JCHR (2024) 14(2), 367-375

Journal of Chemical Health Risks

sanad.iau.ir/journal/jchr

ORIGINAL ARTICLE

A Cytometric Analysis in Circulating Blood Lymphocytes of Water pipe Tobacco Smokers and Non-Smokers by Cytokinesis-block Micronucleus Technique: A Genomic Health Study on Apparent Healthy Premenopausal Women in Tehran

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(Received: 31 December 2022 Accepted: 5 March 2024)

KEYWORDS

Waterpipe smoking;
MNi frequency;
Genomic health;
Tehran

ABSTRACT: According to the association between increased micronuclei (MNi) level in reproductive age women and increased risk of congenital abnormalities, we aimed in this study to find the contribution of WTS to women's health in apparently healthy young cases by comparing the variability of MN values in water pipe smokers and non-smokers. Finding the associations between MNi levels and demographic, socioeconomic, reproductive, and lifestyle factors was the secondary scope of this study. This cross-sectional case-control study was performed on 30 premenopausal women (15 cases and 15 controls) who lived in Tehran, according to our inclusion criteria. The mean MN frequency among waterpipe smokers was 28.53± 7.462, whereas the same parameter in the control group was 6.53 $\pm$ 3.24 (p= 0.001). Non-parametric tests revealed a significant association between MNi and frequency of waterpipe smoking (p = 0.001), age of starting waterpipe smoking (p = 0.003), concurrent use of alcoholic drinks (p= 0.004), and secondhand smoking (p =0.001). In the context of heavy environmental pollution in Tehran, significantly higher MNi frequencies and decreased genomic health in waterpipe smoker women in reproductive age may predispose them to an increased risk of harmful reproductive outcomes. These findings emerge from governmental and non-governmental biomonitoring programs in high-risk women to concern more on unhealthy lifestyles and environmental pollution.

INTRODUCTION

For centuries, older adults in the Middle East, North Africa, and Central Asia have used Waterpipe with the famous names of Shisha, Hookah, Narghile, and Ghelyan as a traditional smoking method. However, its use among young people is increasing globally, and nowadays, Waterpipe tobacco smoking (WTS) has traveled to most countries and has become a prevalent smoking method [1]. Dual Cigarette and Hookah Smoking are associated

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DOI: 10.22034/JCHR.2024.1087903

with adverse perinatal outcomes among pregnant women [2], and growing evidence of WTS indicates it as one of the leading causes of reproductive disorders in both genders [3]. Recent changes in the general pattern of WTS are based on human societies' belief that Waterpipe smoking is not as damaging as cigarette smoking, and this misconception has regained shisha popularity worldwide [4]. Other than different misunderstandings about the health effects of WTS, the availability of varying tobacco products with "appealing flavors" is another reason for Shisha's popularity [4]. Everybody can prepare Waterpipe tobacco easily and rapidly, even by online ordering; moreover, WTS customers never find any label on the product regarding the nicotine content and the toxic effects of additive and flavoring agents [5].

The tobacco use epidemy has expanded its' roots among women of reproductive age and pregnant mothers [6]. The primary health risks of WTS in active or passive smokers have been considered carbon monoxide poisoning, cardiovascular diseases (CVD), lung diseases [7], head and neck cancers [8], endocrine disruption [9], diabetes mellitus, metabolic syndrome and obesity [10]. Despite the lack of human data, experimental studies in lactating rats show the role of WTS on reduced levels of follicle-stimulating hormone (FSH), prolactin, luteinizing hormone, estradiol, and oxidative stress markers in the blood in male progeny [11].

Tobacco smoked in a Waterpipe is a complex matrix of uncontrolled flavoring agents, carcinogenic impurities, and humectants. Exposure to humectant smoke and flavoring syrup in both charcoal-heated whole Shisha and electronic-heated whole shisha smoked in both acute and chronic types of exposure can cause alveolar cell damage and necrosis [12]. Many analytical studies on hookah smoke showed high levels of carbon monoxide, nitric oxide, formaldehyde, furan, nicotine, polycyclic aromatic hydrocarbons, heavy metals (e.g., Zinc, Iron, Cadmium, Vanadium, Aluminum, Lead, Chromium, Manganese, and Cobalt) and nanoparticles [13].

