due to smokeless tobacco and smoking have been well established[6]. Immediate effects of the use of snuff include bad breath, mouth sores, stained teeth, high blood pressure and dizziness[7]. Its use for a long period can cause serious health disorders like leukoplakia, oral cancer and extraoral cancers[8,9]. Henley *et al.*[10] also documented that snuff use and tobacco chewing are associated with an increased risk of all cancers combined including lung cancer and liver cirrhosis.

A quantitative exfoliated cytological procedure for the estimation of nuclear to cytoplasmic size in papanicolaou-stained smear samples by the use of the planimeter method has been reported[11]. Important parameters for the evaluation of oral lesions include the nuclear diameter, cytoplasmic diameter and the nuclear/cytoplasmic ratio[12]. Previous researchers have documented significant cytomorphometrical changes in the exfoliated buccal cells of tobacco users[13-15].

The use of snuff has become a global burden due to its abuse by the young and elderly. Among the Igbos in the South-Eastern States of Nigeria, snuff is commonly used by the elderly who consume the substance either by sniffing through their nostrils or dipping it in the mouth. Numerous data have been documented on the effect of tobacco use in humans, however, there is a paucity of scientific information on the effect of snuff on dealers who are occupationally exposed to the substance during processing and packaging for sales. The present study, therefore, was conducted to evaluate the cytomorphometric parameters of exfoliated cells from the oral mucosa of snuff dealers in a popular market (Ogbete Main) situated within the Enugu Metropolis. The effect of age, sex, alcohol consumption, snuff use and length of working experience on the parameters were also determined.

MATERIALS AND METHODS

Study area: This research work was carried out between September 2016 to November, 2017. The sample collection was carried out in Ogbete Main Market Enugu Metropolis and analysis was done at the University of Nigeria Enugu Campus at the Department of Medical Laboratory Sciences Laboratory.

Ethical clearance: Ethical approval was granted by the Ethical Committee of the University of Nigeria Teaching Hospital, Enugu State. Participation was 100% voluntary and a questionnaire, as well as informed consent, was obtained as either verbal or written depending on the literacy level of individual participants. The study was conducted following the Helsinki declaration.

Subjects: The study was conducted among snuff dealers in the Ogbete Main Market in Enugu Metropolis with a total of twenty snuff dealers as the exposed group and twenty normal subjects as the control. A simple random sampling method was used in the study. Exclusion criteria included subjects with diabetes, previous benign or malignant lesions of the oral mucosa and clinically apparent oral mucosa lesions. This was done to prevent cellular changes associated with these health conditions. Additionally, the control subjects recruited were individuals who are not exposed to snuff. Informed consent was obtained from all recruited individuals. An information sheet was administered to the subjects and the entire non-invasive procedure was explained before the cytological samples were obtained. All required information was collected using an adequately structured questionnaire which included age, gender, working experience (years), smoking habit, alcohol consumption, number of bottles consumed daily, cigarette smoking habit and snuff use.

Sample collection: After a routine examination of the oral mucosa, cell samples were obtained from the inner cheek of the oral cavity. The subjects were first asked to rinse their mouths with drinking water before sample collection, to remove unwanted debris within the oral cavity which will reflect as artefacts on the smears if not removed. Taking all the aseptic precautions, squamous epithelial cells were collected

