

Micronucleus assay in epithelial cells from the oral cavity and urinary tract in female smokers and non-smokers

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ABSTRACT

The aim of the work was to investigate the possible use of epithelial cells from the oral cavity and urinary tract in identifying smoking-related effects in women. Epithelial cells from the oral cavity and urinary tract were collected from 9 smoking and 9 non-smoking women and subjected to micronucleus assay. The DNA damage (cells with micronuclei and nuclear buds), cytogenetic defects (binucleated cells) and cell death (condensed chromatin, karyorrhexis, pyknotic and karyolytic cells) were observed after DNA specific staining. In

pooled analysis of the frequency of binucleated cells and condensed chromatin cells in 18 studied women, statistically significant differences were noted only in epithelial cells from the oral cavity in comparison to those of the urinary tract. Non-pooled results demonstrated no differences in cytogenetic damage frequency in cells collected from the oral cavity and isolated from the urine. The lack of differences in the observed frequencies of micronuclei in buccal and urothelial cells could be an effect of the small size of the sampled group, smoking pattern of the women and the number of cigarettes smoked per day.

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. (Silins and Hogberg 2011; Wogan et al. 2004). Cigarette smoking increases the risk of all histological types of lung cancer (WHO 2007, 2011).

Tobacco smoke is a complex, dynamic and reactive mixture consisting of a gaseous phase and particulate phase. Over 70 components of tobacco smoke produce carcinogenicity in either laboratory animals and/or humans. Sixteen of these are classified by International Agency for Research on Cancer (IARC) as carcinogenic to humans (Group 1) (IARC 2004).

Due to mixed exposures and high carcinogenic potential of many compounds contained in tobacco smoke, human biomonitoring (HBM) is often used for individual risk assessment. HBM is defined as a controlled measurement of chemical or biological markers in available samples from individuals currently, previously or potentially exposed to chemical, physical or biological risk factors in the workplace and/or the environment. Biomarkers used in human health studies are generally divided into three classes: biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility (Manno et al. 2010; Silins and Hogberg 2011).

In human biomonitoring 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 non-invasive way (Fortin et al. 2010; Holland et al. 2008; Knasmueller et al. 2011).

Taking into consideration that up to 90% of all human cancers are of epithelial origin, the use of these types of cells in human monitoring might be a suitable approach (Rosin 1992). 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 (Esteban and Castano 2009). 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 metabolised and/or with their derivatives which are formed after metabolic transformation.

Nowadays exfoliated cells are increasingly used in molecular epidemiological studies for DNA damage assessment. In this biological material it is possible to detect genetic modifications using biomarkers of exposure (DNA adducts), biomarkers of effect (DNA breaks, micronuclei, chromosomal aberration) and biomarkers of susceptibility (gene polymorphism) (Borthakur et al. 2008; Ergene et al. 2007; Hsu et al. 1997; Montero et al. 2003; Rojas et al. 1996; Rupa and Eastmond 1997; Surralles et al. 1997; Thomas et al. 2009; Westphalen et al. 2008). One of the commonly used detection methods is a micronucleus assay, which provides information about DNA damage, cytogenetic defects, proliferative potential and/or cell death (Ceppi et al. 2010; Fenech et al. 2011; Heddle et al. 2011; Holland et al. 2008; Thomas et al. 2009).

The analysis of exfoliated cells, especially after DNA specific staining with Schiff's reagent, provides evidence of micronuclei and nuclear buds and also other abnormalities such as binucleated cells (presence of two nuclei within one cell), condensed chromatin and karyorrhectic cells

(chromatin aggregation leading to nuclear fragmentation), pyknotic cells (shrinkage of nucleus), and karyolytic cells (nuclear dissolution). Formation of binucleated cells is explained as disturbance of cytokinesis caused by spindle apparatus defects, while condensed chromatin, karyorrhectic, pyknotic and karyolytic cells are regarded as indicators of apoptosis and/or cell death (Thomas et al. 2009; Tolbert et al. 1992).

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 female smokers and non-smokers. The identified DNA damage included cells with micronuclei (MN) and nuclear buds (NBUD) and cytogenetic defects observed as binucleated cells (BN), as well as cell death recognised as condensed chromatin (CC), karyorrhexis (KR), pyknotic (P) and karyolytic (KL) cells.

