Research Article

JRAD Journal of Research and Advancement in Dentistry

J Res Adv Dent 2018;9:1:200-208.

Cytomorphometric Analysis of Keretinocytes of Buccal Mucosa of Tobacco Chewers

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ABSTRACT

Aim: The objective of the present study was designed to examine the cellular changes induced by tobacco chewing in keratinocytes of buccal mucosa as revealed by cytomorphometry.

Materials and Method: We compared the cellular diameter (CD), nuclear diameter (ND) and the ratio of nuclear diameter to cellular diameter (ND/CD), as well as the cellular area (CA), nuclear area (NA) and the ratio of nuclear area to cellular area (NA/CA) of buccal mucosa squames of normal subjects (N), with buccal mucosa squames of tobacco users without lesion (A), with tobacco-lime lesion(B), with tobacco chewing habit and premalignant lesion/condition(C), and oral squamous cell carcinoma (D). The study group consisted of 100 patients divided into five groups (N, A, B, C and D) between the ages of 21 and 75 years.

Results: The mean of the cellular diameter of group N, A, B, C, and D was 102.46 ± 1.77µm, 91.55 ± 2.74µm, 87.30 ± 1.10µm 84.84 ± 5.49µm, 60.09 ± 1.33 µm respectively ($p<0.01$). The mean of the nuclear diameter of group N, A, B, C, and D was 11.8 ± 0.22µm, 13.48 ± 0.63µm, 14.01 ± 0.33µm, 13.93 ± 0.52µm, 19.59 ± 0.23µm respectively ($p<0.01$). The mean of the ratio of nuclear diameter to cellular diameter of group N, A, B, C, and D was 0.115 ± 0.000, 0.147 ± 0.008, 0.161 ± 0.005, 0.165 ± 0.007, 0.326 ± 0.008 respectively ($p<0.01$). The mean of the cellular area of group N, A, B, C, and D was 8743.30 ± 311.85µm^2, 7258.72 ± 91.64µm^2, 5546.53 ± 363.12µm^2, 5125.91 ± 347.33µm^2, 4082.08 ± 143.77µm^2 respectively. ($p<0.01$). The mean of the nuclear area (in micrometers) of group N, A, B, C, and D was 104.22 ± 4.38 µm^2, 119.63 ± 2.56 µm^2, 137.50 ± 2.81 µm^2, 145.40 ± 19.23 µm^2, 181.68 ± 3.90 µm^2 respectively ($p<0.01$). The mean of the ratio of nuclear area to cellular area of group N, A, B, C, and D was 0.012 ± 0.001, 0.016 ± 0.000, 0.025 ± 0.001, 0.028 ± 0.003, 0.045 ± 0.001 respectively ($p<0.01$). Univariate analysis of variance (ANOVA) showed a significant group effect for cellular diameter, nuclear diameter ratio of nuclear diameter to cellular diameter, cellular area, nuclear area and ratio of nuclear area to cellular area Multiple comparison tests by Tukey–HSD procedure revealed a significant decrease in the mean cellular diameter, increase in the nuclear diameter and ratio of nuclear diameter to cellular diameter and same with cellular area and nuclear area respectively.

Conclusions: Cytomorphometric changes could be the earliest indicators of cellular alterations. There is progressive decrease in cellular diameter and area, increase in nuclear diameter and area and increase in ratio of nuclear diameter to cellular diameter and nuclear area to cellular area in smears from all tobacco users, as compared to normal subjects. This indicates that there could be cause–effect relationship between tobacco and quantitative alterations.

Keywords: exfoliative cytology, cytomorphometry, oral cancer, tobacco.

Received: Oct. 3, 2018: **Accepted**: Nov. 10, 2018
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pISSN 2278-0076
eISSN 2321-9270

INTRODUCTION

The study of cells, including their formation, origin, structure, function, biochemical activities, and pathologic characteristics is called cell biology or cytology.[12] As per the normal physiology, the oral epithelium renews itself rapidly (probably every 2 weeks). The rationale of oral exfoliative cytology is based on this physiological process.[7] Exfoliative cytology is a simple and noninvasive technique and the smear obtained can be analyzed quantitatively and qualitatively. Qualitative assessment of the exfoliated cells has lost its reputation as a result of a large number of false negative results arising due to the ill-defined diagnostic criteria. In the past exfoliative cytology has not gained wide acceptance due to problems such as inadequate samples, technical errors and the incorrect inter presentation of findings.

