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MICRONUCLEUS ASSAY IN EPITHELIAL CELLS FROM THE ORAL CAVITY AND URINARY TRACT IN MALE SMOKERS AND NON-SMOKERS

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ABSTRACT

The present work was conducted to investigate the possible use of epithelial cells from the oral cavity and urinary tract in identifying smoking-related effects in men. Epithelial cells from the oral cavity and urinary tract were collected from 25 smoking and 25 non-smoking men and subjected to micronucleus assay. The DNA damage (cells with micronuclei and nuclear buds), binucleated cells, condensed chromatin, karyorrhexis, pyknotic and karyolytic cells were observed after DNA specific staining. The analysis revealed the frequency of binucleated cells and condensed chromatin cells in 25 studied men with smoking habit, statistically significant differences were noted only in epithelial cells from the oral cavity in comparison to those of the urinary tract. In non-smokers the results demonstrated no differences in cytogenetic damage frequency in cells collected from the oral cavity and isolated from the urine. The differences in the observed frequencies of micronuclei in buccal and urothelial cells could be an effect of the smoking in the sampled group, smoking pattern of the men and the number of cigarettes smoked per day.

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INTRODUCTION

Exposure to genotoxic agents occurs through a variety of situations, including pollution of the natural environment, medical procedures (chemotherapy, radiotherapy, etc.) as well as life style factors such as work, diet, tobacco smoking, etc. [1-3]. Cigarette smoking increases the risk of all histological types of lung cancer [4]. Tobacco smoke is a complex, dynamic and reactive mixture and produce carcinogenicity in either laboratory animals and/or humans. Due to mixed exposures and high carcinogenic potential of many compounds contained in tobacco smoke, human biomonitoring is often used for individual risk assessment.

In HBM the most commonly used materials are blood and urine. Nowadays epithelial cells are becoming more and more popular as they may be obtained from the oral cavity, bladder or nose in a noninvasive way [5-7].

Potential carcinogens enter the body through dermal penetration, ingestion and/or inhalation. Thus, epithelial cells are usually the first and the most significant barrier to absorption of exogenous factors [8]. Epithelial cells from the oral cavity are in immediate contact with inhaled and ingested genotoxic agents. Urinary cells have contact with dangerous chemicals which are not metabolized and/or with their derivatives which are formed after metabolic transformation.

The aim of the present work was to investigate the potential use of epithelial cells from the oral cavity and urinary tract as biomarkers of early biological effects identified by micronucleus assay in smokers and non-smokers men. The identified DNA damage included cells with micronuclei and nuclear buds and cytokinetic defects observed as binucleated cells, as well as cell death recognized as condensed chromatin, karyorrhexis, pyknotic and karyolytic cells etc.

MATERIAL AND METHODS

Studied groups

General characteristics of the studied groups (middle-aged men: 25 smokers and 25 non-smokers) are presented in Table 1. The differences in mean age and body mass index of smokers and non-smokers were not statistically significant. The smoking participants declared that they had been smoking for at least five years (average smoking period: 25.9 years, around 14.0 cigarettes per day).

The men in this group mainly smoked light and/or flavoured cigarettes. Therefore, the exposed group was described as light smokers (<15 cigarettes per day) [9]. All subjects were informed about the research procedure and signed the agreement for participation in the study. Obtained from the oral cavity, bladder or nose in a non

Buccal cells as well as urine samples were collected at the same time for each person and coded. Buccal cells were collected from left and right cheeks using sterile nylon flocked swabs according to *Thomas* [10]. For each cheek one swab was used and epithelial cells were sampled by a rotating circular motion. Then the buccal cells were released into phosphate buffered saline (PBS) and stored at 4°C for further analyses.

Urine samples were collected into sterile plastic containers [6]. Urothelial cells were isolated from the urine samples by centrifugation immediately after collection (10 minutes, 3000 RPM) at room temperature. Supernatant was removed and cells were suspended in PBS buffer to protect the cells from degradation.

Micronucleus assay in epithelial cells epithelial cells harvesting and slides preparation

Suspended buccal and urothelial cells were purified by centrifugation (several times, 10 minutes, 2000 RPM, room temperature) in fresh PBS buffer. Then cells were homogenised (2-3 minutes, 20000 RPM) by a homogenizer to increase the number of single cells in suspension and filtrated by 100µm nylon filter to remove large aggregates of unseparated cells.

Then, density of the cell suspension was counted by thoma chamber and the cells were fixed at fresh mixture of ethanol: glacial acetic acid (3:1) for 20 minutes. Afterwards, the cells were dropped onto the pre-cleaned microscope slides and allowed to dry.

Staining procedure

Buccal and urothelial cells were hydrated using the following series: 50% ethanol (vol/vol), then Milli-Q water and 20% ethanol (vol/vol), and again Milli-Q water. Then microscopic slides were incubated in 5M HCl for 30 minutes at room temperature for DNA denaturation. One slide was incubated in distilled water as a negative control to check efficiency of the 5M HCl treatment.

