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Cytogenetic Biomonitoring in Buccal Mucosa Cells from Young Smokers

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Key Words
Oral mucosa cells · Cigarette smoke · Micronucleus test

Abstract
Objective: Nowadays, much attention has been focused on the search for new non-invasive methodologies able to predict malignant transformation of oral mucosa cells. The aim of the present study was to comparatively evaluate DNA damage (micronucleus) and cellular death (pyknosis, karyolysis and karyorrhexis) in exfoliated oral mucosa cells from smokers and non-smokers in buccal mucosa cells. ***Study Design:*** A total of 24 young, healthy smokers and 14 non-smokers were included in this setting. Individuals had epithelial cells from the cheek mechanically exfoliated, placed in fixative and dropped in clean slides which were checked for the above nuclear phenotypes. ***Results:*** Smokers presented more ($p < 0.05$) micronucleated oral mucosa cells than non-smokers. Tobacco smoke was not able to increase other nuclear alterations closely related to cytotoxicity such as karyorrhexis, pyknosis and karyolysis. ***Conclusion:*** In summary, these data indicate that the cigarette is able to induce micronuclei in oral mucosa cells, so the micronucleus test is a suitable method for predicting oral cancer risk. © 2016 S. Karger AG, Basel

Introduction

Cancer is defined as a complex genetic disease characterized by altered expression of cell cycle regulatory proteins caused by mutagenic agents and carcinogens and is the common cause of mortality both in developed and in developing countries. Epidemiologic studies have proven that cigarette smoking is the major cause of oral cancer [1, 2]. Therefore, tobacco has been considered the single most important man-made cause of cancer that can be avoided. On a scientific basis, these studies provide convincing evidence of an association between cigarette smoking and oral cancer.

A great deal of attention has been focused on the search for new non-invasive methodologies that enable the prediction of when, and to what extent, the oral mucosa cells undergo malignant transformation as a result of environmental mutagenesis. Herein, the micronucleus test has become the most popular method for detecting structural and numerical chromosomal aberrations caused by environmental mutagenic agents, both in experimental test systems and in human studies [3]. Micronuclei are defined as acentric fragments or whole chromosomes which are not included into the main nu-

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clei of the daughter cells. The formation of micronuclei can be induced by substances that cause chromosome breakage (clastogens), as well as by agents that affect the spindle apparatus (aneugens) [4]. According to Tolbert et al. [5], the specificity of the test to detect genotoxic and cytotoxic effects is improved by analysing other degenerative nuclear alterations indicative of cell death. Among them, pyknosis, karyolysis and karyorrhexis are suitable for this purpose.

Unfortunately, the evaluation of earlier micronucleus studies using buccal cells in smokers has demonstrated that the results are strongly controversial. This is because positive findings were obtained mainly with non-DNA-specific stains or when studying other confounding factors, not specifically focused on the mutagenic outcomes induced by cigarette smoke [6]. For example, Bloching et al. [7] evaluated micronuclei in smokers suffering from cancer or even premalignant lesions of the oral mucosa. An elevated number of micronuclei in these patients was detected, probably due to oral neoplasms, because it is well established that tumours are able to induce mutagenicity as a result of micronucleated cells in oral exfoliated cells. In the study by Stich and Rosin [8], the number of micronuclei of heavy smokers was similar to that of non-smokers. Suhas et al. [9] showed an increased frequency of micronuclei in smokers of beedi, in which concentrations of nicotine, tar, and other toxic agents in smoke from burnt tobacco are higher than in cigarette smoke. The data presented by Wu et al. [10] revealed that cigarette smoking did not increase the number of micronuclei in smokers when compared with controls. Kayal et al. [11] investigated micronuclei in buccal mucosa cells of persons who chewed indigenous products (areca nut, mava, tamol, tobacco with lime, dry snuff, or mashery), but not tobacco cigarettes. Motgi et al. [12] have demonstrated that total numbers of micronucleated cells were significantly lower in non-tobacco users when compared with tobacco users, but such data were obtained by using non-specific DNA stains (Papanicolaou stain). Others have yet revealed a lack of statistical significance for micronucleus frequency between smokers and non-smokers in patients previously submitted to dental X-ray [13, 14]. These data contrast with the current knowledge of cigarette smoke in the risk of oral cancer [15]. It has been discussed whether this procedure may be a reliable method for the detection of human cancer risks as most tumours are of epithelial origin [16]. Such data elicits concerns about the predictive value of the method itself, as it is well documented that the oral cavity is one of the target organs for cancer induction by smoking [17].