MATERIAL AND METHODS

Studied groups

General characteristics of the studied groups (middle-aged women: nine smokers and nine 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: 23.7 years, around 12.0 cigarettes per day). The women in this group mainly smoked light and/or flavoured cigarettes with low tar content. Therefore, the exposed group was described as "light smokers" (<15 cigarettes per day) (Schane et al. 2014). All subjects were informed about the research procedure and signed the agreement for participation in the study.

Table 1. General characteristics of the studied groups of women.

Variable	Smokers	Non-smokers	Total
Number of persons	9	9	18
Age in years (mean±SD)	49.1±14.3	44.1±14.6	46.6±14.3
Body mass index (mean±SD)	22.9±2.2	23.4±3.6	23.1±2.9
Cigarettes per day (mean±SD)	12.0±7.0	NR	NR
Years of smoking (mean±SD)	23.7±13.5	NR	NR

SD – standard deviation. NR – not relevant.

Collection of biological material

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 et al. (2009) and with some modifications (Blaszczyk and Mielzynska-Svach 2012). 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 which originated from the second daily portion of urine from the mid-stream were collected into plastic containers (Fortin et al. 2010). Urothelial cells were isolated from the urine samples by centrifugation immediately after collection (10 minutes, 3 000 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, 2 000 RPM, room temperature) in fresh PBS buffer. Then cells were homogenised (2-3 minutes, 20 000 RPM) by a homogeniser 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. Afterwards, the cells were stained by Schiff's reagent for 90 minutes in the dark and at room temperature 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 x400 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 analysed in 2 000 differentiated cells, but the results were recalculated for 1 000 cells. As for epithelial cells from the urinary tract the analyses were conducted in 1 000 urothelial transitional cells. Microscopic analysis of cytogenetic damage in buccal cells was performed according to scoring criteria recommended by Thomas et al. (2009). The same criteria were used for urothelial cells. It should be emphasised that this type of staining also allows conduction of microscopic analyses with fluorescence microscope.

Statistical analysis

The results were analysed using Statistica for Windows, version 10. Normal distribution was observed 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. Buccal and urothelial cells with micronuclei

Table 2. Frequency of cytogenetic damage in buccal and urothelial cells in 18 studied women (1000 cells-slide⁻¹).

Cell type	Buccal cells (mean±SE)	Urothelial cells (mean±SE)	p-value
Micronucleated cells	0.08±0.04	0.33±0.12	p=0.091
Cells with nuclear bud	0.11±0.06	ND	NR
Binucleated cells	9.53±1.21	1.67±0.36	p=0.001
Condensed chromatin cells	23.86±4.31	9.47±2.96	p=0.012
Karyorrhectic cells	2.89±0.61	2.47±0.47	p=0.975
Pyknotic cells	6.67±0.87	8.87±1.34	p=0.125
Karyolytic cells	4.69±1.01	5.53±2.27	p=0.589

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

and nuclear bud as well as binucleated cells from the urinary tract did not have a normal distribution (Shapiro-Wilk test) even after logarithmic transformation. Other data after their logarithmic transformation achieved a normal distribution. Non-parametric Wilcoxon test was used to detect differences between frequency of cytogenetic damage in buccal and urothelial cells in the 18 studied women. Studied variables with normal distribution concerning 9 smokers and 9 non-smokers were analysed by Student t-test. For variables with non-normal distribution Mann-Whitney test was used.

RESULTS

Micronucleus assay conducted on buccal cells and urothelial cells detected biomarkers of early biological effects. Cytogenetic damages such as micronucleated cells (MN), binucleated cells (BN), condensed chromatin cells (CC),

karyorrhectic cells (KR), pyknotic cells (P) and karyolytic cells (KL) were observed both in buccal and urothelial cells. Cells with nuclear bud (NBUD) were detected only in epithelial cells from the oral cavity (Table 2).

Table 2 shows that frequency of micronucleated cells was higher in urothelial cells as compared with buccal cells. The cells with nuclear bud were found only in material from 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 six-times higher and the level of condensed chromatin cells was two and a half times higher in the oral cavity material in comparison to epithelial cells from the urinary tract. The frequency of karyorrhectic, pyknotic and karyolytic cells from both sources was similar.

Analysis of the results obtained for smokers and non-smokers demonstrated no statistical differences in epithelial cells collected from the oral cavity and isolated from the urine (Table 3).