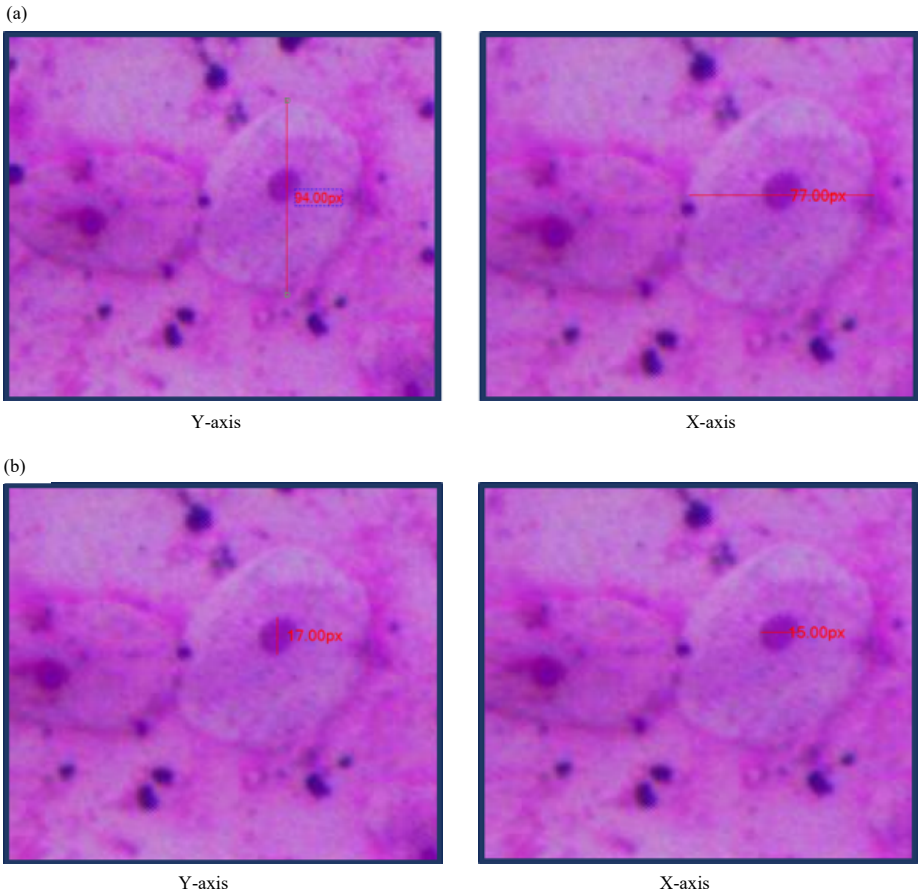

Fig. 1a-b: (a) Cytoplasmic and (b) Nuclear diameter measurements of the buccal cells using the AmScope Image Analysis Software

using a moistened wooden spatula. Cells were scraped from normal-appearing buccal mucosa using a gentle scraping motion and application of little pressure. The cells obtained were transferred to the centre of a clean dry glass slide and spread uniformly and thinly in a circular motion. The samples on the glass slides were immediately fixed with 95% alcohol before cytological staining.

Staining of smears: Giemsa stain was used as the staining procedure before the microscopical examination. The fixed smears were allowed to dry and then stained with freshly prepared Giemsa stain for 20 min. After staining, the slides were rinsed with distilled water and the back of the stained slides were cleaned with cotton wool and left to dry.

Microscopy and photomicrography: The slides were viewed with the olympus binocular microscope with ×10 and ×40 objective lenses. Photomicrographs of several fields of the ×40 objective lens bearing the smeared cells were captured using the AmScope Digital Microscope Eyepiece Camera (MU 300 series model).

Cytomorphometric analysis: The cytomorphometric parameters of the buccal cells which include cellular diameter (CD) and nuclear diameter (ND), were measured using AmScope Image Analysis Software. Measurements were obtained in both axis (x and y) of the cells and nuclei for the cytoplasmic diameter (Fig. 1a) and nuclear diameter (Fig. 1b). Twenty cells were selected from each slide and the CD and ND were measured and recorded. The mean values from both axis were considered as the diameter of that cell and nucleus. Only cells that have clearly defined cellular and nuclear outlines as well as being fully included in the field of vision were selected. Cells with unusually distorted outlines, folded and clumped were not considered for the analysis. The nuclear/cytoplasmic ratio was thereafter determined by dividing the ND with its respective CD.

Statistical analysis: Data analysis was performed by employing the statistical package for social science version 23.0 statistical software (SPSS Inc, Chicago, IL). The significant differences in the means obtained from control and snuff dealers (exposed group) were analysed using an independent

student's t-test, while Pearson's rank correlation analysis tool was used to assess the relationships between the parameters and independent variables (age and length of working experience). Data obtained from the assay were expressed as the mean±standard deviations (SD). Overall effects of age, sex, working experience, alcohol consumption, smoking and snuff use were determined using Analysis of Variance (ANOVA).

RESULTS

General characteristics: Forty individuals were recruited for the study: Twenty for exposed subjects and 20 for control. The mean age of all participants was 41.82 years (standard deviation SD: 18.16: Range 13-70 years, median age: 44.50 years). Table 1 shows the general characteristics of the study groups. The parameters include age, sex, alcohol consumption, number of bottles consumed/day, cigarette smoking status, snuff use and working experience. The number of individuals and percentage for each parameter to the total number of subjects recruited for the study are displayed.