As a result and because of controversial scientific evidence, the aim of this study was to investigate cytotoxicity and mutagenicity in buccal mucosa cells induced by cigarette smoke in young individuals by a micronucleus test using the Feulgen fast green method (specific DNA stain).

Materials and Methods

Subjects

The subjects of this study comprised a total of 24 healthy young adults (10 men and 14 women) with a mean age of 28.8 ± 4.2 years. In this study, the volunteers were considered smokers if they had smoked more than 10 cigarettes/day for at least 5 years. Furthermore, 14 adults (9 men and 5 women) with a mean age of 25.6 ± 4.2 years were included as non-smokers. None of the participants had a history of major illnesses, they were on no medication at the time of the study, they had a body mass index below 27, used illicit drugs less than once a month, and had at least 11 years of schooling. The study was approved by the Human Ethics Committee of the Universidade Federal the São Paulo. Informed consent was obtained from all participants.

Micronucleus Test in Oral Mucosa Cells

After rinsing the mouth with tap water, cells were obtained by scraping the right/left cheek mucosa with a moist wooden spatula. Cells were transferred to a tube containing saline solution, centrifuged (800 rpm) during 5 min, fixed in 3:1 methanol/acetic acid, and dropped onto precleaned slides. Later, the air-dried slides were stained using the Feulgen fast green method, and examined under a light microscope at a magnification of ×1,000 to determine the frequency of micronucleated cells. Two thousand cells were scored from each test person. Samples of smokers were obtained approximately 2 h after the last cigarette.

Data Analysis

Micronuclei were scored according to the criteria described by Beliën et al. [4] as a parameter of DNA damage (mutagenicity). For cytotoxicity, the following nuclear alterations were considered: pyknosis, karyolysis and karyorrhexis. Results were expressed in percentages. This analysis was established in a previous study conducted by our research group [18]. The analysis was evaluated independently by two biomedical doctors in a blinded fashion.

Statistical Methods

The Mann-Whitney non-parametric test was used to compare the frequencies of cytotoxicity among the samples between smokers and non-smokers (control group). Micronucleus frequencies between controls and smokers were evaluated as established by Pereira [19]. The statistical analysis was conducted using BioStat software, version 5.0 (Maringa, PR, Brazil). The level of statistical significance was set at 5%.

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Table 1. Micronucleus incidence in buccal mucosa cells of smokers

Groups	Micronucleus incidence
Control	0.0±0.1
Smokers	0.7±0.8*

* p < 0.05 when compared to control group.

Table 2. Cytotoxicity parameters (karyorrhexis, pyknosis and karyolysis) in buccal mucosa cells of smokers

Groups	Pyknosis	Karyorrhexis	Karyolysis
Control	108.8±37.4	21.6±31.5	17.3±13.4
Smokers	110.0±33.3	16.3±13.8	14.4±22.0

p > 0.05.

Results

Table 1 shows the frequencies of micronucleated cells in buccal mucosa cells of non-smokers and smokers. Significant statistical differences ($p < 0.05$) were obtained, smokers having presented a higher micronucleus incidence.

However, cigarette smoke was not able to increase other nuclear alterations closely related to cytotoxicity such as karyorrhexis, pyknosis and karyolysis of non-smokers and smokers (p values >0.05; table 2).

Finally, exposure to known genotoxins was not investigated. The daily alcohol consumption was not considered in this study, because a recall bias phenomenon had occurred.

Discussion

The aim of this study was to comparatively evaluate chromosome damage and cellular death induced by exposure to cigarette smoking as indicators of genotoxicity and cytotoxicity, respectively. The investigation was conducted using the micronucleus test in oral exfoliated cells in vivo.

