



Micronucleus Assay in Waterpipe Tobacco and Cigarette Smokers: A Comparative Study

¹Prathibha Prasad, ²Mohamed S Hamed, ³Prashant Nahar

ABSTRACT

Aim: Waterpipe tobacco smoking (WTS) has become a global epidemic, especially among youth. WTS has not been studied like cigarette smoking. There is a dire need to study and document health effects of waterpipe smoking in general and specifically on the oral cavity.

Materials and methods: A total sample size of 400 was studied. One hundred exclusive shisha smokers, 100 exclusive cigarette smokers, 100 subjects smoking both cigarette and shisha and 100 non-smokers. We recorded and associated socio-demographical data pertaining to WTS and cigarette smoking in UAE and examining their toxic effects on the oral mucosa at a cytogenetic level by studying the micronuclei (MN) stained by Feulgen and Acridine Orange (DNA specific stains).

Results: A significant association was observed between age distribution and groups. Majority of subjects were males. Arabic nationalities were consuming a higher percentage of Waterpipe and Cigarette separately, and Indian nationality was consuming a higher percentage of waterpipe and cigarette together. Comparison of Micronuclei in Feulgen and Micronuclei in Acridine Orange group between four groups was significant. Mean micronuclei in feulgen was highest for Waterpipe and Cigarette group followed by shisha group and least for Control group. Similarly, Mean micronuclei in acridine orange was highest for waterpipe and cigarette group followed by shisha group and least for control group.

Conclusion: Further epidemiological studies should be undertaken to determine whether WTS is associated with the incidence of lung cancer/oral cancerous lesions.

Clinical significance: The findings of this study could be used to spread awareness that waterpipe smoking, like cigarette

smoking, has the potential to cause genotoxic effects and could eventually lead to carcinogenicity based on duration and frequency.

Keywords: Acridine orange stain, Cigarette smoking, Feulgen stain, Genotoxicity, Micronuclei, Waterpipe tobacco smoking.

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INTRODUCTION

Cigarettes are not the only form of tobacco smoked; in fact, waterpipe/shisha has been used for smoking tobacco for centuries.^{1,2} The water pipe has various names like argile, narghile, shisha, hookah, hubble bubble and varies depending on the locality. It is commonly referred to as shisha in the United Arab Emirates (UAE) and hookah in India. The name WTS is used for the last two decades in the English language scientific literature to refer to any variety of instruments that involves tobacco smoke passing through water before inhalation.³

The use of water pipe is increasing daily, by all age and sex groups. Waterpipe smoking has become a global epidemic by spreading to the western countries, especially among youth.⁴⁻⁶ Girls and women in the Middle East region are increasingly gravitating towards this method of using tobacco.⁷

A rising trend of use of WTS is noted in the region; though many believe tobacco smoking in any form is religiously prohibited. Studies have proved that the narghile smoke contains toxic substances like carbon monoxide, formaldehyde, nitrogen, nitric acid, polyhydrocarbons, chromium, arsenic, lead and volatile aldehydes that are as harmful, if not, more harmful than cigarettes.^{3,8-12}

¹Department of Basic Dental Sciences, College of Dentistry, Gulf Medical University, Ajman, United Arab Emirates

²Department of Oral and Maxillofacial Surgery, College of Dentistry, Gulf Medical University, Ajman, United Arab Emirates

³Oral Medicine Department, Pacific Dental College, Udaipur, India

Corresponding Author: Prathibha Prasad, Department of Basic Dental Sciences, College of Dentistry, Gulf Medical University, Ajman, United Arab Emirates, Phone: 00971557623466, e-mail: pratibhap.79@gmail.com

Acute respiratory diseases and lung impairment are proven health risks. Other health hazards include an increased risk of developing cancers, cardiovascular and chronic respiratory diseases. Indulging in waterpipe smoking during pregnancy can lead to low fetal birth weight. Passive smoking from waterpipe smoke, like cigarettes, poses a serious health risk to non-smokers. Infectious diseases, such as Tuberculosis, might spread when waterpipe is shared among smokers.¹³⁻¹⁵

Waterpipe tobacco smoking (WTS) has not been studied like cigarette smoking. There is a dire need to study and document health effects of waterpipe smoking in general and specifically on the oral cavity. Since the oral mucosa is exposed to the smoke and toxins from WTS directly, studying the effects on a cellular and genetic level will help in a greater understanding of smoke effects. Micronuclei were used as a biomarker of genotoxicity.