WTS should be considered a high-risk behavior because it induces p53 mutation and premalignant lesions in the normal mucosa of the oral cavity [14]. Moreover, a comet assay study showed WTS-associated DNA damaging properties in buccal cells and lymphocytes of smokers [15]. Given that human population studies on the possible toxic effects caused by this unhealthy lifestyle are limited and most studies have focused on cigarette smoking in men, we aimed in this study to monitor the genotoxic impact of shisha smoke by lymphocyte micronucleus (MN) test in young women who continuously exposed to hookah by active smoking in a cross-sectional case-control study. We used the Micronucleus test because all scientific evidence show an association between increased micronuclei (MNi) in lymphocytes and increased risk of cancer, reproductive disorders, and fetal disorders during pregnancy, especially Down syndrome [16] as well as other age-related degenerative diseases [17].

MATERIALS AND METHODS

This research is a population-based cross-sectional study conducted in Tehran to compare the effects of shisha smoking habits and lifestyle on the incidence of Micronucleus in a total population of 30 (15 cases and 15 controls) premenopausal (20-50 years) women.

Inclusion criteria

We enrolled cases and controls from the volunteers who lived in the same zone and were referred to the laboratory for a routine annual check-up. All volunteers were premenopausal women without a clarified history of exposure to hazardous radiation. All of them are exposed to household chemicals during every day housekeeping tasks. Drinking alcohol was considered a usual lifestyle habit among them; therefore, we included alcohol-abuser women and decided to analyze the role of alcohol drinking as a possible synergistic factor in genotoxic effects.

Exclusion criteria

We excluded women with a history of cancer, active cigarette smoking, radiation therapy, and chemotherapy. We also excluded women with a history of recent antibiotic or other drug use (30 days before entering this study). By completing the questionnaire, the study volunteers were homogeneous regarding demographic information, dietary habits, medication regimens, etc., to reduce the effects of other factors on the incidence of Micronucleus as much as possible.

Micronucleus assay

The validated CBMN protocol of Micronucleus (MN) assay based on cytogenetic techniques for evaluating chromosomal damage in human tissues was applied. This protocol uses ex vivo whole blood involving 72 h of culture with the block of cytokinesis at 44 h. CBMN protocol explained this established method for sample processing, slide preparation, and scoring technique [17].

Sample preparation

Blood samples were collected from the volunteers direct after face-to-face interviews from September 2020 to November 2020. Five ml venous blood was obtained during sampling using five cc sterile syringes. After removing the syringe heads, blood samples were poured into preservative-free, lithium-containing heparin tubes and cultured in an RPMI culture medium. According to the standard protocol [17], fetal bovine serum (FBS) and phytohemagglutinin were added to this medium. The flasks were then incubated at 37°C for 44 to 45 hours without the need for CO_2. Then cytochalasin B was added to the medium as a cytokinesis blocker, and the flasks were incubated again for 28 hours (72 hours after the initial culture). The samples were centrifuged for 10 minutes at a speed of 1200 rpm. After this step, the upper solution was gently removed to a volume of 2 ml with the help of a drain pump. Then 3 ml of pre-prepared and refrigerated hypotonic KCl solution was added to the contents of the end of the tube so that the total volume reached 5 ml. The tubes were centrifuged again at 1000 rpm for 7 minutes. The supernatant was drained again by the pump so that the volume of liquid remaining at the end of the tube reached 2 ml. At this point, a new, 5 ml fixative solution consisting of 60 ml of methanol and 10 ml of glacial acetic acid was rapidly added to the contents of the Falcon, and the final solution was kept in the environment for 20 minutes and then centrifuged at 1000 rpm for 7 minutes. In the next step, the upper content was drained again, and 5 ml of fixative was added to the remaining solution at the end of the tube and centrifuged at the same rate for 7 minutes. This step was repeated two times for better cell washing. In the last step, 1 ml of the solution was left in the Falcon tube, and the rest was drained by the pump [18, 19].

