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Original Article

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Micronucleus Investigation in Buccal Mucosal Cells of Young Waterpipe Tobacco Smokers in Tehran

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Abstract

Waterpipe Tobacco Smoke(WTS) is an unhealthy lifestyle that may increase the risk of genotoxic responses and chronic diseases such as cancer. Micronucleus test is a successful and reliable method which is used for screening of genotoxic responses of the whole body and also for screening those people who had already exposed to genotoxic compounds. In this study, specific questionnaires were designed and used for studying the role of shisha smoking on the extent of genotoxic responses and cases were looked for MNs with this biomonitoring method. The study population was 20 young adults (12men and 8 women) who born and lived in Tehran and had continuously smoked shisha more than 2 times weekly for more than 2 years. The associations between all recorded background, environmental and nutritional factors and increased incidence of Micronucleus in buccal cells of all cases were considered by statistical methods. In order to count Micronucleus levels, buccal cells were collected from buccal mucosa of these people with a small-headed toothbrush and was placed the head of toothbrush into buccal cell buffer, slides were prepared and cells were stained with Schiff's reagent and light green.Finally, 1000 differentiated cells were recorded by an optical microscope in each slide and the mean level of MN was determined for each volunteer. All steps were performed according to the buccal micronucleus cytome (BMCyt) assay protocol. Increased incidence of Micronucleus was associated with the extent of shisha smoking per week (p=0.021), alcohol consumption (p=0.021) and BMI (p=0.027). The other effective factor in the occurrence of Micronucleus was gender/sex (p=0.011) but nutritional factors didn't change the level of Micronucleus in our cases. The relationship between other background and environmental factors were not significant too. It seems that long-term consumption of shisha in both genders could increase the risk of genetic toxicity and occurrence of malignancies in human target cells.

Keywords: Buccal mucosa, shisha smoking, micronucleus

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Introduction

Widespread tobacco consumption has become as one of the biggest threats to public health worldwide (Mohamed M, et al, 2006). Tobacco use causes more than 5 million deaths per year globally, and current trends have indicated that tobacco use will cause more than 8 million deaths annually by 2030 (American Lung Association, 2007). Nowadays Waterpipe Tobacco Smoke (WTS) is becoming an increasingly popular method of tobacco use, unfortunately (Maziak W, et al , 2004) and according to the latest information published by the Ministry of Health and Medical Education of Iran, waterpipe use among young Iranian people has become a matter of concern.They announce that 15 percent of the adolescent aged between 13 and 15 are using a water pipe, in the meantime, a total number of 35 hundred tons of tobacco are consumed per year in this regard (Shihadeh A, et al, 2004). As a dramatic fact, recreational use of waterpipe has been reported widespread among university students in Iran by three different studies in 2011 (Shihadeh A, et al, 2004; Rice VH, et al, 2016; Jackson D, Aveyard P, 2008) and another study in 2013 published that 45.1% of adolescents have lifetime uses of waterpipe and 34.2% had ever shared a waterpipe with others (Carroll T, Poder N, Perusc A, 2008) .

Researchers have showed as with cigarette smoke that waterpipe aerosol includes numerous harmful mutagenic and carcinogenic constituents which are chemically synthesized during smoking (e.g., carbon monoxide, CO) as well as constituents which are both transferred and synthesized in situ (eg,polyaromatic hydrocarbons) and different raw material e.g., glycerol, nicotine, tobacco-specific nitrosamines (TSNAs) which could evaporate from the tobacco leaf (Dornelas E, 2012).

Based on a recent meta-analysis on 28 high-quality articles, water pipe could increase the risk of head and neck, esophageal and lung cancers and possibly the risk of stomach and bladder cancer but based mainly on poor-quality studies but larger carefully designed studies in well-defined populations are still requested (Jackson D, Aveyard P, 2008).Due to the increasing rate of shisha tobacco smoking consumption among people in Iran and distribution of tobacco products which contains the flavoring agent and not well standardized, this study tries to examine the harmful effects of shisha tobacco smoking on human health. This study intends to monitor chromosomal aberrations induced by the continuous use of shisha tobacco smoking in the community through a cross-sectional study. In this research, to investigate the relationship between shisha tobacco smoking and an increased risk of genetic damage, the micronucleus test was used.