In exfoliative cytology, the quantitative parameters are objective and reproducible; they are important adjuncts in making a cytopathological diagnosis. One such quantitative parameter is morphometry.[5] Morphometry may find application in distinguishing normal tissue from abnormal. Cellular morphology reflects the biologic behaviour of the tissue and the host on one hand and the genetic and molecular biology of the cells themselves on the other. The changes in morphology of cells can be measured by cytomorphometric analysis.[3,8]

Cowpe et al (1985). Presently with advanced imaging techniques, computerized systems and the use of quantitative techniques to verify the reliability of cytomorphometric analysis, this method is achieving credit once again. Cowpe et al (1985) pointed out that the application of quantitative techniques to cytology could markedly improve its diagnostic sensitivity.[7]

The variations obtained in these parameters have been attributed to exposure to carcinogenic agents like tobacco through their end-products.[5] There is little mention in the literature to quantitative cytologic assessment of the effects of tobacco chewing upon normal oral mucosa. The morphology of the exfoliated cells depends on the nature of alterations taking place in the keratinocytes of epithelial layer.[1] Cytomorphological parameters such as cellular diameter (CD), nuclear diameter (ND), nuclear area (NA), cytoplasmic area, NA/CA ratio, nuclear shape, nuclear membrane continuity, optical density and nuclear texture have shown meaningful results in the diagnosis of oral lesions. Of these parameters, the nuclear and cytoplasmic areas and the nuclear-to-cytoplasmic ratio have been shown to be significant in the diagnosis of oral lesions[14]

Thus, the aim of the study was to analyze cytomorphometrically all six parameters ND, CD, ND:CD, NA, CA, NA:CA of the keratinocytes in the buccal mucosal smears of tobacco chewers and compare with those of normal healthy controls.

MATERIALS AND METHODS

Patient selection:-

The present study was conducted in Department of Oral and Maxillofacial Pathology, Karnavati School of Dentistry, Uvarsad, Gandhinagar. 100 patients were randomly selected from Department of Oral Medicine and Radiology, Karnavati School of Dentistry, Uvarsad, Gandhinagar and Gujarat Cancer Research Institute (GCRI), Civil hospital, Ahmedabad during duration of 2011 to 2013.

Patients were divided into 5 groups:

Group N consists of 20 subjects without any habit and any lesion
Group A consists of 20 patients with tobacco chewing habit but without any lesion,
Group B consists of 20 patients with tobacco-lime lesion,
Group C consists of 20 patients with tobacco chewing habit and premalignant lesion/condition.(Leukoplakia and OSMF ,Histopathologically proven cases)
Group D consists of 20 subjects with tobacco chewing habit and oral squamous cell carcinoma (Histopathologically proven cases)

20 patients of **group N** (patients without any habit or any lesion) served as control. Tobacco chewing was defined as consumption 5 or more tobacco quids per day for minimum of 10 years.

Exclusion Criteria: Patients with smoking habit, alcoholic, anemic, diabetic, history of any recent systemic illness and those on medication were

excluded from the study group to avoid cellular changes associated with these conditions.

After taking written consent of all patients, detailed history including habit of tobacco chewing (duration and frequency) was recorded. Each patient underwent routine hematologic examination to determine the hemoglobin levels. The patients found to be anemic (i.e., female patients with the hemoglobin concentration of less than 11 mg/dL and male patients with the hemoglobin concentration of less than 12 mg/dL) were excluded from this study.

Preparation of cytological Smears:

Exfoliated cells were obtained using gentle scraping motions exerting little pressure with a wooden spatula moisten with normal saline. The scrapings were smeared onto the centre of glass slide and spread over a large area preventing clumping of cells, than immediately fixed with 95% ethyl alcohol (commercially available Biofix sprey) atleast 15 minutes. The smears were stained with the Papanicolaou stain.

