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Tobacco use increase the number of aneuploid nuclei in the clinically healthy oral epithelium

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BACKGROUND: The most important risk factor linked to the development of oral leukoplakia (OL) and oral squamous cell carcinoma (OSCC) is tobacco use. Tobacco contains carcinogens that influence the DNA repair, cell cycle control and may produce chromosomal aberrations. The loss or acquisition of one or more chromosomes is defined as aneuploidy.

METHODS: Aneuploidy was determined by means of the DNA-content included in cells obtained by exfoliative cytology and Feulgen's staining. The cells were collected from the clinically healthy lateral margin of the tongue of non-smokers without oral lesions, smokers without oral lesions, smokers with OL, and smokers with OSCC, using the CytoBrush®. Each group was composed of 20 individuals. A Carl Zeiss image analyzer system and the KS300 software were used. Statistical analysis was performed with BioEstat® software.

RESULTS: The mean percentage of aneuploid nuclei was statistically higher in the smokers (79.65%), smokers with OL (68.4%), and smokers with OSCC (93.65%), as compared to non-smokers (39.3%) ($P < 0.05$). A trend toward an increase in the aneuploidy of the smokers with OSCC group ($P = 0.02$), as compared to the non-smoker group, could be observed. No significant difference could be observed as regards the mean percentage of aneuploid nuclei in relation to duration of tobacco use or the number of the cigarettes smoked.

CONCLUSIONS: Tobacco use is responsible for an increased number of aneuploid nuclei in the oral epithelium.

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Introduction

There is a strong relation between tobacco use and the development of oral squamous cell carcinoma (OSCC). Tobacco use is the most important risk factor linked to the development of oral leukoplakia (OL) and OSCC, in addition to it being currently the most evident cause of disease and death worldwide (1). Tobacco contains carcinogens that are related to the carcinogen metabolism, DNA repair, cell cycle control, and chromosomal aberrations in OSCC (2). Moreover, prior literature reports on the evaluation of the effect of tobacco use on clinically healthy oral epithelial cells of smokers with or without OL/OSCC. Histochemistry techniques used in exfoliative cytology on clinically healthy oral epithelial cells from smokers have demonstrated the following changes: (i) increased nuclear area (3, 4); (ii) an increase in the number of keratinized cells (5); and (iii) a higher epithelial cell proliferation (6–9). The increased cell proliferation of the clinically healthy oral epithelium was determined by an AgNOR count (1, 7–9).

Exfoliative cytology is a painless, non-invasive technique which extracts intact cells from different layers within the epithelium (10). Sampaio et al. (7) and Sujathan et al. (11) suggest that by using smears, AgNOR studies are more precise, since the entire nucleolus can be analyzed, rather than only part of it as occurs in histological sections. Exfoliative cytology can also be a useful tool for the detection of initial changes in smokers and for the adequate monitoring of second tumors in patients treated for OSCC (1). Carcinogens can create a vast, genetically altered area in the epithelium. The concept of field cancerization was conceived by Slaughter (1953), based on a histological examination of dysplastic epithelium attached to an invasive oral cancer, which was responsible for the occurrence of a local tumoral recurrence (12).

A great change in the DNA-content could be observed in human tumors. DNA-image cytometry (DIC) allows for the quantification of the DNA-content in nuclei obtained by exfoliative cytology and Feulgen's staining. According to existing evidence, the amount of Feulgen's stain is proportional to the quantity of DNA in the cell nuclei. The loss or acquisition of one or more chromosomes is known as *aneuploidy*, and aneuploid cells tend to present an altered DNA-content (13, 14). During mitosis, the parent cells divide into two daughter cells with a diploid number of chromosomes. An unequal distribution of the chromosomes in the daughter cells generates aneuploidy (14). To date, studies have not evaluated the effect of tobacco use on the DNA-content of clinically healthy oral epithelial cells. The goal of this study is to verify the effect of tobacco use on the DNA-content of oral epithelial nuclei collected by exfoliative cytology from the clinically healthy lateral margin of the tongue of non-smokers without oral lesions, smokers without oral lesions, smokers with OL (SOL), and smokers with OSCC (SOSCC).

Patients and methods

Patients

This study was approved by the local Committee on Bioethics from Universidade Federal de Minas Gerais (UFMG). The samples were obtained from the School of Dentistry at UFMG for dental treatment or treatment/diagnosis of OL or OSCC. The samples included 20 non-smokers (22–66 years of age, mean 40.8 years), 20 smokers (25–59 years of age, mean 44.05 years), 20 SOL (22–77 years of age, mean 47.35 years), and 20 SOSCC (40–68 years of age, mean 54.2 years). OL was diagnosed according to that proposed by Axell et al. (15). The diagnosis of OSCC criteria proved to be in accordance with that proposed by the WHO-2005 (16). Smokers were defined as those patients who smoked over 10 cigarettes per day over a 10-year period (8). Only smokers of filtered cigarettes were included in this study. Alcohol use was not assessed in this study because we had difficulty to find individuals that only used tobacco or alcohol, and in all evaluated groups there are some patients using alcohol. Also, use alcohol was extremely variable. Patients with alcohol use in the non-smokers were nine (45%), smokers without any oral lesion were 10 (50%), SOL were 10 (50%) and OSCC were 15 (75%). Before the smears were collected, each patient underwent a clinical examination of the oral cavity and the medical history was documented. Patients with diabetes mellitus were excluded from this study (17). However, some patients did mention having hypertension.

