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RESEARCH ARTICLE

Cytological multimarker screening using BMCyt test in waterpipe smokers: an integrative study of cell damage, toxicological and cancer risk

MARUHEN A. D. SILVEIRA*, ALAN S. ANTONELLI, BRUNO O. FIORELLI and LUCIANA P. G. D'ARCE

Centre of Biological Sciences and Health, College of Biological Sciences, State University of West Paraná (UNIOESTE), Universitária Street, 2069, Cascavel, Paraná 85819-110, Brazil
*For correspondence. E-mail: maruhensilveira@gmail.com.

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Abstract. Waterpipe smoking is an ancient method of tobacco smoking practiced worldwide. There is a common belief that waterpipe smoking is a safer alternative to cigarette, but many studies showed that some toxicants were associated with cancer risk, significantly higher in waterpipe smoking. Thus, this study aimed to evaluate the status of waterpipe smoker's buccal cells and its cancer risk using the buccal micronucleus cytome test. Forty waterpipe smokers (noncigarette smokers) were recruited and paired by gender, age and alcoholic habits with 40 control subjects. One-thousand cells from each individual were analysed and the number of pyknotic cells (PYC), karyolitic cells (KYL), karyorrhetic cells (KHC), condensed chromatin (CC), binucleated cells (BN), basal cells (BC), nuclear buds (NBUD) and differentiated cells (DIFF) were counted. Additionally, 2000 differentiated cells were analysed counting micronucleated cells (MNi) and nuclear buds. We observed an increasing $P < 0.05$ in all waterpipe smoker's cell parameters, except DIFF (fold-decrease). Only CC showed no differences between groups. The interference in the cell cycle plus DNA damage observed in this study could be responsible for the high number of damaged cells and in death process, showing the importance of our study and the high risk in waterpipe smoking.

Keywords. cytogenetic biomarkers; cancer risk; DNA damage; micronuclei; waterpipe smokers.

Introduction

Waterpipe smoking (*shisha*, *hookah* or *narghile*) is a Middle East ancient tobacco smoking methods that became a global trend in the 1990s, mainly as a local phenomenon among youth (Maziak 2011). It is the second smoking tobacco method worldwide (Akl *et al.* 2011; American Lung Association (ALA) SCP Hookah smoking: a growing threat to public health 2012 (http://www.lung.org/assets/documents/tobacco/hookah-policy-brief-updated.pdf); Aslam *et al.* 2014; Maziak *et al.* 2015). Precisely, trends from the global youth tobacco survey (GYTS; 1999–2008) involving more than half a million participants worldwide showed that while cigarette smoking is either stable or declining, other forms of tobacco smoking, such as waterpipe smoking, showed a current increasing (Warren *et al.* 2009).

This method involves placing the tobacco in a bowl surrounded by burning charcoal. Water is used in a boil and the process of passing the smoke through water leads to a common belief that water acts as a filter, and therefore, waterpipe is a safer smoking alternative (Smith-Simone *et al.* 2008; Cobb *et al.* 2010). However, the smoke still includes many volatilized and pyrolysed tobacco products, carbon monoxide and charcoal components with high toxic effects (Al Rashidi *et al.* 2008; Monzer *et al.* 2008; Eissenberg and Shihadeh 2009; Sepetdjian *et al.* 2010; Schivo *et al.* 2014). Al Rashidi *et al.* (2008) reported presence of high toxic substances such as formaldehyde, acetaldehyde and acrolein in the waterpipe smoking. This is a disturbing data, once the levels of these toxicants were significantly higher than in cigarette smoke and all were classified by IARC as group 1 or group 2, regarding cancer risk. Further, Akl *et al.* (2010) and Koul *et al.* (2011) showed association between waterpipe smoking and human cancer, mainly lung cancer.