Table 3. Frequency of cytogenetic damage in buccal and urothelial cells (1000 cells·slide⁻¹) in 9 female smokers and 9 non-smokers.

Cell type	Buccal cells			Urothelial cells		
	Smokers (mean±SE)	Non-smokers (mean±SE)	p-value	Smokers (mean±SE)	Non-smokers (mean±SE)	p-value
Micronucleated cells	0.05±0.05	0.11±0.07	p=0.730 ^a	0.33±0.21	0.33±0.17	p=1.000 ^a
Cells with nuclear bud	0.17±0.12	0.05±0.05	p=0.666 ^a	ND	ND	NR
Binucleated cells	7.89±1.90	11.17±1.38	p=0.182	1.67±0.56	1.67±0.50	p=0.864 ^a
Condensed chromatin cells	24.44±6.93	23.28±5.53	p=0.897	7.17±1.66	11.0±4.86	p=0.785
Karyorrhectic cells	2.83±1.10	2.94±0.60	p=0.721	2.83±0.31	2.22±0.76	p=0.541
Pyknotic cells	5.44±0.72	7.89±1.54	p=0.231	10.00±2.42	8.11±1.63	p=0.512
Karyolytic cells	2.89±0.80	6.50±1.70	p=0.167	2.33±0.84	7.67±3.65	p=0.703

SE – standard error. ^a Mann-Whitney test. ND – not detected. NR – not relevant.

DISCUSSION

In our study the average frequency of micronuclei in buccal cells was $0.08 \pm 0.04 \cdot 1000^{-1}$ cells (Table 2). According to Holland et al. (2008) 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 reported by Bolognesi et al. (2013). In the case of urothelial cells, the average frequency of micronuclei in the studied group of women was $0.33 \pm 0.12 \cdot 1000^{-1}$ cells (Table 2). The obtained frequencies of micronuclei in urothelial cells were in the range for women (0.09-10.46 MN·1000⁻¹) documented by Fortin et al. (2010).

Our data showed that differences between frequency of micronuclei and nuclear buds in buccal cells of female

smokers and non-smokers were not statistically significant (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. According to Haveric et al. (2010) no essential differences in the frequency of micronuclei in buccal cells of young female smokers from Bosnia and Herzegovina compared with the control group of non-smokers were observed. In the same materials, differences in frequencies of micronuclei between male smokers and non-smokers occupationally exposed to polycyclic aromatic hydrocarbons were not observed (Karahalil et al. 1999). In the study concerning carpet fabric workers, in the control group no significant effects of tobacco smoking on micronuclei and nuclear buds level in exfoliated

epithelial cells from the oral cavity in men were recorded (Diler and Celik 2011). 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 (Gabriel et al. 2006). Celik et al. (2003) study of petrol station attendants indicated higher frequency of micronuclei in male smokers in comparison to non-smokers. 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 (Celik et al. 2010; Ergene et al. 2007). A higher frequency of micronuclei was recorded in the group of male smokers from India aged 41 and above, smoking for more than 20 years (Chandirasekar et al. 2011). Nersesyan et al. (2011) 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.

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 women (Table 3). The lack of statistically significant differences in the frequency of the BN, KR and KL in oral cells of smokers and non-smokers was also reported by Celik et al. (2003) and Diler and Celik (2011). Other research has observed a remarkably higher frequency of BN, KR and KL in buccal cells collected from smokers than non-smokers (Celik et al. 2010; Ergene et al. 2007). 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 frequency of pyknotic cells was similar in smokers and non-smokers in the study of Nersesyan et al. (2011).

In our experiment no significant differences in frequencies of cytogenetic damage in urothelial cells of female smokers and non-smokers were observed (Table 3). In other studies a statistically higher frequency of micronuclei in urothelial cells was detected in male smokers in comparison to non-smokers (Burgaz et al. 1995; Zamani et al. 2011). No papers describing the analysis of the other cytogenetic damage (BN, CC, KR, P, KL) than micronuclei in urothelial cells have been found. Therefore, comparison of our results with other work is currently not possible.

Most studies applying micronucleus assay use buccal cells 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. 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.

In our study the lack of differences in the obtained 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 women

and the number of cigarettes smoked per day. The examined group consisted of 18 women, nine 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. (2011) 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.

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