Sample preparation and staining

Smears were collected from the clinically healthy lateral margin of the tongue using a CytoBrush® (18). The area where the sample was to be collected was cleaned of excessive saliva and surface debris. The first sample collected was discarded to avoid cells in degeneration. In patients with SOL or OSCC located on the tongue, the smears were obtained on the opposite clinically healthy lateral margin of the tongue. The collection of cells was transferred to a dry glass slide and then set in Zenker's solution for 3 h. Next, the smears were washed in water and stained by Feulgen's stain. Briefly, hydrolysis was performed using 1 N of hydrochloric acid at 60°C for 15 min, followed by rising with distilled water. Shift's reagent was applied for 1 h and 30 min. The smears were washed in running waters for 5 min and then dehydrated.

DNA-image cytometry (DIC)

DNA-image cytometry was performed using an image analysis system. The images were viewed through a JVC TK-1270/RGB camera and immediately digitalized at 400× magnification. The Carl Zeiss image analyzer system and the software KS300 (Carl Zeiss, Oberkochen, Germany) were used. The DNA-content in the nucleus of the healthy cell, in a given organism, were denominated the 2c or 'diploid amount' of DNA. The DIC results in integrated optical density (IOD) values, which are equivalent but not identical to the DNA-content. The measurement of the DNA-content requires the obtaining of the DNA index (DI). The DI is obtained by dividing the IOD of the nuclei from the samples by the IOD of the lymphocyte nuclei, which are considered diploid control cells. A DI of 2.0 corresponds to the diploid cells (2c), while a DI of 4.0 corresponds to the tetraploid cells (4c). Samples with a DI = 2.0 ± 0.2 or a DI = 4.0 ± 0.4 are considered diploid or tetraploid, respectively. Values of DI below or above this interval are considered aneuploid (14, 19).

The DNA-content of 100 randomly selected nuclei were selected from each patient. Nuclear debris, as well as pycnotic, folded, or overlapping nuclei, were manually discarded. The mean percentage ± standard deviation (SD) of aneuploidy cells in each group was determined. As the percentage did not conform to a normal distribution, the Kruskal–Wallis test was used for statistical analysis. The samples were defined as aneuploid when more than 10% of the nuclei were not diploid (2c) (20). The non-smoker, smoker, SOL, and SOSCC groups were compared according to their diploid and aneuploid statuses. The Fisher test was used for statistical analysis, and the Bonferroni correction was applied. Smokers were divided into group 1: smoking up to 20 years; group 2: smoking between 21 and 40 years; and group 3: smoking between 41 and 60 years. They were also divided into group A: smoking up to 19 cigarettes per day; group B: smoking between 20 and 30 cigarettes per day; and group C: smoking more than 31 cigarettes per day (1). The correlation between time of smoking (groups 1, 2, and 3), number of cigarettes smoked per day (groups A, B, and C) and the mean percentage of the aneuploid cells was determined by parametric analysis with Pearson correlation. The alpha level was set to 0.05. Statistical analysis was performed using the BioEstat® software (21).

Results

The nuclei that underwent Feulgen's staining presented a purplish color (Fig. 1). The mean percentage ± SD of

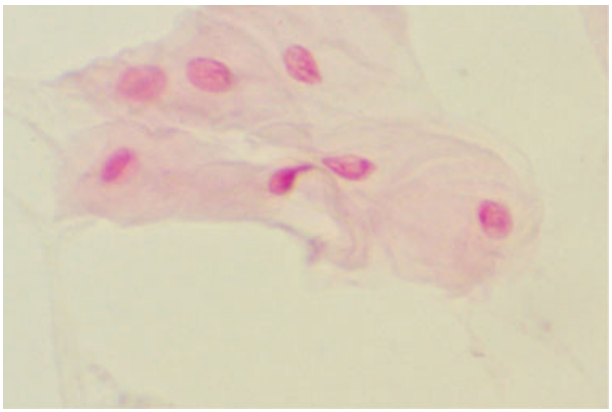

Figure 1 Feulgen's stain in exfoliative cytology obtained from the clinically healthy lateral margin of the tongue. The oral epithelial nucleus has a purplish color (Feulgen's stain, 400× magnification of original).