Materials and methods

Subjects

The study was carried out on 20 shisha users with similar consumption patterns. Shisha smoking more than 2 times per week was considered as our criteria sample selection. They were asked to complete a questionnaire to obtain necessary information on their environmental and nutritional factors and also personal factors (age, BMI, alcohol addiction, smoking habits, and health, etc.).

Buccal cell sampling, preparation, and staining

All of the procedures were performed in accordance with the buccal micronucleus cytome (BMCyt) assay protocol (Thomas P. et al, 2009).

Buccal cell originates from a multilayered epithelium that lines the oral cavity. Before buccal cell collection, the mouth of the subject rinsed twice thoroughly with 100 ml of water to remove excess debris. The small-headed toothbrush was used to obtain cell samples from buccal mucosa. The buccal cells were transferred into two falcon tubes labeled LC (left cheek)

and RC (right cheek), each containing 10 ml of buccal cell buffer at ph. 7.0 and centrifuged for 10 min at 581 rcf. The supernatant was removed and replaced with 5 ml of fresh buccal cell buffer and centrifuged for 10 min at 581 rcf.
This process was repeated twice because this buffer helped inactivate endogenous DNAases present in the oral cavity and remove bacteria and cell debris that could complicate scoring. The supernatant was removed and replaced with 5 ml of fresh buccal cell buffer. The pool of the cells from the left and right tubes into one falcon tube and centrifuged for 10 min at 581 rcf and the supernatant was removed. the cells were suspended in 1 ml of buccal cell buffer. To further aid cellular disaggregation and obtain slide preparation with clearly separated cells, 50 μl of DMSO was added. 120-150 μl of cell suspension was poured onto the clean and dry microscopic slides and allowed to air-dry for 10 min before staining.
Slides were immersed for 1 min in 50% and 20% ethanol, respectively and then washed for 2 min with milli-Q water. Slides were placed in 5 M HCL for 30 min and then rinsed in running tap water for 3 min. in the next step, slides were stained with Schiff 's reagent for 60 min and rinsed in running tap water for 5 min and then rinsed well in milli-Q water. In the end, slides were immersed in 2% Light Green for 20-30 s and rinsed well in milli-Q water, air dried and viewed under a light microscope.

Scoring method

One slide was prepared from each sample. The number of micronuclei was determined according to Thomas P. et al guideline (Thomas P. et al, 2009).In our study, 1000 differentiated cells were scored in order to determine the frequency of micronuclei. The nuclei and the micronuclei were magentas in color, whereas the cytoplasm was blue/green. Cells were scored by using light microscopy.

Statistical analysis

The slides were coded during processing and decoded at the time of statistical analysis. Statistical analyses were performed with SPSS for Windows, version 21. Mean ± SD was calculated for background factors and frequency (%) was determined by environmental and nutritional factors. The methods used to calculate p-value were included Student T-test, Cross Tabs, and Correlation. The level of $p<0.05$ was considered to be statistically significant (two tails).

Results

Characteristics of sample population:

The main characteristics of the exposed group including gender, age, BMI, smoking shisha, duration of smoking shisha, smoking cigarette, alcohol addiction, disease, family history of cancers, severe stress were summarized in Table 1. The mean age of our cases was 25.2 (3.67) years and their mean BMI was 23.01 (3.42).
As we explained the frequencies(%) of other variables in table 1 25% of subjects smoked shisha 2-4 times per week, 30% of them 4-8 time per week and 45% more than 12 times per week. The total duration of smoking shisha in 30% of subjects was 1-4 years, in 20% of them was 4-8 years and in the rest of subjects was more than 8 years. Out of total samples,20% of subjects were smokers and 45% of them were addicted to alcohol too. Moreover, 10% of subjects had an underlying medical condition and 20% had a family history of cancer. In the population studied, 60% of people exposed to stress in their lives (heavy car accidents, divorce, loss of loved ones etc.) and 20% of them had night shift jobs.