MATERIALS AND METHODS

A total sample size of four hundred was studied. One hundred exclusive shisha smokers, 100 exclusive cigarette smokers, 100 subjects smoking both cigarette and shisha and 100 non-smokers. Along with the Arab population, the Indian population residing in UAE were also studied as they are influenced by the increasing trend of waterpipe smoking in the country. A total of 195 Arabs and 105 Indians were studied. After a brief explanation of the study, and obtaining a written consent signed from the study participants, a questionnaire was administered to collect data. A detailed case history was taken followed by a clinical examination. A buccal swab was taken from the subjects. The exfoliated cells were transferred onto two glass slides which were immediately fixed using a spray fixative. These slides were later stained by Feulgen stain and Acridine Orange to examine the micronuclei.

The micronucleus assay being valid and sensitive, yet a very simple technique was adopted as a biomarker of the genotoxicity/genetic damage. The exfoliated cells of oral mucosa not only come in direct contact with the carcinogenic substances in the smoke; the systemic effects of the smoke will also be surely exhibited by these cells.¹⁶⁻²⁰ Slides stained by feulgen stain were observed under a compound light microscope for micronuclei. Slides stained by acridine orange stain were examined under the fluorescent microscope which highlights the micronuclei. This was done to rule out other secondary nuclear deformities.²¹ Micronuclei (MN) either originate from fragments of a chromosome or whole chromosomes which are not included in the main daughter nuclei during nuclear division. Thus, Micronuclei assay provides us with a measure of chromosome loss and chromosome breakage. It has been documented to be as sensitive as

classical metaphase chromosomal analysis in serving as an indicator of chromosome damage. Micronuclei frequency was checked in 1000 cells. They were scored according to Tolbert's criteria.²²⁻²⁴ Terms waterpipe and shisha are used in the description interchangeably.

Statistical Analysis

Collected data were entered in an Excel chart and was analyzed by Statistical Package for Social Sciences (SPSS) version 22 software. Frequencies and proportions were used to categorize the data of the study. The Chi-square test was used to determine the significant difference between the frequencies in one or more categories. Descriptive statistics were represented as mean and standard deviation. Analysis of variance (ANOVA) was used to analyze variation among and between groups. A *p* value below 0.05 was considered as statistically significant.

RESULTS

Table 1 shows that most subjects in exclusive shisha smokers group, cigarette, and control groups were in the age group below 25 years. In group smoking, both shisha and cigarette majority of them were in the age group 25 to 30 years. Subjects older than 35 years were also seen in this category of subjects compared to other groups. A significant association was observed between age distribution and groups, implying age may play a role in the selection of tobacco product. Younger age group was consuming Shisha and Cigarette separately.

Table 2 depicts the gender distribution and in our study, the majority of subjects were males in all the four groups. 89% of exclusive shisha smokers group, 100% of exclusive cigarette smokers group, 90% of group smoking both shisha and cigarette and 88% of control group subjects were males.