Staining and scoring methods

In order to prepare the microscopic slides, the slides were first cooled by placing them on an ice pack. The remaining solution in the Falcon tube was then dripped onto the slides from a distance of 10 cm with the help of a Pasteur pipette and dried. Then Giemsa solution with a concentration of 10% was poured on the slides with the help of a dropper, and the slides were floated in this solution for 20 minutes. They were then washed with distilled water and left in the environment for 24 to 48 hours and scored by YS100 light microscope. Two slides were prepared from each sample, and the number of micronuclei was determined according to the protocol. In the present study, 1000 differentiated cells were scored to determine the frequency of micronuclei three times in each slide, and the mean $\pm$ standard deviation was reported[17, 18].

Statistical analysis

The slides were coded during processing and decoded at the time of statistical analysis. The baseline characteristics between the case and the control groups were compared using Student's t-test or Mann-Whitney U test for parametric and non-parametric variables, respectively, which were presented as mean (± SD) values for parametrical distribution or median (interquartile range, IQR) for non-parametrical distribution, and chi-square tests for categorical variables, which were expressed as number (percentage) . All data were analyzed using SPSS for Windows (version 21; IBM® SPSS® Statistics, Armonk, NY, USA), and a two-sided $p < 0.05$ was considered statistically significant [20].

RESULTS

Demographic and reproductive characteristics

Table 1 compares the demographic, reproductive, and background factors between cases and control groups. The mean ages of cases and control groups (29.53 ±7.05 *vs.* 31.4±8.67, p=0.52) were matched without any significant difference. The results of the student t-test show no meaningful difference in BMI, height, and weight of case and control groups. Similar patterns in

menstrual disorders, the incidence of ovarian dysfunction, and the history of birth control methods were seen in cases and controls. Other mentioned factors, sociodemographic, dietary, and lifestyle factors were practically similar in cases and control groups.

Table 1. Demographic and Reproductive factors of the study population

Characteristics	Control group (n=15)	Case group (n=15)	*P* value
Age(Years)	31.4 ± 8.68	29.53 ±7.05	0.52
BMI(Kg m^{2-1})	23.71 ± 5.4	24.95 ± 4.2	0.345
Height(cm)	162.26 ± 4.36	166.73 ± 5.07	0.055
Weight(kg)	63.26 ±14.76	69.26 ± 11.69	0.226
History of background disease			
(yes)	4	6	0.179
Menstrual disorders (yes)	2	2	1.00
History of ovarian disease	1	1	1.00
Active Smoking	0%	6.7%	1.00
Passive Smoking	20%	73.3%	0.009***
Alcohol	26.7%	80%	0.009***

Micronucleus levels in the study sample

Figure 1 shows six micrographs of binucleated cells with increased micronuclei (MNi), which means MN, 2MNs, and 3MNs compared with control. In our study samples (N=30), the mean MN level was 17.53 ± 12.53 (0-40), the mean 2MN level was 3 ± 0.66 (0-3), but 3MN was seen in one person only. MN levels were not associated with demographic and some lifestyle factors, including age, height, weight, BMI, history of background disease, education, employment occupation, night shift jobs, and exposure to household chemicals, in total study participants (n=30).