The buccal micronucleus cytome (BMCyt) assay was largely used in occupational, environmental, lifestyle and

Maruhen A. D. Silveira, Alan S. Antonelli and Bruno O. Fiorelli contributed equally to this work.

nutritional studies (Holland *et al.* 2011; Khan *et al.* 2015). This assay is useful for assessing cancer risk from exposure to genotoxic carcinogens (Bonassi *et al.* 2011), once the parameters evaluated are biomarkers of DNA damage, cytokinetic defects, proliferative potential and cell death; and so, important parameters to characterize the status of the squamous epithelium and predisposition to cancer (Thomas *et al.* 2009). It is a cost-effective and minimally invasive test to evaluate genomic damage, cell death and cytostatic effects (Thomas *et al.* 2009; Bonassi *et al.* 2011) and was chosen due to the fact that it is a multimarker screening of many cytological abnormalities that predicts cancer risk.

Given the current lack of studies evaluating the cell effects caused by waterpipe smoking, this study aimed to evaluate the status of waterpipe smokers that are not cigarette or ex-smokers and its relation with cancer predisposition.

Materials and methods

Study population

All subjects signed the informed consent approved by the Ethics Committee (CAAE: 30857614.5.00000.0107) and completed a questionnaire. Waterpipe smoker's group consisted of 40 waterpipe smokers paired by gender, age and alcoholic habits with 40 nonsmoker's controls.

Questionnaire

Subjects filled out a questionnaire containing identification, family history, smoking habits, alcohol consumption, medical history and genetics history before collection. Two groups were made in which only individuals without familial history of cancer or any other chronic disease were carefully included. Waterpipe smokers (with no association with other kinds of tobacco smoke) group included waterpipe smokers that smoke at least 1 h per day for 1–2 days per week for at least one year. All cigarette smokers and ex-smokers were excluded from the analyses. Cigarette smokers were defined as those who had smoked at least 100 cigarettes during their lifetime, or were at the time of recruitment, smoking occasionally or every day; ex-smokers were those who had stopped smoking for at least one year prior to collection (CDC 2008). To evaluate alcohol consumption, subjects were classified into three categories: no consumer, defined as any alcohol consumption or social consumption; moderate drinking, defined as consuming up to one cup (about 100 mL) of alcohol per day, or more than a glass on weekends; high consumption, defined as the consumption of more than 1 L of light alcoholic beverage (beer, wine, or cider), or two glasses of spirit (rum, vodka, or whiskey) per day, for at least six years (Gontijo *et al.* 2002). High consumers of alcohol were excluded from the analyses. Table 1 shows the demographic data of the studied population.

BMCyt

Collection and retention of the oral mucosa were performed according to Thomas *et al.* (2009), with slight modifications. Briefly, cells of the buccal mucosa were collected by scraping the inner cheek with a sterile swab and placed in a Falcon tube containing 3 mL of saline solution. Samples were centrifuged at 1000 rpm for 5 min at 25°C and fixed using glacial acetic acid and methanol (1:3, v/v) solution, before another centrifugation at 1000 rpm for 5 min at 25°C. Then, the same fixing solution was added and the falcon tube were stored under refrigeration for 24 h. For staining, suspended cells in the fixing solution were dropped onto clean and cool slides, which were allowed to dry at room temperature for 24 h. HCl (5 N) was added to the slides for 30 min and distilled water was used to wash the slides. After air drying, slides were stained with Schiff's reagent for 90 min, and counter-stained with Fast Green 0.5% for 3 min. One thousand cells from each individual were analysed using an optical microscope; the number of pyknotic cells (PYC), karyolitic cells (KYL), karyorrhetic cells (KHC), condensed chromatin (CC), binucleated cells (BN), basal cells (BC) and differentiated cells (DIFF) were first evaluated. There after 2000 more differentiated cells, in two slides, were analysed for counting micronucleated cells (MNi) and nuclear buds (NBUD).

Statistical analysis

T-test was used to evaluate the means of PYC, CC, KYL, KHC, MNi and BC. Wilcoxon test was used to evaluate

Table 1. Demographic characteristics of the studied population.

		Gender		Age (year)	Alcohol consumption			
					NC		MC	
	N	M	F		M	F	M	F
Waterpipe smokers	40	20	20	22.55 ± 3.02	2	5	18	15
Control	40	20	20	20 ± 3.15	2	4	18	16

M, male; F, female; NC, no consumer; MC, moderate consumer.

Table 2. Mean and standard error of the results of collections per 1000 cells/** per 2000 differentiated cells.