Table 1 The mean percentage of aneuploid cells in non-smokers, smokers without any oral lesion, smokers with oral leukoplakia and with oral squamous cell carcinoma

Group	*Values ± SD*
Non-smokers	39.3 ± 35.21*
Smokers without any oral lesion	79.65 ± 30.68*
Smokers with oral leukoplakia	68.40 ± 39.47*
Smokers with oral squamous cells carcinoma	93.65 ± 12.19*

SD, standard deviation; *$P < 0.05$ (Kruskal–Wallis test).

aneuploid nuclei from 20 cases in each group is presented in Table 1. No tetraploid cells could be observed. The mean percentage of the aneuploid nuclei was statistically higher in the smokers, SOL, and SOSCC groups when compared to non-smokers. No significant difference in the mean percentage of aneuploid nuclei among smokers, SOL, and SOSCC was determined. The percentage of diploid and aneuploid samples from 20 cases in each group is presented in Table 2. The percentage of diploid and aneuploid samples proved to be statistically different ($P = 0.02$) among the non-smoker, smoker, SOL, and SOSCC groups. The significance level ($P = 0.016$) was obtained by Bonferroni correction. A trend toward an increase in the aneuploid status of the SOSCC group ($P = 0.02$), when compared to the non-smoker group, could be observed.

Table 2 Percentage of diploid and aneuploid samples among groups of non-smokers, smokers without any oral lesion, smokers with oral leukoplakia and smokers with oral squamous cell carcinoma

Group	*Diploid (%)*	*Aneuploid (%)*	*Total (%)*
Non-smokers	6 (30)	14 (70)	20 (100)
Smokers without any oral lesion	1 (5)	19 (95)	20 (100)
Smokers with oral leukoplakia	4 (20)	16 (80)	20 (100)
Smokers with oral squamous cell carcinoma	0 (0)	20 (100)	20 (100)

Table 3 The duration of the smoking habit and the mean percentage of aneuploid cells in smokers without any oral lesion, smokers with oral leukoplakia and with oral squamous cell carcinoma

Group	*Patients (n = 60)*	*Mean duration of the smokering habit (years) ± SD*	*Mean percentage of aneuploid cells ± SD*
1	9	25.05 ± 9.68	64.44 ± 42.81*
2	40	29.86 ± 6.26	82.32 ± 29.40*
3	11	49.36 ± 5.81	86.00 ± 29.26*

Group 1: smoking up to 20 years; group 2: smoking between 21 and 40 years; group 3: smoking between 41 and 60 years.
SD, standard deviation; *$P > 0.05$ (Pearson correlation).

Table 4 The number of the cigarettes smoked per day and the mean percentage of aneuploid cells in smokers without any oral lesion, smokers with oral leukoplakia and with oral squamous cell carcinoma

Group	*Patients (n = 60)*	*Mean of the smoked cigarettes per day ± SD*	*Mean percentage of aneuploid cells ± SD*
A	30	16.5 ± 6.5	81,06 ± 30.48*
B	24	16.95 ± 8.99	77.95 ± 34.26*
C	6	22.1 ± 14.9	88.50 ± 21.01*

Group A: smoking up to 19 cigarettes per day; group B: smoking between 20 and 30 cigarettes per day; group C: smoking more than 31 cigarettes per day.
SD, standard deviation; *$P > 0.05$ (Pearson correlation).

There was no significant correlation between the time of smoking (groups 1, 2, and 3) (Table 3) and the number of cigarettes smoked (groups A, B, and C) (Table 4) when evaluating the mean percentages of aneuploid nuclei in smoker's groups.

Discussion

It could be observed that smokers with or without OL/OSCC presented a higher percentage of aneuploid nuclei than did non-smokers. In addition, a trend toward an increase in the aneuploid status of the SOSCC group, when compared to the non-smoker group, could be observed. Despite the frequent presence of aneuploidy in cancer, the exact role of aneuploidy in carcinogenesis is still not well-understood. According to Dey (14), aneuploidy represents an important marker of the malignant transformation of precursor lesions. Furthermore, DNA aneuploidy should be regarded as a consequence of chromosomal instability, which appears to contribute to the genesis and progression of cancer (22, 23).

The cells of oral mucosa healthy clinically are diploid (24, 25). In non-smokers group, it was observed 39% aneuploid cells and 70% of aneuploidy in the oral mucosa healthy clinically. The explanation for this unusual finding may well be the fact that the majority of previous studies that assessed DNA-ploidy in clinically healthy oral mucosa investigated nuclei which was isolated from formalin-fixed, paraffin-embedded, archival material, whereas the present study used the assess-

ment of the DNA-content of cytological samples. McCullough and Farah (26) observed a more highly abnormal DNA-content in the nuclei present in cytological control specimens than in histopathologically proven abnormal samples. Also, in the cytological samples where the nuclei are obtained from the superficial layer with a large number of degenerated cells, the DNA-content may be modified. This fact was minimized by collecting a second cytological smear. Additionally, external factors unevaluated, as the alcohol use, may be contributory to this fact. Future study with randomly population of the patients with use alcohol is important.