The cells - stained by Schiff's reagent for 90minutes in the dark and at roomtemperature and then counterstained in 0.2% (wt/vol) Light Green solution for 30 seconds in the case of buccal cells and 60 seconds in the case of urothelial cells. Dehydration in absolute ethanol protected cells against fading. Dried microscopic slides were mounted in DPX medium.

Scoring procedure

Microscopic analysis was conducted with a transmitted light microscope at x 400 and x1000 magnification. Cytogenetic damage was observed in the main nucleus stained in magenta colour and pale blue/green staining cytoplasm. In the case of epithelial cells from the oral cavity the frequency of micronuclei and other types of cells was analyzed in 2000 differentiated cells, but the results were recalculated for 1000 cells.

Epithelial cells from the urinary tract the analyses were conducted in 1000 urothelial transitional cells. Microscopic analysis of cytogenetic damage in buccal cells was performed
according to scoring criteria recommended by Thomas [10]. The same criteria were used for urothelial cells.

Statistical analysis

The results were analysed Statistically for the following types of cells: binucleated cells and condensed chromatin cells in material collected from the oral cavity as well as karyorrhectic and pyknotic cells obtained from the urinary tract. Even after logarithmic transformation, the buccal and urothelial cells with micronuclei, the nuclear bud and the urinary tract binucleated cells did not have a normal distribution. Other data after their logarithmic transformation achieved a normal distribution. Studied variables with normal distribution concerning 12 smokers and 12 non-smokers were analyzed by Student t-test. For variables with non-normal distribution Mann-Whitney test was used.

RESULTS

Micronucleus assay is a technique which detects biomarkers of early biological effects on buccal and urothelial cells. Cytogenetic damages such as MN cells, BN cells, CC cells, KR cells, P cells and KL cells were observed both in buccal and urothelial cells. Cells with NB were detected only in epithelial cells from the oral cavity (Table 2).

The frequency of micronucleated cells was found to be higher in urothelial cells as compared with buccal cells. The cells with nuclear bud were found only in the oral cavity. Statistically significant differences in frequency of binucleated cells and condensed chromatin cells were observed in buccal versus urothelial cells. Frequency of binucleated cells was eight-times higher and the level of condensed chromatin cells was one and a half times higher in the oral cavity material as compared to urinary tract epithelial cells. The frequency of karyorrhectic, pyknotic and karyolytic cells from both sources was more or less similar.

The results obtained for smokers and nonsmokers men demonstrated no statistical differences in epithelial cells collected from the oral cavity and isolated from the urine sample (Table 3, 4). Our results showed that cigarette smoking did not have any significant impact on the frequency of other cytogenetic damage (BN, CC, KR, P, KL) in buccal cells of the investigated men (Table 3).

Table 1: General characteristics of the studied groups of men.

Variable	Smokers	Non-smokers	Total
Number of persons	25	25	50
Age in year	48.5 ± 2.05	45.2 ± 4.10	46.85 ± 3.075
Body mass index (mean±SD)	32.6 ± 3.12	25.8 ± 1.06	29.2 ±2. 09
Cigarettes per day (mean±SD)	14.0 ±1.30	NR	-
Years of smoking (mean±SD)	25.9 ± 3.05	NR	-

SD – standard deviation. NR – not relevant.

Collection of biological material

Table 2: Frequency of cytogenetic damage in Buccal and Urothelial cells in 50 studied Men (1000 cells·slide-1).

Cell type	Buccal cells (Mean ±SE)	Urothelial cells (Mean ±SE)	p-value
Micronucleated cells	0.10 ±2.03	0.63± 1.52	p=0.051
Cells with nuclear bud	0.17± 0.62	ND	NR
Binucleated cells	11.43±1.51	3.07±1.06	p=0.001*
Condensed chromatin cells	28.26±1.25	11.05±1.05	p=0.001*
Karyorrhectic cells	3.09±1.01	2.72± 1.04	p=0.884
Pyknotic cells	6.36±1.59	9.07±1.42	p=0.345
Karyolytic cells	6.09±1.10	7.65± 3.12	p=0.870

* Statistically significant (p), SE – Standard error. ND – not detected. NR – not relevant.

Table 3: Frequency of cytogenetic damage in Buccal cells (1000 cells·slide-1) in 25 Male smokers and 25 non-smokers.

Cell type	Smokers (Mean ±SE)	Non- Smokers (Mean ±SE)	p-value
Micronucleated cells	0.08 ±1.22	0.13 ±1.05	p=0.179
Cells with nuclear bud	0.18 ± 3.02	0.09 ±1.25	p=0.287
Binucleated cells	9.19 ±1.08	15.57 ± 2.13	p=0.173
Condensed chromatin cells	26.15 ± 2.23	15.08 ± 2.05	p=0.590
Karyorrhectic cells	3.03 ± 3.16	3.14 ± 1.20	p=0.408
Pyknotic cells	6.82 ± 1.26	9.27 ±1.45	p=0.189
Karyolytic cells	4.09 ± 1.20	8.10 ±0.15	p=0.326

SE – standard error.