Cytomorphometric analysis: The results obtained from the cytomorphometric analysis of the exfoliated buccal cells of exposed (snuff dealers) and control subjects are indicated in Table 2. No statistically significant difference was observed in the cytoplasmic diameter (CD), nuclear diameter (ND) and nuclear/cytoplasmic ratio (N/CR) between the exposed and control groups (p>0.05).

Table 1: General characteristics of study groups showing the age groups, sex, alcohol drinking, number of bottles/day, and working experience

Characteristics	Exposed subjects (n = 20)	Control (n = 20)	Total
Age groups (years)			
<46	5 (25%)	16 (80%)	21 (52.5%)
46-70	15 (75%)	4 (20%)	19 (47.5%)
Total	20 (50%)	20 (50%)	40 (100%)
Sex			
Male	14 (35%)	15 (37.5%)	29 (72%)
Female	6 (15%)	5 (12.5%)	11 (27.5%)
Total	20 (50%)	20 (50%)	40 (100%)
Alcohol drinking status			
Yes	17 (42.5%)	12 (30%)	29 (72.5%)
No	3 (7.5%)	8 (20%)	11 (27.5%)
Total	20 (50%)	20 (50%)	40 (100%)
Number of bottles consumed/day			
1-2 bottles	15 (37.5%)	13 (32.5%)	28 (70%)
3 bottles and above	5 (12.5%)	7 (17.5%)	12 (30%)
Total	20 (50%)	20 (50%)	40 (100%)
Cigarette smoking status			
Yes	0 (0%)	2 (5%)	2 (5%)
No	20 (50%)	18 (45%)	38 (95%)
Total	20 (50%)	20 (50%)	40 (100%)
Snuff use			
Yes	14 (35%)	-	14 (35%)
No	6 (15%)	20 (50%)	26 (65|%)
Total	20 (50%)	20 (50%)	40 (100%)
Working experience			
<21	6 (30%)	-	-
21-29	5 (25%)	-	-
30 and above	9 (45%)	-	-
Total	20 (100%)		

Table 2: Cytomorphometric analysis results showing the cytoplasmic diameter, nuclear diameter and nuclear/cytoplasmic ratio of exposed (snuff dealers) and control subjects

Groups	Cytoplasmic diameter	Nuclear diameter	Nuclear/cytoplasmic diameter
Snuff dealers	84.63±6.64	13.99±1.12	0.17±0.02
Control	84.43±7.13	14.40±0.76	0.17±0.02
F-Ratio	0.008	1.807	0.901
Significance	p>0.05	p>0.05	p>0.05

Table 3: Effects of age, sex, alcohol drinking habit, snuff use and working experience on CD, ND and N/CR of the exposed group (snuff dealers) and control

Parameters	Cytoplasmic diameter	Nuclear diameter	Nuclear/cytoplasmic ratio
Age groups			
Exposed			
<46 years	82.99±8.08	13.93±1.21	0.17±0.01
46-70 years	85.17±6.32	14.02±1.13	0.17±0.02
Control			
<46 years	83.75±6.92	14.35±0.83	0.17±0.02
46-70 years	87.15±8.37	14.61±0.26	0.17±0.01
Significance	p>0.05	p>0.05	p>0.05
Pearson's correlation (r)	0.213 (NS)	-0.095 (NS)	-0.220 (NS)
Sex			
Exposed			
Male	85.29±6.51	13.97±1.14	0.17±0.02
Female	83.07±7.28	14.05±1.15	0.17±0.01
Control			
Male	85.34±7.96	14.47±0.78	0.17±0.02
Female	81.69±2.69	14.21±0.74	0.17±0.01
Significance	p>0.05	p>0.05	p>0.05
Alcohol consumption			
Exposed			
Yes	85.42±6.42	13.96±1.18	0.16±0.02
No	80.10±7.32	14.20±0.85	0.18±0.01
Control			
Yes	87.23±6.55	14.47±1.89	0.17±0.01
No	80.21±6.07	14.29±1.53	0.18±0.02
Significance	p>0.05	p>0.05	p>0.05
Snuff use			
Exposed			
Yes	86.04±7.94	13.81±1.29	0.16±0.03
No	84.02±6.24	14.07±1.08	0.17±0.02
Control			
Yes	-	-	-
No	84.53±6.80	14.20±0.76	0.17±0.02
Significance	p>0.05	p>0.05	p>0.05
Working experience (snuff dealers only)			
<21	86.96±8.41	14.00±1.10	0.16±0.01
21-29	80.26±4.59	14.73±0.70	0.18±0.01*
30 and above	85.49±5.76	13.59±1.21	0.16±0.02
Significance	p>0.05	p>0.05	p<0.05
Pearson's correlation (r)	0.156 (NS)	-0.041 (NS)	-0.126 (NS)