Biopsy:

Punch or incisional biopsies were taken from the selected lesional area of buccal mucosa of the precancerous and malignancy patients. Sections of 4-5 um thickness were stained with H and E stain. Mounted sections were evaluated under light microscope for histopathological diagnosis for Group C [Leukoplakia OSMF and Group D OSCC Patients]

Cytomorphometric Analysis:

All the cytological smears stained with PAP stains were analyzed using Light microscope in 10x magnification for screening. Fifty cells with defined borders were selected in 40x magnification in zigzag pattern to avoid selection of the same cell again. Images of the fifty cells were captured with the help of a digital camera (Canon Power shot a2200 Hd) under high power objective lens of 40x magnification in fix zoom property. The images were uploaded to computer for analysis. The cytomorphometric analysis was carried out using the Mshot Digital imaging system v1.0.1. Only clearly defined cells were measured, avoiding clumped or folded cells and unusually distorted nuclei and cells.

Nuclear and cellular area

For measurement, the nuclear and cellular outlines were traced on the screen and the software automatically calculated the cellular area and nuclear area. Measurement was recorded in μm^2.

Nuclear and cellular diameter

Following calculations for the area, the same cells were subjected for calculations of diameter. Values were obtained in both axes of the cells and nuclei. Mean value of diameters were taken as a final diameters value in Measurement was recorded in µm.

Nuclear-to-cellular diameter ratio

Nuclear-to-cellular diameter ratio was calculated using following formula
Nuclear to cellular diameter ratio=Nuclear diameter Cellular diameter

Nuclear-to-cellular area ratio

Nuclear-to-cellular area ratio was calculated using following formula
Nuclear to cellular area ratio=Nuclear area Cellular area

Statistical analyses

Statistical analyses were carried out by the, Analysis of variance (ANOVA) & Tukey HSD test using SPSS 18.0 software.

RESULTS

Table 1: Mean of Cellular Diameter and Mean Of Cellular Area In Different Groups of Study.

Groups	No of patients	CA (μm^2)	CD (µm)
		Mean ± Std. Deviation	Mean ± Std. Deviation
Group N	20	8743.30 ± 311.85	102.46 ± 1.77

Group A	20	7258.72 ± 91.64	91.55 ± 2.74
Group B	20	5546.53 ± 363.12	87.30 ± 1.10
Group C	20	5125.91 ± 347.33	84.84 ± 5.49
Group D	20	4082.08 ± 143.77	60.09 ± 1.33

Table 2: Comparison of Mean Difference of Cellular Diameter (CD) and Mean Difference of Cellular Area (CA) Between Different Groups. (Tukey-HSD test).

Group compared		CD (µm)		CA (µm²)	
		Mean Difference	Sig.	Mean Difference	Sig.
N	A	10.91	<0.0001*	1484.57	<0.0001*
	B	15.16	<0.0001*	3196.76	<0.0001*
	C	17.61	<0.0001*	3617.38	<0.0001*
	D	42.36	<0.0001*	4661.22	<0.0001*
A	B	4.24	<0.0001*	1712.19	<0.0001*
	C	6.70	<0.0001*	2132.81	<0.0001*
	D	31.45	<0.0001*	3176.64	<0.0001*
B	C	-2.45	0.074	-420.61	<0.0001*
	D	27.20	<0.0001*	1464.45	<0.0001*
C	D	24.74	<0.0001*	1043.83	<0.0001*

* p-value < 0.05: clinical significant difference

Table 3: Mean Of Nuclear Diameter And Mean Of Nuclear Area in Different Groups of Study.

Groups	No of patients	ND (µm)	NA (µm²)
		Mean	Mean
N	20	11.8 ± 0.22	104.22 ± 4.38
A	20	13.48 ± 0.63	119.63 ± 2.56
B	20	14.01 ± 0.33	137.50 ± 2.81
C	20	13.93 ± 0.52	145.40 ± 19.23
D	20	19.59 ± 0.23	181.68 ± 3.90

Table 4: Comparison of Mean Difference of Nuclear Diameter (CD) and Mean Difference of Nuclear Area (CA) Between Different Groups. (Tukey-HSD test).