Cell types	Waterpipe smokers	Control	*P* value
Basal	141.387 ± 9.804*	101.188 ± 9.385	< 0.001
Condensed chromatin	52.350 ± 7.623	51.413 ± 6.889	< 0.560
Karyolytic	105.463 ± 11.049*	81.225 ± 6.649	< 0.001
Karyorrhetic	41.175 ± 10.609*	25.925 ± 4.855	< 0.001
Micronucleated**	9.075 ± 1.587*	1.638 ± 0.531	< 0.001
Pyknotic	7.225 ± 2.066*	3.600 ± 1.798	< 0.001
Differentiated	1634.925 ± 15.397*	1731.600 ± 12.585	< 0.001
Binucleated	7.987 ± 1.923*	3.675 ± 1.016	< 0.001
Nuclear Buds**	0.438 ± 0.426*	0.113 ± 0.240	< 0.001

*$P < 0.05$.

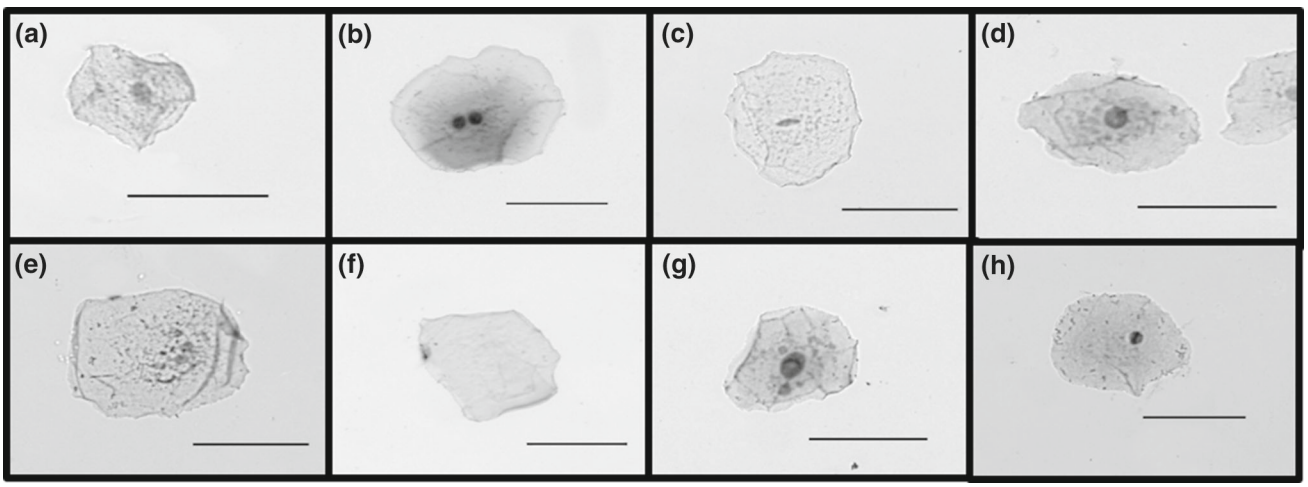

Figure 1. Main cell types observed on analyses. (a) Basal cell. (b) Binucleated cell. (c) Condensed chromatin cell. (d) Differentiated cell. (e) Karyorrhetic cell. (f) Karyolitic cell. (g) Cell with nuclear bud. (h) Pyknotic cell.

the mean of BN, DIFF and NBUD. All the analyses considered $P < 0.05$. SigmaPlot for Windows ver. 11.0 (Systat Software, Chicago, USA) was used to perform the statistical analyses.

Analyses considered gender and alcohol consumption as confounding variables. Paired *t*-test was used, considering a 5% of significance level.

Results

Table 1 shows the demographic characteristics of the studied population. There were no statistically differences in the results including age, gender and alcohol consumption in the analyses (data not shown). We observed an increase in all the cell parameters in the waterpipe smoker's group, except DIFF and CC. A fold-increase (1.39 in basal cells; 1.29 in KYL; 1.58 in KHC; 5.54 in MNi; 2.00 in PYK; 2.17 in BN and 3.87 in NBUD) were observed in this group ($P < 0.05$). Moreover, DIFF decreased (0.94 fold-decrease; $P < 0.05$) in the waterpiper smokers group and CC showed no differences between groups (figure 2). Figure 1 shows some abnormalities observed in our study. The mean and standard error of all parameters in both groups (waterpipe smokers and paired controls) are provided in table 2.