*p-value less than 0.05 when compared to other groups

Table 4: Effects of alcohol drinking habit and snuff use in all participants

Alcohol consumptions	N	Cytoplasmic diameter	Nuclear diameter	Nuclear/cytoplasmic ratio
Consumption status				
Yes	29	86.17±6.04	14.17±1.08	0.17±0.02
No	11	80.19±6.42	14.27±0.59	0.18±0.01
	p-value	0.011	0.783	0.025
	Significance	p<0.05	p>0.05	p<0.05
Number of bottles/day				
1-2 bottles	17	85.99±7.24	14.10±1.18	0.17±0.02
3 bottles and above	12	86.43±5.34	14.29±0.97	0.17±0.01
	Significance	p>0.05	p>0.05	p>0.05
Snuff use				
Yes	6	86.04±7.94	13.81±1.29	0.16±0.03
No	34	84.26±6.68	14.27±0.90	0.17±0.02
	Significance	p>0.05	p>0.05	p>0.05

Effects of age, sex, alcohol drinking habit, snuff use and working experience on CD, ND and N/CR of the exposed group (snuff dealers) and control: As shown in Table 3, no significant difference was observed with the buccal cells' cytomorphometric parameters to age, sex, alcohol drinking habit and snuff use between the exposed subjects and control (p>0.05). Among the snuff dealers, a statistically significant increase in the N/CR was observed based on length of working

experience (p>0.05) for an individual in the 21-29 years' category compared to <21 years and 30 years and above. Pearson's correlation analysis did not reveal any relationship between the parameters and age or length of working experience.

The effects of alcohol consumption and snuff use on the parameters for all participants are shown in Table 4. A statistically significant increase in CD and N/CR was observed in participants who consume alcohol when compared to those who do not (p<0.05). However, these changes were not affected by the number of alcohol bottles consumed daily (p>0.05). More so, no change was noted with snuff use (p>0.05).

DISCUSSION

Cytomorphometric analysis of buccal cells in the present study revealed no significant difference between control subjects and snuff dealers who do not directly use snuff but are exposed to it during processing, dispensing and packaging. However, among the snuff dealers, significant differences were observed in association with length of working experience and alcohol drinking status. These findings suggest that obvious risks to the buccal epithelial cells due to snuff exposure may be dependent on some factors.

Smokeless tobacco (snuff), is commonly inhaled through the nasal cavity but has been reported to cause oral and extraoral cancers[8,9]. Due to the increased and prolonged use of tobacco, a magnitude of associated risks has been reported in a large number of investigations[13,15,16]. Various forms of tobacco have been known to cause cellular changes and even oral cancers, because the product is kept in the oral cavity for a long period, making contact with the mucosal cells[17,18].

Cytomorphometry serves as an easy and valuable tool to assess the effect of tobacco on oral exfoliated cells[19]. Cytomorphologic alterations could serve as early indicators of dysplastic changes which may result from actions induced by certain health conditions or chemical contents of genotoxic agents on the buccal mucosa. In the evaluation of oral lesions, significant changes are observed in the nuclear size, cytoplasmic size and their ratio[20]. Increased nuclear diameter (ND), decreased cytoplasmic diameter (CD) and resultant increased ratio of the ND to CD are significant alterations observed in oral lesions[21]. A previous study by Suvarna *et al.*[22] documented that an increase in the nuclear size and a decrease in CD are the significant morphological changes that are observed in cells that are actively proliferating. Increased ND which is due to increased DNA content required for replication[23], has been attributed to cellular adaptation in response to lesions in oral epithelium[15].

Snuff contains many chemical agents and is confirmed as a class 1 carcinogen since it causes cancer of the oral cavity and pancreas and may be linked to an increased risk of oesophageal cancer[24]. Many substances which cause genetic mutations, chromosomal abnormalities and micronuclei induction are present in snuff. Tobacco-specific nitrosamines are among the major constituents in snuff which have been shown to induce oral tumours in rats[25].