Group compared		ND (µm)		NA (µm²)	
		Mean Difference	Sig.	Mean Difference	Sig.
N	A	−1.68	<0.0001*	- 15.41	<0.0001*
	B	−2.21	<0.0001*	- 33.28	<0.0001*
	C	−2.13	<0.0001*	- 41.18	<0.0001*
	D	−7.78	<0.0001*	- 77.46	<0.0001*
A	B	−0.53	0.001*	- 17.86	<0.0001*
	C	−0.44	0.010*	- 25.76	<0.0001*
	D	−6.10	<0.0001*	- 62.04	<0.0001*
B	C	−0.08	0.971	7.89	.059
	D	−5.57	<0.0001*	- 44.18	<0.0001*
C	D	−5.65	<0.0001*	- 36.28	<0.0001*

Table 5: Mean Ratio Of Nuclear And Cellular Diameter (ND:CD) And of Mean Ratio Of Nuclear And Cellular Area NA:CA in Different Groups of Study.

Groups	No of patients	ND:CD	NA:CA
		Mean	Mean
N	20	0.115 ± 0.000	0.012 ± 0.001
A	20	0.147 ± 0.008	0.016 ± 0.000
B	20	0.161 ± 0.005	0.025 ± 0.001
C	20	0.165 ± 0.007	0.028 ± 0.003
D	20	0.326 ± 0.008	0.045 ± 0.001

Table 6: Comparison of Mean Difference of Nuclear And Cellular Diameter (ND:CD) and Mean Difference of Nuclear And Cellular Area NA:CA Between Different Groups. (Tukey-HSD test).

Group compared		ND:CD		NA:CA	
		Mean Difference	Sig.	Mean Difference	Sig.
N	A	0.032	<0.0001*	- 0.004	<0.0001*
	B	0.045	<0.0001*	- 0.012	<0.0001*
	C	0.049	<0.0001*	- 0.016	<0.0001*
	D	0.210	<0.0001*	- 0.032	<0.0001*
A	B	0.013	<0.0001*	- 0.008	<0.0001*
	C	0.017	<0.0001*	- 0.011	<0.0001*
	D	0.178	<0.0001*	- 0.028	<0.0001*
B	C	0.004	0.265	0.003	<0.0001*
	D	0.165	<0.0001*	- 0.019	<0.0001*
C	D	0.161	<0.0001*	- 0.016	<0.0001*

* p-value < 0.05: clinical significant difference.

In the Group N, Group A, Group B, Group C and Group D the mean **Cellular Area** (CA) with standard deviation were 8743.30 ± 311.85µm2, 7258.72 ± 91.64µm2, 5546.53 ± 363.12µm2, 5125.91 ± 347.33µm2 and 4082.08 ± 143.77µm2 respectively. **(Table 1)** In Group N, Group A, Group B, Group C and Group D the mean **Cellular Diameter** (CD) with standard deviation were 102.46 ± 1.77 µm, 91.55 ± 2.74 µm, 87.30 ± 1.10 µm 84.84 ± 5.49 µm and 60.09 ± 1.33 µm respectively. Mean CD and CA values illustrated progressive decrease from Group N to Group A to Group B to Group C to Group D. These findings suggested that the cellular parameters i.e cellular diameter and cellular area decreases from normal to tobacco lime lesion group to premalignant groups to OSCC group. **(Table 2)**

Similarly in the present study, mean **nuclear area** (NA) of keratinocytes of buccal mucosa Group N was 104.22 µm^2, Group A was 119.63µm^2, Group B was 137.50 µm^2, Group C was 145.40µm^2 and Group D was 181.68µm^2 which showed progressive increase and statistically significant difference with each other group (P<0.0001) except between tobacco lime lesion and premalignant lesion and condition. **(Table 3)**

In present study, mean **nuclear diameter** (ND) of keratinocytes of buccal mucosa of Group N was 11.8µm, Group A was 13.48µm, Group B was 14.01µm, Group C was 14.03µm, and Group D was 19.59µm. Which shows progressive increase and statistically significant difference with each other group(P< 0.0001) except between tobacco lime lesion and premalignant lesion and condition.**(Table 4)**

In present study, mean **nuclear diameter to cellular diameter ratio** (ND:CD) of keratinocytes of buccal mucosa of Group N was 0.115, Group A was 0.147, Group B was 0.161 Group C was 0.165, and Group D was 0.326. This shows progressive

increase and statistically significant difference with each other group (P< 0.0001) except between tobacco lime lesion and premalignant lesion and condition. **Table 5**

