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Genotoxic Effects of Waterpipe Smoking on the Buccal Mucosa Cells

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Abstract

Waterpipe smoking is popular in many parts of the world. Micronuclei (MN) evaluation in the exfoliated oral cells of smokers is a non-invasive technique for evaluation of possible tobacco harm. We aimed to assess whether MN levels are higher in waterpipe smokers than in never smokers. We examined oral smears of 128 adult male waterpipe smokers and 78 males who never smoked tobacco in rural Egypt. The total number of MN per 1000 cells per subject, and the number of MN-containing cells per individual were compared. We observed a higher level of total MN in waterpipe smokers (10 ± 4) than in never smokers (4 ± 2 , $p < 0.001$). A similar difference was found for the mean number of affected cells per individual (8 ± 3 vs. 4 ± 1.62 , $p < 0.001$). MN

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levels were not significantly dose related. This study is among the first to assess the association between waterpipe smoking and a cytogenetic measure of tobacco harm. The two-fold increase in MN level is consistent with previous reports of MN in cigarette smokers. More research is needed to determine if such MN levels are predictive of future health consequences.

Keywords

Waterpipe smoking; tobacco; buccal cells; micronuclei; cytogenetics; tobacco harm

Introduction

Waterpipe tobacco smoking is widely practiced in the Middle East, parts of Asia, and more recently in Europe and North America. Biomarker studies may be useful to characterize the exposure levels and possible health risks resulting from waterpipe smoking. One of the most rapid and efficient techniques to study the impact of such environmental factors on genetic stability in human cells is the micronucleus (MN) test¹. Investigations of MN frequencies support the widely accepted assumption that the MN are products of early events in human carcinogenic processes especially in the oral cavity, which is directly exposed to cigarette and waterpipe smoke^{2,3,4}. Epidemiological studies have demonstrated an increase in MN among smokers with oral carcinoma^{5,6}. The MN test has therefore been used for early identification of the carcinogenic process^{3,7,8,9}.

MN in exfoliated human oral cells is a small intranuclear DNA structure separated from the main nucleus of the basal epithelial layers¹⁰. The daughter cells containing the micronuclei migrate up through the epithelium and are exfoliated into the oral cavity¹¹. The average reported healthy population MN frequency is 1–3 per 1000 cells¹². Increases in the MN frequency in exfoliated oral cells have been observed as a result of exposure to pesticides, neoplastic drugs^{13,14}, radiotherapy^{15,16}, coke oven emissions^{17,18}, cigarette smoking^{13,19,20,21}, arsenic in drinking water²², chronic infection²³, and different occupational exposures^{8,9,24}.

This study aimed to assess whether waterpipe smoking increases MN levels in their exfoliated oral cells relative to MN levels in the oral cells of persons who never smoked tobacco products. Secondly, we aimed to determine the extent to which this cytogenetic damage is influenced by smoking behaviors (intensity, duration, symptoms of nicotine addiction, and inhalation of waterpipe smoke).

Methods

Participants

The Egyptian Smoking Prevention Research Institute (ESPRI) previously conducted a baseline household smoking prevalence survey²⁵. A random sample of 2358 households was selected from 9 villages in Qalyubia governorate in the Nile delta region in Egypt. A total of 10,161 subjects, participated in the study. Subjects for the present study were recruited from these participants if they met the following eligibility criteria: adult (18 years of age and above), males (note that only 1.2% of rural women smoke), either current waterpipe smokers (defined as those who smoked waterpipe at least once per week and smoked less than 100 cigarettes in their life time) or never smokers (defined as those who never tried waterpipe or cigarettes in their life time). We recruited 128 current waterpipe smokers and 78 never smokers in the year 2004–2005. After providing their written informed consent to participate in this study, all individuals were interviewed using an administered questionnaire to collect demographic data (age, marital status, education and occupation),

smoking behavior (frequency of waterpipe smoking, numbers of hagers [waterpipe tobacco units] smoked daily and weekly, and symptoms of nicotine dependence), quitting behavior, the degree of inhalation of tobacco smoke, current exposure to occupational chemicals and pesticides, and body weight and height.

Sample collection, staining and evaluation

Exfoliated cells of the buccal mucosa were obtained by scraping the buccal mucosa with a wooden spatula^{1,26,27}. For each individual, two slides were prepared by smearing the cells immediately onto the center of clean glass slides. A modified Papanicolaou method was used for staining²⁶. For this method the smears fixed in 95% ethyl alcohol, and then we performed hydration by running a tap water wash. We applied nuclear stain (DNA specific) Meyer's hematoxylin for 4 minutes, followed by a rinse in stream of gently running water 15 minutes. Dehydration by 70% and 95% ethyl alcohol was then performed for ten dips each. Cytoplasmic stain (orange green) was applied for 1 minute, followed by a rinse in 95% ethyl alcohol for 5 minutes. Cytoplasm and nucleolar stain (RNA specific) by EA polychrome was applied for 1.5 minutes, followed by a rinse by 95% ethyl alcohol (X2), ten dips each. The next step was dehydration by absolute ethyl alcohol (X2) for ten dips, and then clearing by absolute ethyl alcohol and xylene (1:1) for 1 minute. The preparation was mounted by using a standard mounting medium.