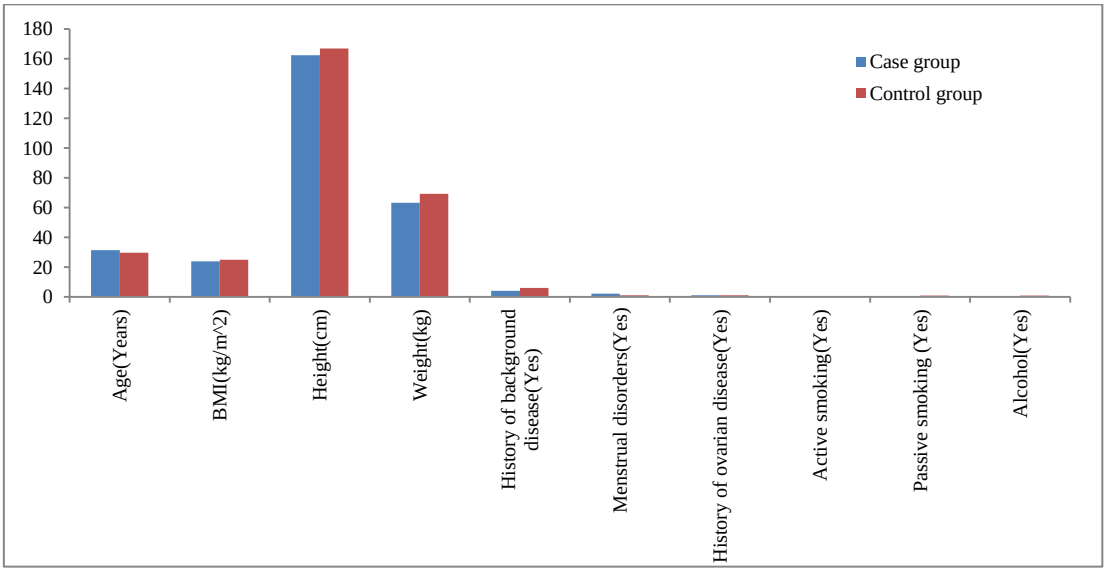

Figure 1. Demographic and Reproductive factors of the study population.

Micronucleus levels in cases and controls

Table 2 shows a highly significant difference (p=0.001) between the mean micronucleus levels of cases (28.533 ± 7.46292) and controls (6.53 ± 3.24844).Other than this essential difference, the level of binucleated cells with 2MN was also significantly higher in cases (1.06 ± 1.0328) compared to controls (0.26± 0.45) (p=0.041) (Figure 2).

Table 2. Number of MNs seen in cases and control groups (mean ± SD).

Variables in cases and control		Mean	Std. deviation	*P*-value
Total MN	Control	6.53	3.24844	0.001***
	Case	28.533	7.46292	
2 MN	Control	0.26	0.45774	0.041*
	Case	1.06	1.03280	
3 MN	Control	0.00	0.0000	0.539
	Case	0.133	0.35187	

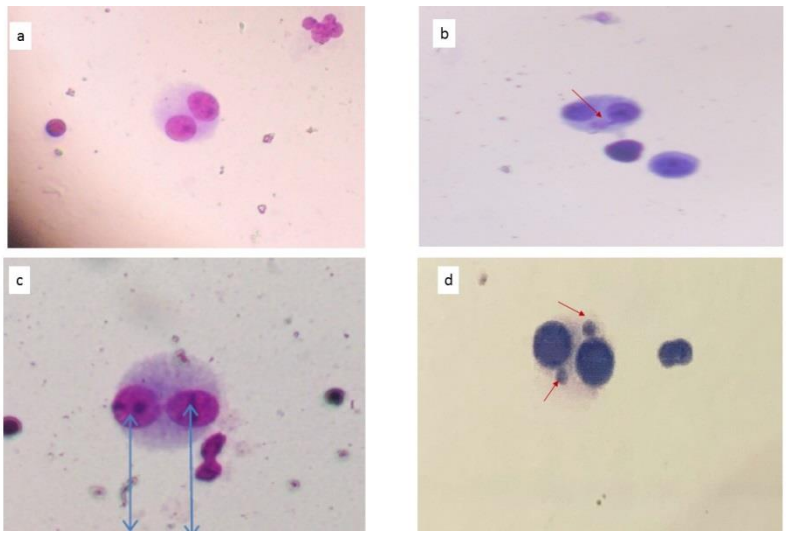

Figure 2. Micrograph of binucleated lymphocytes without and with different numbers of micronuclei. 2a: binucleated lymphocyte without micronuclei in the control group; 2b: binucleated lymphocyte with cytoplasmic micronuclei in the control group; 2c: binucleated lymphocyte without micronuclei in a woman from case group;2d: binucleated lymphocyte with 2 micronuclei (2MN) in a woman from case group