A significant association was observed between nationality and groups as shown in Table 3. Arabic

Table 1: Age distribution of subjects with respect to different groups

		Group							
		Shisha + Cigarette				Control			
		Shisha Count	Shisha %	Cigarette Count	Cigarette %	Control Count	Control %	Control Count	Control %
Age	below 25 years	39	39.0	37	37.0	22	22.0	44	44.0
	25 to 30 years	36	36.0	28	28.0	31	31.0	40	40.0
	31 to 35 years	11	11.0	7	7.0	18	18.0	6	6.0
	above 35 years	14	14.0	28	28.0	29	29.0	10	10.0

$\chi^2 = 32.42$, df = 9, *p* below 0.001*

Table 2: Gender distribution of subjects with respect to different groups

	Group							
	Shisha		Cigarette		Shisha + Cigarette		Control	
Gender	Count	%	Count	%	Count	%	Count	%
Male	89	89.0	100	100.0	90	90.0	88	88.0
Female	11	11.0	0	0.0	10	10.0	12	12.0

$\chi^2 = 12.25$, df = 3, $p = 0.007^*$

Table 4: Comparison of mean micronuclei in feulgen and micronuclei in acridine orange group

		Micronuclei in Feulgen		Micronuclei in Acridine Orange	
		Mean	SD	Mean	SD
Group	Shisha	12.57	6.49	8.22	5.52
	Cigarette	11.74	6.52	8.02	5.35
	Shisha + Cigarette	17.88	7.73	13.07	6.86
	Control	2.03	0.89	1.10	0.72
<i>p</i> value		below 0.001*		below 0.001*	

nationalities were consuming a higher percentage of shisha and cigarette separately, and Indian nationality was consuming a higher percentage of shisha + cigarette together.

Mean micronuclei distribution is shown in Table 4. Mean micronuclei in Feulgen was highest for group smoking both shisha and cigarette and was least for the control group. Similarly, mean Micronuclei in Acridine Orange was highest for Group smoking both Shisha and Cigarette and was least for Control group. This difference in mean Micronuclei in Feulgen and Acridine Orange was statistically significant. Images of Micronuclei with respect to Feulgen and Acridine orange stain is shown in Figures 1 and 2 respectively.

As shown in Table 5, with respect to Micronuclei in Feulgen, majority of subjects in exclusive shisha smokers group (57%) and in exclusive cigarette smokers group (58%) had micronuclei of 4 to 12, majority in group smoking both shisha and cigarette (71%) had micronuclei of above 12, and majority in Control group had micronuclei of 0 to 3. This observation was statistically significant between the four groups. With respect to micronuclei in acridine orange, majority of subjects in exclusive shisha smokers group (59%) and in exclusive cigarette smokers group (60%) had micronuclei of 4 to 12, majority in group smoking both shisha and cigarette (51%) had micronuclei of above 12 and 100% in control group had micronuclei of 0 to 3. This observation was statistically significant between the four groups.

DISCUSSION

A deep concern for the splurge in popularity of waterpipe smoking worldwide and especially among teenagers and adolescents as seen in our country made us study

Table 3: Distribution of subjects with respect to nationality in different groups

	Group							
	Shisha		Cigarette		Shisha + Cigarette		Control	
Nationality	Count	%	Count	%	Count	%	Count	%
Arabic	75	75.0	70	70.0	50	50.0	82	82.0
Indian	25	25.0	30	30.0	50	50.0	18	18.0