Micronucleus Levels:

Baseline frequencies for micronucleated cells in the BM were usually within the 0.5-2.5 MNi/1000 cells range. Minimum and a maximum of micronucleus in exposed groups were respectively 0 and 8 numbers. The aver-

Table 1: General characteristics of exposed group

No	Variables	Frequencies (%) in exposed persons (n=20)
1	Gender	
	Males	12(60%)
	Females	8(40%)
2	Shisha Smoking Pattern	
	2-4 times /week	5(25%)
	4-8 times / week	6(30%)
	>8 times/week	9(45%)
3	Total Period of Shisha Smoking	
	1-4 years	6(30%)
	4-8 years	4(20%)
	> 8 years	10(50%)
4	Cigarette Smoking	
	Yes	4(20%)
	No	16(80%)
5	Alcohol Consumption	
	Yes	9(45%)
	No	11(55%)
6	History of Background Diseases	
	Yes	2(10%)
	No	18(90%)
7	Familial History of Cancer	
	Yes	4(20%)
	No	13(65%)
8	History of Severe Stress	
	Yes	12(60%)
	No	5(25%)
9	History of exposure to chemicals	
	Yes	2(10%)
	No	18(90%)
10	History of Night Shift Jobs	
	Yes	4(20%)
	No	16(80%)

age number of micronucleus in this study was 4.9(2.1).

Micronucleus levels and Shisha Smoking:

As we described in tables 2, the relationship between the frequency of shisha smoking and mean of micronucleus was significant (p=0.021) although we didn't find a close correlation between duration of shisha smoking and mean of micronucleus levels (Table 2).

Clinicopathological Significance of Micronucleus levels

The statistical associations between other factors and the number of micronuclei were listed in Table 3. Among these factors, gender (p=, alcohol consumption (p=) and BMI (p=0.027) showed statistical associations with the number of micronuclei but no significant association was observed between other factors and the number of micronuclei.

Table 2: Association between the mean of micronucleus levels and shisha smoking factors

Mean of Micronucleus	**Frequency of Shisha Smoking**			**p-value**
1	2-4time /week	4-8 times / week	> 8 times per week	
<4/9 >4/9	4 1	2 4	3 6	0.021*
Total Duration of Shisha Smoking				
	> 8 years	4-8 years	1-4 years	
<4/9	3	2	4	0.726
>4/9	3	2	6	

Table 3: Relationship between some background and environmental factors and MN levels

No	**Variables**	**MN Levels**	**p-value**
1	Age		0.833
	<25(2.24)years	4.24(1.13)	
	>25(2.24)years	5.84(1.93)	
2	BMI		0.027*
	21.19(3.42)	<4.9	
	24.50(2.74)	>4.9	
3	Gender		0.007*
	Males	5.91(1.83)	
	Females	3.37(1.84)	
4	Cigarette Smoking		0.046*
	Yes	5.33(1.15)	
	No	3.00(2.12)	
5	Alcohol Consumption		0.021*
	Yes	6.11(1.69)	
	No	3.90(2.11)	
6	History of Background Diseases		0.948
	Yes	5.00(1.41)	
	No	4.88(2.29)	
7	Familial History of Cancer		0.910
	Yes	5.00(2.16)	
	No	5.15(2.37)	
8	History of Severe Stress		0.895
	Yes	5.16(2.40)	
	No	5.00 (2.12)	
9	History of exposure to chemicals		0.748
	Yes	2(10%)	
	No	18(90%)	
10	History of Night Shift Jobs		0.176
	Yes	6.25(2.36)	
	No	4.56(2.09)	

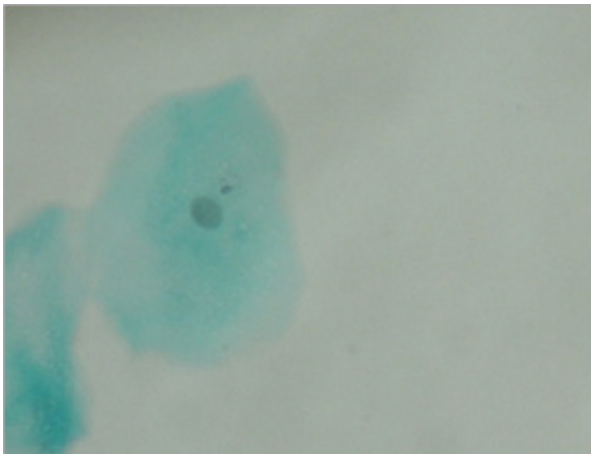

Figure 1: Oral buccal mucosal cells having MN

Nutritional factors and Micronuclei levels: Nutritional factors including the frequency and extent of dairies, vegetables, fruits, supplements and others factors consumption were assessed. They didn't affect the level of micronucleus levels in our cases. Since a large number of dietary factors have been investigated and no association of these factors was found with the micronucleus levels, details were omitted.