There are a number of possible ways to develop aneuploidy, such as: (i) defective formation of the mitotic spindle, (ii) defective attachment of chromatids, (iii) fusion bridging abnormality, (iv) defective cytokinesis, or (v) mitotic checkpoint defects (14). Exposure to tobacco carcinogens can act as an aneuploid agent by disabling mitosis proteins, either physically or chemically. In fact, 99% of the chemical carcinogens are the polycyclic aromatic hydrocarbons that bind or react to these proteins (27, 28). As the effect of tobacco use is accumulative (29), it was expected that individuals who had smoked for the longest period of time and who had consumed the largest amount of cigarettes per day would in turn present the highest percentage of aneuploid nuclei (29). However, according to the present study, it was not possible to determine these significant correlations. Nevertheless, when considering an increase in the length of time smoking and the amount of cigarettes consumed per day, a trend toward an increase in the mean percentage of aneuploid nuclei could be observed. These results suggested that the length of time smoking and the amount of cigarettes smoked per day may in fact be responsible for increased aneuploid nuclei in smokers. Still, AgNOR analyses were unable to demonstrate a direct correlation to the number of cigarettes consumed per day and the length of time referent to the patient's smoking habit (1, 8).

In the current study, the distribution of gender and age was not considered. However, it could be demonstrated that gender and age present no correlations with alterations in oral epithelial cells (7, 30–32). Also, no difference was noted between the sexes and the AgNOR count of the clinically healthy oral epithelium of smokers, SOL, SOSCC, and non-smokers (8, 33). In this study, the smears were collected from the clinically healthy lateral margin of the tongue, because: (i) it is the most frequent site for the OSCC (34) and (ii) the carcinogens can create a vast area of genetically altered cancer fields in the epithelium (12, 35). In the patients with OL or OSCC, the smears were collected from the opposite clinically healthy lateral margin of the tongue to avoid cell alterations of the leukoplakia or squamous cell carcinoma (36, 37).

Alcohol was not considered in this study, because it was observed a great variation in the time, quantity and type of drink consumed for each patient. The influence of alcohol on patients who are at risk of contracting oral cancer has been assessed in prior literature through the use of quantitative exfoliative cytology (38–40). A statistically significant reduction in the mean of the cytoplasmatic and nuclear areas was found for the alcohol group when compared to the controls (39). However, Ogden et al. (38) evaluated the DNA-profile of buccal cells from alcohol-dependent patients and reported a diploid profile. Further studies are warranted to evaluate an association among smoking, alcohol, and the DNA-content.

Mehrotra et al. (41) consider that changes occur at the molecular level before viewing under a microscope and before clinical changes occur. Identification of high-risk oral pre-malignant lesions and intervention at pre-malignant stages may well constitute one of the keys to reducing mortality, morbidity, and the cost of treatment associated with OSCC. Exfoliative cytology appears to be a useful tool to detect and monitor initial alterations (1) as well as to establish an appropriate treatment for smokers (4, 8). Exfoliative cytology is a complementary diagnostic method which presents several advantages, such as rapid and easy execution, low cost, diagnostic safety, efficacy, and noninvasiveness. In addition, it can be repeated several times (1, 4). The DNA-content is increasingly coming into practice for assistance in the diagnosis and grading of malignant tumors. Most human neoplasms reveal light microscopically detectable chromosomal aberrations. Chromosomal aneuploidy is defined as numeric and/or structural aberrations and may be used as a marker for neoplasms. DNA aneuploidy is the cytometric equivalent of chromosomal aneuploidy. Detection of chromosomal or DNA aneuploidy serves as a neoplasm marker (42–44). However, the DNA-content of the clinically healthy oral epithelial nuclei in exfoliative cytology, used to verify the influence of tobacco use on DNA aneuploidy, has yet to be performed. Studies regarding the use of the AgNOR method in exfoliative cytology have shown significant increases in the AgNOR count in clinically healthy oral epithelial cells of smokers with or without OL or OSCC, demonstrating that cigarette smoking does in fact influence the proliferative activity of these cells (1, 7–10). The results of the current study also demonstrated that tobacco use causes alterations in the DNA-content in clinically healthy oral epithelial nuclei in smokers with or without OL and OSCC when compared to non-smokers.

Tobacco use is responsible for an increased number of aneuploid nuclei in clinically healthy oral epitheliums. This finding represents a contributory fact regarding an increase in the relation between tobacco use and the development of pre-malignant or malignant oral lesions.

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