$\chi^2 = 26.61$, df = 3, p below 0.001*

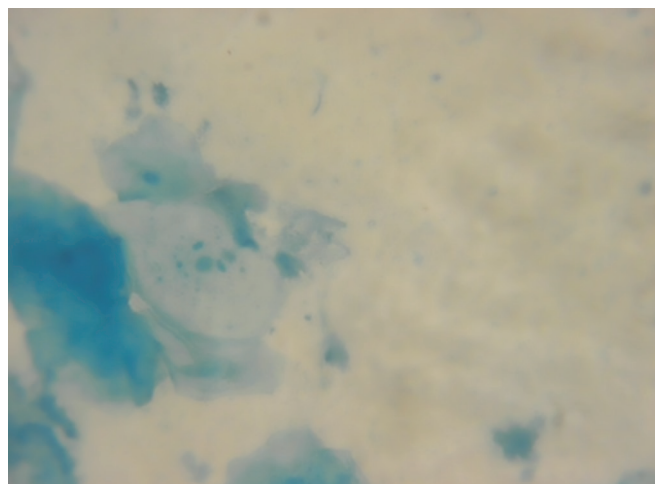
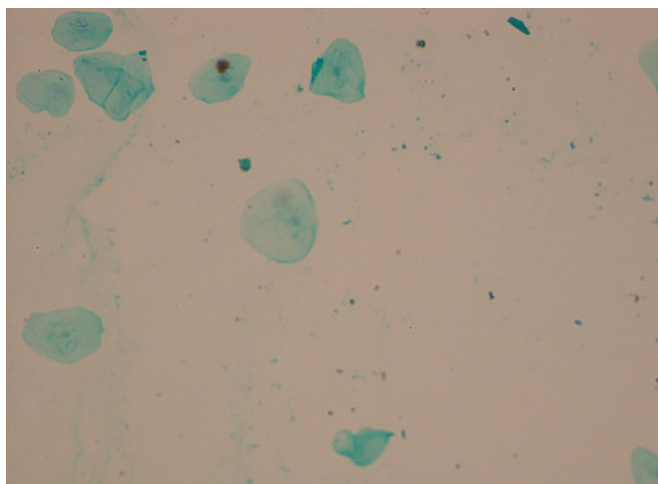
this issue. There have been many surveys done, and many hypotheses proposed the proof is not compelling enough to show the ill effects on health caused by WTS. This could be one of the main reasons for the lack of fear in its usage. The WTS is associated with coolness factor, a very mysterious and enigmatic effect because of its history and tradition.

Majority of subjects in exclusive shisha smokers group, exclusive cigarette smokers group and control groups were in the age group below 25 years. In group smoking both shisha and cigarette majority of them were in the age group 25 to 30 years. Higher percentages of subjects were above 35 years in group smoking both shisha and cigarette than other groups. According to a study done by Saadawi et al. from American Chemical Society, it's the younger people who are prominent users of hookah.²⁵ Our results were concurrent with the study carried out in 2008 by Smith et al., who inferred that hookah use in California was much higher among young adults and a significant association was observed between age distribution and groups, i.e., age can play a role in the selection of tobacco product. Younger age groups were predominantly Shisha users whereas older age groups were predominantly cigarette users with an occasional indulgence of shisha.²⁶

Majority of subjects were males in all the four groups. 89% of exclusive shisha smokers group, 100% of exclusive cigarette smokers group, 90% of group smoking both shisha and cigarette and 88% of control group subjects were males. Smith studied hookah smoking in two different age groups in the USA and found that it was much higher among men compared to women in both the age groups classified as younger and older adults.²⁶ Our results also correlated with the survey conducted by Mohammed Jawad prevalence of smoking among men was the highest in Vietnam and contrary among women in Russia.²⁷ Our study results were similar to Anand NP who studied WTS in India and observed that male subjects consumed hookah twice as much as females.²⁸ A cross-sectional study among dental students in a university in Jordan by Obeidat et al. was a very good example of social acceptance of WTS. In their study, females were the predominant users of WTS rather than

Table 5: Comparison of micronuclei in feulgen and micronuclei in acridine orange group between four groups

		Group								χ^2 value, df, p value
		Shisha		Cigarette		Shisha + Cigarette		Control		
		Count	%	Count	%	Count	%	Count	%	
Micronuclei in Feulgen	0–3	3	3.0	7	7.0	0	0.0	99	99.0	382.96, 6, below 0.001*
	4–12	57	57.0	58	58.0	29	29.0	1	1.0	
	above 12	40	40.0	35	35.0	71	71.0	0	0.0	
Micronuclei in Acridine Orange	0–3	20	20.0	20	20.0	8	8.0	100	100.0	262.32, 6, below 0.001*
	4–12	59	59.0	60	60.0	41	41.0	0	0.0	
	above 12	21	21.0	20	20.0	51	51.0	0	0.0	