Similarly in the present study, mean **nuclear area to cellular area ratio** (NA:CA) of keratinocytes of buccal mucosa Group N was 0.012, Group A was 0.016, Group B was 0.025, Group C was 0.028 and Group D was 0.045. Which was show progressive increase and statistically significant difference with each other Group(P< 0.0001) except between tobacco lime lesion and premalignant lesion and condition. **Table 6**

DISCUSSION

The malignancies of the oral cavity arise from premalignant lesions, such as leukoplakia, oral submucous fibrosis and tobacco lime lesion. During transformation of normal tissue to premalignancy or malignancy, cellular changes occur at the molecular level before they are seen under the microscope and much before clinical changes become evident. Cellular morphology reflects the biological activity of cell. John K. Frost opines that general biological activity is reflected best in nucleus and functional activity is reflected in cytoplasm.[6] Identification of high risk oral premalignant lesions and intervention at premalignant stages could constitute one of the keys in reducing the mortality and morbidity. Diagnostic technique could increase the chances of earlier detection of premalignant and malignant lesions.[10]

Exfoliative cytology, which is a simple, noninvasive diagnostic technique, could increase the chances of earlier detection of premalignant and malignant lesions. In exfoliative cytology, the quantitative parameters are objective and reproducible; they may be important aids in the making of a cytopathologic diagnosis in such situations. One such quantitative parameter is morphometry, with advance technology, more reliable quantitative techniques like cytomorphometry, histometry etc., can be made using computer assisted image analyzer. The smear obtained by exfoliative cytology can be analyzed quantitatively and qualitatively. With advancements in the field of quantitative oral exfoliative cytology, various parameters such as nuclear size, cell size, nuclear-to-cytoplasmic ratio, nuclear shape, nuclear discontinuity, optical density and nuclear texture can be evaluated collectively in order to confirm the diagnosis. Of these parameters, the nuclear size, cytoplasmic size and their ratio have been shown to be significant in the evaluation of oral lesions. The variations obtained in these parameters have been attributed to exposure to carcinogenic agents like tobacco. The concept of cellular or nuclear alteration on exposure to varying forms of tobacco can be best explained by reviewing the nature of cellular response to stimuli from the end products of different types of tobacco usage.[5]

Lebert (1851) emphasized the altered size of cells and nuclei as a basis of diagnosing cancer.[16] Since no single structural change is diagnostic by itself, a combination of several abnormalities is always necessary.

Hence Ogden et al. (1997) and Cowpe and Longmore (1981), suggested that quantitative techniques, based on the evaluation of parameters such as nuclear area (NA), cytoplasmic area (CA) and nuclear to cytoplasmic area ratio (NA:CA) may increase the sensitivity of exfoliative cytology for early diagnosis of oral cancer.[17] Ramesh et al. (1998) assessed the morphometric changes of cell diameter (CD) and nuclear diameter (ND) of squames from oral premalignant and malignant lesions.[14]

Taking this into consideration, the present study has been carried out to analyze the effect of tobacco chewing on keratinocytes of buccal mucosa using parameters like cellular area (CA), nuclear area (NA), NA:CA Ratio, cellular diameter (CD), nuclear diameter (ND), ND: CD ratio.

Present study includes tobacco lime lesion as a separate study group (i.e. Group B) and premalignant lesion/condition (leukoplakia and OSMF as Group C) as a separate study group and all the patients were non anemic and none of the patients have any history of radiotherapy/chemotherapy, alcohol drinking and any systemic diseases. Thus any compounding effects of these parameters on cytomorphology of keratinocytes of buccal can be ruled out.[13,9,2,7]

Various stains used in cytology like PAP, Giemsa stain, May-Grunwald Giemsa stain.[8] PAP stain is

still a big hit in the cytology staining procedures. The advantages of PAP staining lies in the fact that the dehydration and cleaning solution helps in causing cellular transparency. This detects the overlapped cells and their individual morphology better. The second significant advantage is the differentiating stain of PAP, OG-6 and EA-50 provides a subtle range of green, blue and pink hues to the cell cytoplasm according to the amount of keratin content and the maturation pattern of the cell.[7,16] In the present study, in PAP stained smears 50 unfolded cells were analyzed in a stepwise manner.