Discussion

The prevalence of shisha smoking among adolescents and young generations varies in different parts of the world. The current study aimed to survey the lifestyle, nutritional, and family characteristics related to adolescent lifetime shisha smoking among 20 Iranian persons aged 25.2(3.67) years old who started shisha smoking for more than 1 years and used it minimally twice weekly.At the next step, we tried to find genotoxic damages by Micronucleus(MN) in buccal cells of the exposed population. The buccal cell micronucleus (MN) assay is a noninvasive and valuable tool for biomonitoring DNA damages(Holland N. et al, 2008), first proposed by Stich et al. , as a useful biomarker of environmental induced genetic damages e.g. smoking and alcohol consumption , micronutrient deficiencies , exposures to environmental pollutants (e.g. pesticides, arsenic, formaldehyde), medical procedures (e.g. radio- and/or chemotherapy), as well as, inherited genetic defects in DNA repair (Stich H.F, Rosin M.P, 1983; Stich H.F, et al, 1984) which were considered in our survey. Unfortunately, we were not able to do the same biomonitoring on age and gender matched healthy population but analysis of the role of a wide range of background factors and their associations with MN frequencies could be considered as a great strength point of present work.

This study showed for the first time the role shisha smoking in raising the micronucleus levels in buccal mucosal cells of young Iranian people who smoked shisha for more than 2 years routinely in comparison to unexposed healthy people according to other studies (Holland N. et al, 2008.). Our results present also the significant role of shisha smoking intervals on increased MN frequencies in young people($p=0.021$). Moreover, each shorter intervals caused higher levels of MN especially in men with a history of alcohol consumption and higher BMIs. The genotoxic damages by shisha smoking showed higher frequencies in males, alcohol abusers and people with higher BMI levels. Although the roles of alcohol(Benassi-Evans B, Fenech M. 2011) and BMI (Donmez-Altuntas H,et al, 2014) on increased MN frequencies have been previously described, gender dependency of

MN is still controversial (Milosević-Djordjević O, et al, 2010; McHugh MK, et al,. 2013; Nefic H, et al, 2013)

Of the factors listed in Table 3, the correlation between gender and alcohol consumption was significant. Micronucleus frequency was in men more than women, maybe because the extent of smoking shisha was higher in men (Table 5). Ethanol is one of the chemicals related to the development of oral malignant neoplasms. In a study, 40 alcoholic individuals who did not smoke and 20 alcohol and tobacco abstainers were selected. The frequency of micronuclei in buccal mucosa cells was higher in the group of alcoholic individuals when compared to the control group, although there was no significant difference ($p > 0.05$) (Donmez-Altuntas H,et al, 2014), the age factor appears to be associated with a micronucleus frequency (Milosević-Djordjević O, et al, 2010). In the present study, there was no significant correlation between age and micronucleus frequency, maybe because all the samples were about the same age. According to this study, the mean number of micronucleus was higher in subjects who had higher BMI. So far, there has been no direct study done on the effect of increased BMI on increased micronucleus. Obesity has been reported to cause an increase in free radicals and the action of free radicals can increase and cellular DNA damage. In obesity, oxidative stress can arise in many ways. No relation to other factors listed in Table 5 with the mean number of micronucleus, was found.

Conclusion

From the present study, an increase in MN frequency seems to be related to shisha smoking in both genders. Limitations in this study were small sample size and the lack of control group and also its cross-sectional design. It is advisable to confirm or reject the effects of alcohol consumption and BMI on increase in MN frequency by doing separate studies with larger cases and control groups in the future.

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