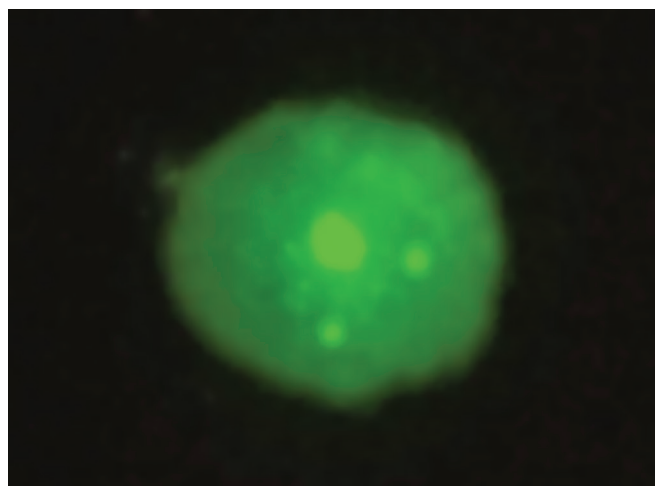
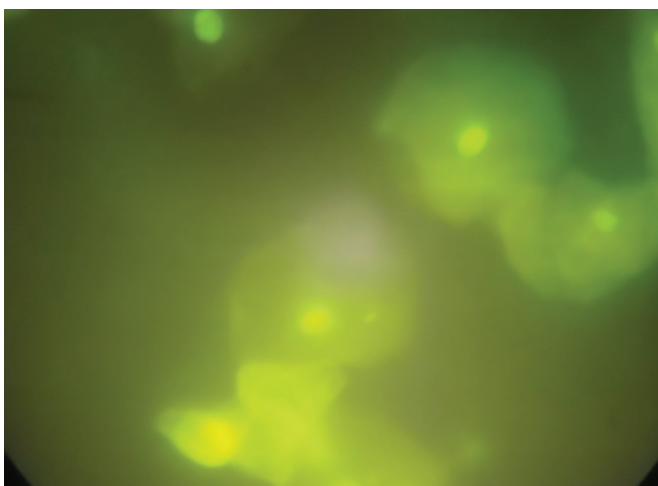
**Figs 1A and B:** (A) 10x view of micronuclei in feulgen stained slides; (B) 40x view of micronuclei in feulgen stained slides

cigarettes.²⁹ As a cigarette is considered a taboo in the society, women are seen to be inclined towards smoking shisha, which is considered to have a traditional and cultural value; however, the numbers were still high for males. These results were contrary to ours, and the difference could be related to the sample size and a bigger expat community in UAE.

A significant association was observed between nationality and groups, i.e., a higher percentage of subjects of Arab nationality were using shisha and Cigarette separately and a higher percentage of subjects of Indian nationality were using Shisha and Cigarette together.

Mohammed Jawad conducted the Global Adult Tobacco Survey (GATS) which monitored adult tobacco use. The survey conducted in 2008–2010 concluded that cigarette users indulged in waterpipe smoking more than non-cigarette smokers in India and Russia, but in Egypt, the scene was the exact opposite. Smoking both cigarette and waterpipe were higher in Russia followed by Egypt and Vietnam. Waterpipe smoking was virtually non-existent in Mexico, Philippines, and Thailand.²⁷

The micronucleus assay was adopted as a biomarker of the genotoxicity/genetic damage. It is a valid and sensitive technique yet very simple. Micronuclei frequency more than 1–3 per 1000 cells was seen in

**Figs 2A and B:** (A) 10x view of micronuclei stained by acridine orange in an easily spottable bright fluorescence; (B) 40x view of the same