Waterpipe Tobacco Smoking (WTS) pattern

-Age of onset: The mean starting age of WTS was 20.53± 4.257 in cases, and a significant association between starting age and the number of MNs was detected (p=0.003).

-Frequency: Out of all the 15 cases, 46.7% of women smoked shisha once a week, 33.3% two times per week, 6.7% three times a week, and 13.3% smoked shisha four times per week. Mac Nemar's nonparametric test showed a highly significant association between increased micronuclei (MNi) and frequency of shisha smoking (p=0.001).

-Duration: The history of shisha smoking ranged from 3 to 7 years (9 ± 5.12). Logistic regression analysis showed a higher level of MN in shisha smokers with a long

history of WTS (p=0.003).

Drinking alcohol

Occasionally all women in the WTS group used to drink alcohol in the recreational model for more than three straight years, but few women from the control group used to drink alcohol. Mac Nemar's nonparametric test showed a highly significant association between alcohol abuse patterns and increased MN levels in the total study population (p=0.004).

Active and passive smoking

Although infrequent smoking was not associated with MN (p=0.2), 2MN (p=0.267), and 3MN (p=0.933), close associations were found between passive smoking and MN (p=0.001) and 2MN (p=0.038) in our total study population (p=0.004).

Nutritional factors

The association between dietry habits and increased micronuclei (MNi) wa assessed with linear regression analysis in the total study population (p=0.062). The dietry factors analyzed included consumption patterns of red meat, white meat, seafood, different vegetables and fruits in detail, egg and dairy, and some particular habits, including vegetarianism and special diets.

DISCUSSION

Despite the role of WTS on genome instability, very few national programs have been conducted in the Middle East against WTS, especially for women [21]. Genome stability is a key determinant factor for a healthy pregnancy and normal fetal development [22], and genome instability in human lymphocytes could be associated with pre-eclampsia (PE), intrauterine growth restriction (IUGR), high-risk pregnancies [23] and postnatal mental retardation [24]. We conducted this study to compare genomic health between water pipe smokers and nonsmokers women who decided to get pregnant through their family planning program. We also performed this study to find any possible association between increased micronuclei (MNi) and shisha smoking patterns, drinking alcohol habits, women's reproductive health, occupation, and dietary patterns.

We selected age-matched study participants with similar background factors according to our inclusion and exclusion criteria to achieve these goals. We determined the level of genomic instability by Micronuclei (MN) assay in peripheral blood lymphocytes as a reliable method for screening prevalent environmental induced reproductive and endocrine disorders in women [25].

According to our study results, the average frequency of MN in an entire group of 30 women in reproductive ages were all born and lived in Tehran was 17.53±12.53(0-40). This MN frequency was generally comparable with the average frequency of MN in 130 healthy Iranian people in 2016 (6- 21 MN per 1000 binucleated cells) regardless of their history of smoking [26], MNi frequency among female children (0-15 years) according to a survey in Turkey was 4.12 ± 1.867 [27]. Although the effects of age and gender on MNi were suggested for the first time in the mid-1980s and the early 1990s and later by many other laboratories around the world [17,18], we did not find any age-dependent difference in MNi frequency in this small group of women in the similar age range.