The snuff dealers in the present study are exposed to the snuff aerosols during production and sales and as such, they passively inhale the substance. It, thus, may be presumed that the level of exposure was not enough to exert any obvious changes in the oral mucosal cells of all dealers when considered collectively. However, based on the length of working experience (in years), an increased N/CR in subjects who have worked between 21-29 years was observed which suggests an obvious alteration of the mucosal cells' cytomorphometry. It is not well understood how similar changes were not observed with dealers who have worked more than them (30 years and above), but this observation calls for further investigation.

Contrary to our report on snuff use, a previous study documented changes in cytomorphological features leading to an increase in nuclear/cytoplasmic ratio (N/CR) in association with an increase in frequency and duration of tobacco usage[16]. Although a few participants (snuff dealers) acknowledged the use of snuff as a habit, no cytomorphometric variation was noted. Perhaps the reason for this could be due to the fewer subjects who use snuff when compared to those who do not. Studies involving a higher population of individuals who use smokeless tobacco have reported increased oral cancer incidence[26,27], however, some other researchers recorded no such association[28,29].

Mathew[13], documented that the ND and CD of the oral mucosal cells in cigarette smokers and other participants who chewed betel quid, or are practising both habits, was significantly increased and decreased, respectively than those obtained in the control group. Similarly, another research that evaluated the effect of smoking and betel quid chewing on the buccal mucosal cells, documented an increase in ND and a decrease in CD in smokers and individuals who practised both habits[14]. Similar changes have also been reported by Khot *et al.*[15], who investigated individuals with different forms of tobacco usage. It thus implies that deleterious exfoliated cytological changes may only be observed in individuals who

actively or directly use tobacco products either by smoking, snuffing or chewing. This active and direct usage of the tobacco products may pose more threats than the passive inhalation experienced by the snuff dealers in the present study.

Significant sex (gender) and age-related variations in CD, ND and N/CR were not observed in the present study. For gender, variations are usually observed in the female population which have been attributed to hormonal influences[30]. Based on age, the findings in this work are in the contrast with those of previous researchers who documented decreased CD and ND with increasing age, suggesting a negative correlation of the parameters to age[31,32]. Perhaps this finding was not evident in the present study due to the smaller sample size, however, a positive correlation was observed with CD while ND and N/CR were negatively correlated with the age of all participants combined (though non-significant). Age-related variation is often attributed to cellular senescence[33,34]. Recent studies have shown that by cytomorphometry of exfoliated buccal cells, it is possible to estimate the age of an individual which can be beneficial in forensic medicine and medicolegal issues[33,35].

Alcohol consumption significantly increased the CD and N/CR of all participants (both control and exposed subjects) in the present study. Surprisingly, however, when a reference was made to snuff exposure, no significant alteration was observed. This finding emphasizes the deleterious effects of alcohol consumption on the oral mucosal cells. Previous studies have documented cytomorphometric changes in buccal mucosal cells in the alcoholics[36,37]. Although the mechanism associated with oral mucosal changes due to alcohol intake is poorly understood, the carcinogenic metabolite of alcohol, acetaldehyde, has been implicated. This metabolite is produced by oxidation catalysed by several enzymes leading to its accumulation in the oral cavity[38]. Acetaldehyde directly damages the DNA by forming DNA adducts and crosslinks[39,40] which can subsequently result in malignant transformation[38]. The consumption of alcoholic beverages such as wine, beer and liquor has often been reported to increase the risk of oral, pharynx, larynx and oesophagus cancers[37,41]. Even though the use of both tobacco and alcohol has synergistically increased oral cancer risk[42], alcohol on its plays a role in oral carcinogenesis. The changes observed in the present study, therefore, provide evidence of the detrimental effect of alcohol consumption on the participants rather than their exposure to snuff.

CONCLUSION

In the present study, exposure to snuff resulting from handling in production and sales activities exerts changes on cytomorphometric parameters of exfoliated cells of the buccal mucosa, especially with increased years of occupational exposure. Variations due to alcohol drinking habits were also observed in both snuff dealers and control subjects.

SIGNIFICANCE STATEMENT

The present study discovered that although occupational exposure to the aerosols of snuff did not significantly alter cytomorphometric parameters of buccal cells in snuff dealers compared with unexposed control subjects, length of working experience and alcohol drinking status altered the parameters among the snuff dealers. This may serve as an eye-opener to the researcher and therefore calls for further investigation. The study will therefore help researchers to discover the occupational exposure effects of smokeless tobacco on dealers that many researchers were not able to explore.

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