The big advantage of the micronucleus assay is the relative ease of scoring, the limited costs and person-time required, and the precision obtained from scoring larger numbers of cells. The measurement of the frequency of micronuclei induced in cells by mutagen agents is widely used for cytogenetic biomonitoring [4]. Micronuclei contain genetic material that is lost from the whole DNA during mitosis, as a result of clastogen or aneugen events [4]. Hence, there will arise bigger micronuclei from whole chromosomes as a follow-up to damaging of the spindle apparatus of the cell (aneugen). Smaller micronuclei are the result of structural aberrations and consist of chromosomal breakage. Damages that lead to the formation of micronuclei take place in the basal layer of the epithelial tissue, where cells undergo mitosis. Programmed turnover of epithelial tissues brings the cells to the surface where they exfoliate, and therefore it is possible to detect them.

Genomic damage plays a pivotal role during carcinogenesis. It has been well established that genomic damage is produced by environmental exposure to mutagens, carcinogens as well as to genetic factors such as defects in the xenobiotic metabolism and DNA repair deficiency [20]. Micronucleated cell frequencies predict genomic instability [21]. The detection of an elevated frequency of micronuclei in a given population indicates an increased risk of cancer [8]. However, cell types that repair DNA damage efficiently are likely to show lower levels of residual damage than cells less proficient in DNA repair [22]. Buccal cells have been shown to have limited DNA repair capacity relative to peripheral blood lymphocytes, and therefore may more accurately reflect genomic instability events in epithelial tissues [4].

Tobacco is known to contain various genotoxic chemicals, and smoking is a well-documented cause of cancer including the oral cavity [23]. Our results demonstrated an increase in micronucleus frequency in buccal cells from smokers using a small sample, indicating that this technique is sensitive to these changes. In fact, several works have failed to show any positive mutagenic effect of smoke. Some works have reported no differences in the induction of micronuclei between smokers and non-smokers [6], while others have shown that smokers had less DNA damage than non-smokers [24]. Exposure to nicotine caused a statistically significant increase in micronucleus frequency in human gingival fibroblasts in vitro [25]. However, it has been demonstrated that nicotine inhibits the action of nitrosamines which is catalyzed by P450 2E1 [26, 27], and also it participates in the conversion of benzo[a]pyrene to DNA-reactive metabolites [28]. Nitrosamines and polycyclic aromatic amines are considered important chemical agents able to promote genetic insult as far as carcinogenesis [15]. In this regard, it has been postulated that nicotine attenuates the acute toxic effects of tobacco condensate in cultured human epithelial cells [29].

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To monitor cytotoxic effects, the frequencies of karyorrhexis, karyolysis and pyknosis were included into this experimental design. Our results showed that in young smokers tobacco use did not induce cytotoxicity as depicted by no statistically significant differences ($p > 0.05$) between smokers and non-smokers. Some authors have argued that nicotine is able to prevent apoptosis in human gingival fibroblasts in vitro [25]. Conversely, others have suggested an increase in apoptosis after cigarette smoke extract stimulation in rat alveolar cells [30]. Curiously, it has been shown that nicotine has anti-oxidant properties [31, 32], so it could interfere with positive results of cytotoxicity. Notwithstanding, explanations for the diverging results may be found in differences pertaining to methodology and/or population characteristics, as well as the sample size. Moreover, it is important to stress that variations in the nicotine levels of the cigarettes smoked by the participants play a crucial role in the human health effects [6]. This issue requires further investigation.

Besides the power of the statistical analysis as a critical factor for the determination of putative outcome, various additional explanations (including seasonal and regional differences) for the reported discrepancies have been proposed [6]. Particularly, some confounding factors are relevant and must be considered when studying biomonitoring human cytogenetic studies. Viruses, alterations in the immune system, failures in the DNA repair system and interindividual variations have already been associated with increased frequencies of chromosome aberrations [33]. Furthermore, an age-related increase in micronuclei has been postulated [34]. Due to the homogeneity in casuistics, it was not possible to correlate the frequency of micronucleated cells with the age in this setting.

In conclusion, the results of the present study suggest that smokers are a high-risk group for developing oral cancer since positive mutagenicity was found. Moreover, we conclude that the micronucleus assay in buccal mucosa cells is a suitable method for predicting the oral cancer risk.

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Disclosure Statement

There is no conflict of interest declared.

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