Discussion

Our work is the first to perform a multimarker screening of cytological parameters in waterpipe smokers that are not associated with other kinds of tobacco smoke. Moreover, the BMCyt showed to be an excellent tool, cheaper and minimally invasive, and were observed reliable differences in almost all parameters evaluated (BC, KYR, KHC, MNi, PYK, DIFF, NBUD and BN cells). The use of a multimarker approach combining the analysis of buccal cell types and nuclear abnormalities reflects potential alterations in cellular kinetics, metabolism, the structural buccal mucosa profile and genomic stability events (Bolognesi *et al.* 2013).

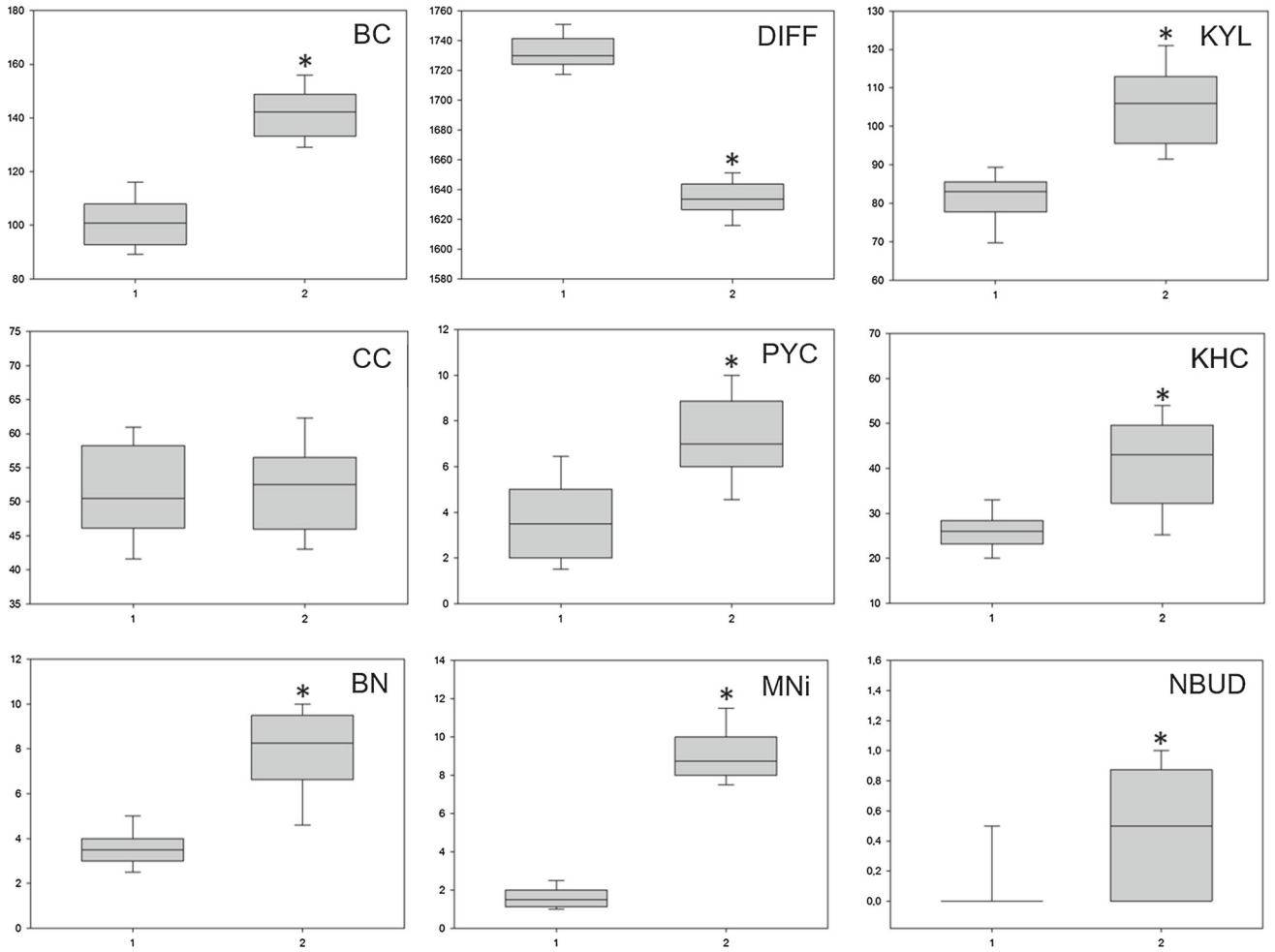

Figure 2. Mean and standard error of the results of collections per 1000 cells and per 2000 differentiated cells. 1, waterpipe smokers group; 2, control group; $^{*}P < 0.05$; BC, basal cells; DIFF, differentiated cells; KYL, karyolytic; CC, condensed chromatin; PYC, pyknotic; KHC, karyorrhetic; BN, binucleated cells; MNi, micronucleated cells; NBUD, nuclear buds.

First of all, BC and DIFF reflect the proliferative activity in buccal cells. Increase in BC was the first evidence of cell cycle abnormalities on the waterpipe smoker's group. PYK, KYR and KHC are indicative of cell death (necrosis and apoptosis) (Thomas *et al.* 2009) and all three parameters were increased in waterpipe smokers group. The induction of apoptosis could be related to significant DNA damage (Fenech 2006) and may occur by specific endogenous and exogenous stimuli under normal physiological conditions or by genotoxic agent exposure. Derka *et al.* (2006) observed in animal models that cellular proliferation and apoptosis rates are higher in early stages of tumourigenesis. Additionally, BN is indicative of cytokinesis failure and susceptibility to aneuploidy (Bolognesi *et al.* 2013). It is known that the presence of micronuclei (MNi), NBUD and other nuclear abnormalities in oral cavity cells may be associated with exposure to genotoxic and mutagenic agents (Holland *et al.* 2008). Nevertheless, these increase in DNA damage and cell death parameters indicates genome instability, cytokinesis defects and early stages of tumourigenesis in the waterpipe smoker's group. In a similar study, El-Setouhy *et al.* (2008) showed high micronuclei frequencies in oral cells of waterpipe smokers, but with no analyses of other cytological parameters.