Table 4: Frequency of cytogenetic damage in urothelial cells (1000 cells·slide-1) in 25 female smokers and 25 non-smokers.

Cell type	Smokers (mean±SE)	Non- Smokers (mean±SE)	p-value
Micronucleated cells	0.40 ±0.11	0.38 ±1.06	p=1.200
Cells with nuclear bud	ND	ND	NR
Binucleated cells	1.58 ± 1.15	1.56± 1.50	p=0.692
Condensed chromatin cells	6.85±0.16	10.25 ± 2.16	p=0.805
Karyorrhectic cells	3.03±0.25	2.56 ±1.06	p=0.462
Pyknotic cells	12.10± 1.64	9.85 ±0.26	p=0.516
Karyolytic cells	2.64 ±1.25	8.07± 2.05	p=0.813

SE – standard error. ND – not detected. NR – not relevant.

DISCUSSION

The average frequency of micronuclei in buccal cells was observed to be 0.10 ±2.03·1000-1 cells (Table 2) in the present investigation. According to Holland [5] our results are within the baseline range of 0.05 MN·1000-1 cells and below the newest baseline frequency range of 0.30-1.70 MN·1000-1 cells as reported by Bolognesi [11]. In the case of urothelial cells, the average frequency of micronuclei in the studied group of men was 0.63± 1.52. 1000-1 cells (Table 2).

The differences between frequency of micronuclei and nuclear buds in buccal cells of male smokers and non-smokers were found statistically insignificant (Table 3). Other results also confirm the lack of effects of tobacco smoking on the frequency of micronuclei in material collected from the oral cavity.

Haveric *et al* in their study on young female smokers from Bosnia and Herzegovina observed no essential differences in the micronuclei frequency in buccal cells as compared with the control group of non-smokers [12]. Karahalil *et al* study also recorded no differences in frequencies of micronuclei between male smokers and non-smokers, who are occupationally exposed to polycyclic aromatic hydrocarbons were not observed [13]. Similarly Diler and Celik in their study on control group of carpet fabric workers, recorded no significant effects of tobacco smoking on micronuclei and nuclear buds level in exfoliated epithelial cells from the oral cavity in men [14]. However, twice the frequency of micronuclei in buccal cells was identified in smokers than non-smokers in the studies of chronic cigarette smoking in mixed male and female population [15]. But Celik *et al* study of petrol station attendants revealed higher frequency of micronuclei in male smokers in comparison to non-smokers [16].

In participants exposed to industrial paints and to pesticides, statistically higher frequencies of micronuclei and nuclear buds in oral epithelial cells were detected in male smokers than in non-smokers [17, 18]. Chandirasekar *et al* reported the occurrence of higher frequency of micronuclei in the age group of 41 and above male smokers from India, who was smoking for more than 20 years [19]. Nersesyan *et al* observed a higher frequency of micronuclei in oral mucosa cells of male smokers, but significant differences were measured only in individuals smoking non-filtered cigarettes [20].

The present experiment observed no significant difference in frequencies of cytogenetic damage in urothelial cells of male smokers and non-smokers (Table 4). In other studies a statistically higher frequency of micronuclei in urothelial cells was detected in male smokers in comparison to nonsmokers [21, 22].

Other research has observed a remarkably higher frequency of BN, KR and KL in buccal cells collected from smokers than non-smokers [17, 18]. Significant increase of BN, CC, KR and KL in oral cells of smokers using medium filter and non-filtered cigarettes as compared with non-smokers was recorded.

The lack of differences in the present results concerning the frequency of micronuclei in buccal and urothelial cells may be contributed to the small size of the investigated group, smoking behaviour patterns of men and the number of cigarettes smoked per day. The examined group consisted of 24 men, twelve of whom declared that they smoked about 12 cigarettes per day. The obtained data suggested that the examined group could be defined as light smokers. Moreover, Bonassi *et al*., based on the project HUMNxl results, concluded that the significant increase of micronuclei in buccal cells was associated with heavy smoking exceeding 40 cigarettes per day [23].

Ewa Blaszczyk, and Danuta Mielzynska-Svach in their studies concluded that most of the studies applied micronucleus assay for buccal cells analyses rather than urothelial cells. This is probably due to the fact that urothelial cells require isolation from the urine and they are characterised by degenerative properties as well as heterogeneity [24]. Isolated epithelial cells from urine mainly consist of transitional and squamous cells. Moreover, there are essential differences between materials obtained from male and female, which additionally complicates the microscopic analyses. The present investigation is in consistent with the above findings.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

LIST OF ABBREVIATIONS

HBM	:	Human Biomonitoring
MN	:	Micronucleated
BN	:	Binucleated
CC	:	Condensed Chromatin
KR	:	Karyorrhectic
P	:	Pyknotic
KL	:	Karyolytic
NB	:	Nuclear Bud
WHO	:	World Health Organization
DNA	:	Deoxy ribonucleic acid
HCl	:	Hydrochloric acid

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