Figs 3A and B: Speckled lesion seen on the right and left buccal mucosa of a subject who smoked shisha exclusively

smokers in a healthy population. The exfoliated cells of oral mucosa not only come in direct contact with the carcinogenic substances in the smoke; but also exhibit the features due to systemic effects of the smoke. Mean micronuclei in feulgen was highest for group smoking both shisha and cigarette and least for control group. Similarly, mean micronuclei in acridine orange was highest for group smoking both shisha and cigarette and was least for control group. This difference in mean micronuclei in feulgen and acridine orange was statistically significant. With respect to micronuclei in feulgen. Majority of subjects in exclusive shisha smokers group (57%) and in exclusive cigarette smokers group (58%) had micronuclei of 4 to 12, the majority in-group smoking both shisha and cigarette (71%) had micronuclei of above 12, and a majority in Control group had micronuclei of 0 to 3. This observation was statistically significant between the four groups. With respect to micronuclei in acridine orange majority of subjects in exclusive shisha smokers group (59%) and in exclusive cigarette smokers group (60%) had micronuclei of 4 to 12, majority in group smoking both shisha and cigarette (51%) had micronuclei of above 12 and 100% in control group had micronuclei of 0 to 3. This observation was statistically significant between the four groups. This proves that shisha/hookah/WTS is as dangerous if not more dangerous than cigarette smoking and it is exceptionally dreadful to have a habit of smoking both cigarette and shisha. Even though several studies have raised concerns over the lack of research on oral health effects of shisha smoking, only scant literature is available to compare the results of this study. It was certainly a difficult task to compare our results with other studies also because of the differences in methods, like collecting swabs, techniques of staining, and the abnormality in cell/nucleus studied. Yadav et al. have established that hookah smoke could be genotoxic for

human beings.³⁰ Ghada et al. studied the metabolites of Tobacco-Specific Nitrosamine Exposures and established that levels of NNAL{4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol} were higher in cigarette smokers when compared to waterpipe smokers.³¹ Noushin and Mona, Iran have confirmed that the cytotoxic effect of cigarette and waterpipe smoking on buccal mucosa cells was significantly higher when compared with the non-smokers. The cellular death by smoking cigarette was higher than with water pipe.³² El-Setouhy et al. in Egypt examined micronuclei in oral smears and tried to establish that the cytogenetic effect by waterpipe smokers was higher than non-smokers.³³ Our study correlates with their finding of WTS having more micronuclei compared to non-smokers. Our study has also, in addition, compared it with cigarette smokers and those who smoke both cigarette and waterpipe. An extremely interesting finding in results of this study was speckled lesion seen on right and left buccal mucosa of a subject who smoked shisha exclusively for the last 14 years after moving to Dubai (Fig. 3). The subject was a follower of a strict vegetarian diet and didn't consume alcohol or smoke cigarettes. He is well educated and is working as a senior engineer in a reputed company. He is third generation of well-educated family and both parents are doctors. The reason he started smoking shisha is the hype it has created in the Middle East. Since his first puff he was so hooked on to it and continued to smoke it every weekend for hours. One of the other reasons quoted by the subject was that it is the most convenient form of entertainment. The lesion was clearly dysplastic but unfortunately the patient returned to his home country and investigators could not follow up regarding the biopsy results. Most of the previous studies have shown that 99% of waterpipe smokers were also cigarette or other form of tobacco like beedi/cigar smokers, which has made it impossible for researchers to isolate the effect of waterpipe smoking.³⁴

A study by Koul and others has established the association of smoking Hookah which is a form of WTS to lung cancer in a population of Kashmir.^{35,36} El Hakim in Egypt has proposed that WTS has adverse effects on general health and causes oral squamous cell carcinoma.³⁷ The finding of our study could be substantial evidence that WTS alone has the potential to cause deleterious health effects and lead to morbidity and mortality.

CONCLUSION

Hopefully, this study paves way for more data collection to establish the degree of risk of genotoxicity, developing epigenetic and dysplastic changes associated with WTS use, which would help eventually to curb the progression of at-risk lesions to malignancy. Further epidemiological studies should be undertaken to determine whether WTS is associated with incidence of lung cancer/oral cancerous lesions. By creating awareness among the public, it is also hoped that more strategies of primary prevention may be implemented.

CLINICAL SIGNIFICANCE

The findings of this study could be used to spread awareness that waterpipe smoking, like cigarette smoking, has the potential to cause genotoxic effects and could eventually lead to carcinogenicity based on duration and frequency.

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