Screening for MN was performed under an oil immersion lens (X1000), followed by phase contrast microscopy for confirmation of MN according to established methods^{19,26,28,29}. At least 1000 intact epithelial cells per individual were scored to achieve the average percent micronucleated cells. The opaque extranuclear-intracytoplasmic bodies seen under oil immersion lens and phase contrast were considered micronuclei, whereas binucleated cells, fragmented nuclei (karyorrhexis), karyolysis and nuclei-like broken eggs were not counted as MN (Figures 1, 2, and 3). Total micronuclei (TMN) was defined as the total number of MN per 1000 cells per subject. Cells with micronuclei (CMN) was defined as the number of cells containing MN per 1000 cells per subject (note that some cells may have multiple MN^{8,26,27}.)

Statistical analysis

Descriptive data analysis was conducted to examine the distribution of TMN and CMN. Body mass index (BMI) was calculated as $\text{weight (kg)}/[\text{height (m)}]^2$, and was dichotomized as underweight or normal weight vs. overweight or obese³⁰ (CDC, 2007). The Mann-Whitney U and Kruskal-Wallis tests were used initially to assess differences in TMN and CMN levels by smoking status. The Shapiro-Wilk normality test was applied to TMN and CMN, which were subsequently log-transformed for regression analysis. Linear regression was used to assess the impact of waterpipe smoking on the dependent variable TMN controlling for potential confounders (age, education, BMI, occupational exposures). All statistical tests were two sided, using $\alpha=0.05$. Statistical analyses were performed using SPSS (release 13.0) for Windows.

Results

The mean TMN and CMN were significantly higher (more than 2 folds) among waterpipe smokers as compared to never smokers: mean TMN 10.2 ± 3.9 vs. 4.1 ± 1.9 , $p < 0.001$; mean CMN 8.0 ± 3.2 vs. 3.7 ± 1.6 , $p < 0.001$ (Figures 4 and 5). In the never smokers, the range for TMN was 1 to 9, whereas for waterpipe smokers the TMN ranged from 2 to 26.

Waterpipe smokers were significantly younger in age than never smokers (mean age (SD) 47.2 (14) vs. 53.4 (11), $p < 0.001$), were more likely to report exposure to agricultural

pesticides (52.7% vs. 31.2%, $p<0.05$), and had a lower BMI (mean (SD) 25.8 (4.2) vs. 28 (5.1), $p<0.001$). However, as shown in table 1, neither these nor the other questionnaire variables had a statistically significant effect on TMN levels in either smokers or non-smokers.

Table 2 shows the impact of waterpipe smoking behaviors on the levels of TMN; the results for CMN were almost identical and therefore they are not shown separately. There was no statistically significant increase in TMN among those who smoked at least 5 hags per day compared to less than 5 hags, nor for weekly use of more than 28 hags compared to less than 28 per week. TMN levels were not significantly associated with typical addiction indicators, including the duration of smoking, minutes to the first use of waterpipe in the day, smoking even when ill, or the tendency to inhale tobacco smoke into the chest.

Linear regression analysis of the log-transformed TMN levels showed that waterpipe smoking (regression coefficient 1.18 and 95% CI 1.01– 1.34) independently increased the level of TMN, even after controlling for age, education, BMI, and occupational exposures (model $R^2=0.54$; $P<0.001$).

Discussion

Our study demonstrated more than a two fold increase of MN frequency in waterpipe smokers compared to never smokers. Although we are not aware of a comparable study in the waterpipe literature, these results were in concordance with those that compared cigarette smokers to non-smokers^{21,31,32,33}. Sarto and his colleagues, for example, detected a 2-fold increase in the number of MN in smokers as compared with non-smokers¹³. The higher MN frequency in cigarette smokers has been attributed to benzo(a)pyrene, a component of tobacco smoke that consistently induced MN in a linear fashion, using an in vitro system^{34,35}.

Some researchers did not detect any increase in the TMN frequency of lymphocytes in cigarette smokers^{27,36,37}. A pooled analysis of 24 data sets from the Human Micronucleus Project confirmed that smokers do not have an overall increase in MN frequency in their lymphocytes, although when the interaction with occupational exposure is taken into account, heavy smokers were the only group showing a significant increase in genotoxic damage⁷. These studies evaluated the MN frequency in the lymphocytes, in contrast to our study of the oral epithelial tissues which are in immediate contact with tobacco, and hence might be a better surrogate tissue than lymphocytes for assessing genotoxic damage.