All these data are very disturbing once Montazeri *et al.* (2017) published an excellent review and meta-analysis associating waterpipe smoking and cancer; they described more than 50 papers that reported association between waterpipe smoking and human cancer, especially lung and oesophageal cancer. Further, Jacob *et al.* (2011) enrolled 16 waterpipe smokers and measured several markers of tobacco smoke exposure in blood and urine before and after one waterpipe smoking session. Metabolic biomarkers as tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and polycyclic aromatic hydrocarbon (PAH) were increased in urinary excretion after session.

A systematic review of health effects of waterpipe smoking studies showed that waterpipe smoking doubles the risk of lung cancer (Akl *et al.* 2010). Koul *et al.* (2011) showed that waterpipe smoking is associated with a

six-fold increase in lung cancer risk compared to no smoking. However, this study did not control any other exposures (second-hand smoke, occupational exposures), or even socio-economic factors. In our work, no confounding variable had statistically effects on data. Recently, Walters *et al.* (2017) showed positive correlation between waterpipe smokers and epigenetic changes in the small airway epithelium, demonstrating disturbances in molecular and epigenetic levels in waterpipe smokers too.

In general, the cell cycle deregulation plus the DNA damage observed in our study indicates that the cellular homeostasis was committed. Further, disturbing data based on: high levels of oral and oropharyngeal cancers in some countries of the Middle East compared to other parts of the world; extended exposure to the carcinogenic smoke content; oral complications of waterpipe smoking; indicative of early carcinogenic processes and association with cancer (El-Setouhy *et al.* 2008; Dar-Odeh and Abu-Hammad 2009; Rastam *et al.* 2010) show us the importance of our study and the emerging risk that all waterpipe smokers are implied. Thus, the market and use should be reviewed, especially for uninformed youth.

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