In present study there were 84% male and 16% female. There was a predominance of male population (84%) because tobacco chewing habit has become a cultural characteristic and generally men start practicing this habit early in age. Our finding of male predominance is also seen in other studies conducted by Ramesh T et al. (1998& 1999), Hande A et al. (2010) and Mollaoglu et al. (2001).[11,14,15,5]

From the observations in present study and all the above mentioned studies we could infer that with increase of severity of disease cellular parameters (area and diameter) decrease. The significant reduction in CD from normal through premalignant lesions to squamous cell carcinomas shows that the reduction in cell size could be an early indication of malignant change, as suggested by Cowpe et al (1988).[4]

Carcinogens reduce the ability of the cytoplasm to mature, so that there is a greater immaturity to the cytoplasm of the cell with greatly increased activity of the nucleus leading to decrease in cellular parameters. These morphometric changes in tobacco chewers may be attributed to mechanical injury, chronic inflammation, constant exposure to carcinogens released from the smokeless tobacco, generation of reactive oxygen species (ROS), depletion of protective enzymes in the cells, nutritional deficiencies secondary to the habit.[1]

Along with reduction in cellular parameters, our study has also found progressive increase in nuclear parameters. (Area and Diameter) The mean of NA and ND as observed by Khandelwal S. et al.(2010), Acharya S. et al.(2013) and Joshi P et al.(2013) in their respective studies showed progressive increase from normal mucosa of healthy individual to OSCC cases.[7 ,1]

This increase in nuclear dimension is related to an increase in the nuclear contents required for replication and signifies increase nuclear activity. Increase in nuclear size could be produced as an inflammatory change due to chronic irritation of keratinocytes of buccal mucosa caused by tobacco habits.[1] Nuclear parameter in present study did not show statistically significant difference between group of tobacco lime lesion and group of premalignant lesion and condition. Same was the finding for cellular diameter this indicates that cellular content and nuclear activities in both these groups are similar. These cytomorphometric findings suggested that tobacco lime lesion and leukoplakia/OSMF similar potential to progress to OSCC.

Cowpe JG et. al(1988), Franklin CD and Smith CJ (1980) reported that the **N:C ratio** has the advantage of relating nuclear volume to cytoplasmic volume and possibly represents the significant changes that occur in the cell, more accurately at a morphological level.[5] In present study NA:CA ratio and ND:CD ratios showed progressive increase from group N to A to B to C to D. This observation was seen due to significant elevation in nuclear area and diameter and significant reduction in cellular area and diameter. The ratio of NA:CA and ND:CD was highest in OSCC group. Similar finding of mean NA:CA ratio and mean ND:CD ratio were given by Khandelwal S. et al.(2010) and Acharya S. et.al.(2013)[7,1]

From all the above observation of all the studies we could observed that there was difference in all the values obtain for mean of ND, mean of NA, mean of CA, mean of CD, mean of ND:CD ratio and Mean of NA:CA ratio, which can be attributed to different study population selected, different content of tobacco used by the population and different software used. Present study thus elucidated the importance of early recognition of cellular alterations for identification of individuals who require early intervention even in the absence of visible changes of mucosal surface. Because the basic defect of any cell alteration begins at the molecular level, and triggers a series of reactions

which affect the entire cell system. This is ultimately reflected in the cellular morphology.

Application of quantitative techniques of smear of keratinocytes obtained from buccal mucosa of oral premalignant and malignant lesions, suspicious normal area and squamous cell carcinoma can possibly improve the diagnostic value of oral exfoliative cytology and also assist in monitoring the status of the mucosa of tobacco chewers.

Further, as the acceptance in the reliability of measurable values increases, this method can also aid in motivating individuals to withdraw from tobacco use. Hence,we emphasize that cytomorphology is an invaluable parameter to assess the influence of tobacco on buccal mucosa. Our study restricts itself to performing linear measurements on exfoliative cytology and hence, we propose that large scale studies can be carried out to further confirm the efficacy of this procedure in detection of early changes of buccal mucosa using automated image analysis. This method can also be utilized for routine screening procedure of patients to detect any changes from the normal mucosa.

CONCLUSION

Cytomorphometric changes could be the earliest indicators of cellular alterations. There is progressive decrease in cellular diameter and area, increase in nuclear diameter and area and increase in ratio of nuclear diameter to cellular diameter and nuclear area to cellular area in smears from all tobacco users, as compared to normal subjects. This indicates that there could be cause–effect relationship between tobacco and quantitative alterations.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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