It was unexpected that, among waterpipe smokers, we did not detect associations of TMN or CMN with well known indicators of nicotine addiction, such as the lifetime duration of smoking, time to first waterpipe smoke of the day, and number of hags per day or per week. It should be noted that these types of variables are commonly used in studies of cigarette smokers, whereas waterpipe smoking may represent a completely different paradigm requiring the development of new variables and new tools to assess possible addiction. Similarly, whereas for cigarettes the dose is conveniently estimated from asking smokers about the number of cigarettes smoked per day, no such validated dose estimators exist for waterpipe smoking. It is not clear, for example, whether the number of hags per day adequately captures dose information, nor is it clear how hags and cigarettes can be directly compared for dose-response studies. Several researchers are beginning to address these concerns with biological markers of exposure, indicating that, despite differences in exposure and behavior, the biological effects may be similar^{38, 39}.

Several methodological considerations should be noted when comparing our results to previous work by others. It is difficult to compare results between studies due to such

methodological differences as staining techniques, microscopic magnification level, the number of cells counted, and techniques for collecting cells^{10, 14, 26}. Populations living in urbanized areas affected by air pollution may have different background rates of MN than other populations. It is also important to note that in our study, despite the fact that some subjects reported being exposed to pesticides, only a few of them were working regularly in agriculture, and this may have diluted the potential effects of pesticides on MN levels. For example, 11 of 128 never smokers reported agriculture as their occupation, while 24 total never smokers reported pesticide exposures. The larger number of exposed persons was likely due to reports of those who help their families seasonally on farms, and who would therefore be expected to have lower chronic exposure levels than farmers.

In summary, waterpipe smoking increased the frequency of MN due to possible genotoxic action of substances present in the waterpipe tobacco smoke. Further research is needed to quantify the amount of tobacco exposure resulting from waterpipe smoking, to enable the further evaluation of cellular and tissue level biological damage.

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Figure 1.
Buccal smear shows micronuclei Pap stained. X1000.

Figure 2.
Buccal smear shows karyorrhexis (fragmented nucleus). Pap stained. X1000.

Figure 3.
Buccal smear shows micronuclei (opaque with phase contrast). Pap stained. X1000.

Figure 4.
The mean number of TMN and CMN in never smokers and waterpipe smokers.

Figure 5.
The mean number of TMN in never smokers and waterpipe smokers

Table 1

The average TMN in never smokers and waterpipe smokers, by demographic and occupational variables.

Characteristics	Total number of micronuclei/1000 cells/subject					
	Never smokers N=128			Current waterpipe smokers N=78		
	N	Mean	SD	N	Mean	SD
Age						
45 years	22	4.7	1.9	68	10.9	4.0
>45yrs	56	3.9	1.7	60	9.6	3.7
						.09
Educational level						
no formal education	47	4.1	1.8	79	9.7	3.7
formal education	26	4.3	1.8	40	11.1	3.5
						.06
Occupation						
not working	18	4.0	1.7	12	8.9	2.8
high administrative and technical	20	4.9	1.8	13	11.7	3.3
services and trade	20	4.4	1.6	38	10.6	4.0
agriculture	11	3.2	2.4	50	10.0	4.2
chemical and dusty jobs	7	4.6	1.3	15	10.3	3.6
						.27**
Exposure to pesticides						
no	53	4.3	1.9	60	10.6	4.0
yes	24	3.8	1.8	67	10.0	3.8
						.45
BMI (kg/m ²)						
<18.5-24.9	23	3.9	2.1	61	10.9	4.4
25 and above	55	4.3	1.7	66	9.9	3.1
						.27

* Mann-Whitney test

** Kruskal-Wallis test

Table 2

Mean TMN and smoking behavior in waterpipe smokers (n=128).

Characteristics	N	Mean	(SD)	* p-value
Number of hagers /day				
4 hagers	66	10.5	3.9	
> 4 hagers	62	10.1	3.9	.31
Number of hagers/week				
28 hagers	67	10.2	3.9	
>28 hagers	59	10.3	3.7	.71
Age of smoking initiation				
20years	79	10.3	4.0	
>20years	49	10.3	3.7	.63
Duration of smoking				
14years	70	10.6	3.7	
>14years	58	9.9	4.1	.30
Minutes to first waterpipe of the day				
1 hour	57	10.2	3.7	
> 1 hour	68	10.2	3.9	.71
Smoking when ill				
no	111	10.0	3.6	
yes	16	12.6	4.9	.06
Inhalation of tobacco smoke				
no	104	10.4	3.9	
yes	24	9.7	3.7	.27

* Mann-Whitney Test