The background frequency of micronuclei of 200 subjects from the average-aged Croatian general population in 2019 (38.28 ± 12.83 years) was 5.06 ± 3.11 per 1000 nucleated cells with confirmed association with age, sex, and several lifestyle factors [28]. The MN level of our nonsmoker healthy women was 6.53 ± 3.24844, which was slightly higher than the Croatian population in both genders (5.06 ± 3.11) and young Turkish girls (4.12 ± 1.867). This higher MN frequency suggests the possible role of ambient air pollution in Tehran [29, 30] or other exposure resources, or increased level of depression, stress and anxiety in Iranian women during the outbreak of COVID-19 [31] based on anxiety-associated genotoxic effects**.**

One of the most important scientific achievements of this study was the highly significant difference (p=0.001) between the MNi levels of waterpipe smokers (28.533 ± 7.46292) and nonsmokers (6.53 ± 3.24844), which highlights the importance of WTS on increased levels of MN in women as a sensitive study population. Our cases showed higher levels of binucleated cells with 2MN (1.06 ± 1.0328) compared to controls (0.26 ± 0.45) (p=0.041). However, in these healthy WTS women,

increased micronuclei (MNi) were neither associated with demographic factors (e.g., age, BMI) nor with socioeconomic factors (education, job). History of background diseases and dietary habits also did not change the MNi level in the total population of women. However, smoking-related factors, including age at WTS onset (p=0.003), frequency of WTS (p=0.001), and more extended History of WTS (p=0.003), increased MN levels in a highly significant manner.

Excessive alcohol consumption was another unhealthy lifestyle factor in apparently healthy WTS women. The specific association between alcohol abuse and increased risk of female reproductive cancers and disorders has been a consistent finding in numerous studies because alcohol induces chromosome instability according to cytokinesis-block micronucleus cytometry assay in an in vitro study [32]. The prevalence of alcohol drinking in our control group was 26.7%, while the majority in WTS women was 80% (p=0.009). Mac Nemar's non-parametric test showed a significant association between alcohol abuse patterns and increased MN levels in the total study population (p=0.004). This is the first study that reports and compares increased micronuclei (MNi) between alcohol drinkers and non-drinkers women.

Although our study subjects were non-active smokers and infrequent smoking was not associated with MN (p=0.2), 2MN (p=0.267), and 3MN (p=0.933), close associations were found between secondhand smoking and MNi (p=0.001) and 2MNi (p=0.038) in our total study population (p=0.004). Environmental tobacco smoke (ETS), or secondhand smoke, is a significant source of exposure to many substances that are hazardous to women's health [33]. In parallel to WTS and alcohol drinking, involuntary smoking caused highly significant increased micronuclei (MNi) compared to women with a healthier lifestyle. The evidence of the present study does not support the possible protective role of the dietary factor on genotoxic events (p=0.062). However, more extensive surveys of several hundred women with different nutritional habits may change our opinion from these initial findings.

CONCLUSIONS

Our results showed increased micronuclei (MNi) as an indicator of chromosome instability frequencies in apparently healthy women who were Waterpipe smokers. MNi was influenced by smoking patterns and other exogenous factors especially drinking alcohol and secondhand smoking. Increased micronuclei (MNi) frequencies in childbearing women who decide to get pregnant emerge the necessity of public awareness about this dangerous lifestyle, its developmental risks, and the possible risk of cancer in WTS women. We evaluated the MNi frequency in a sample belonging to the non-occupationally exposed population of Tehran. However, we tried to detect the possible effect of dietary habits, demographic, socioeconomic, and reproductive health factors on genomic damage, but we did not find any meaningful statistical correlation. To improve women's health, especially at reproductive ages, we suggest conducting the same study on MNi frequencies concerning other cities in Iran, its association with the high level of environmental pollution, unhealthy lifestyle, and reproductive factors. We hope our study achievements in the present work could be considered a good stimulus for conducting future bio-monitoring surveys in high-risk people with unhealthy lifestyles living in polluted cities like Tehran.

ACKNOWLEDGEMENTS

The authors are thankful to Novin Medical Radiation Institute for providing lab equipment and a standard study environment. The authors also acknowledge all blood donors who kindly support this survey voluntarily.

ETHICAL CONSIDERATION

This study was approved by the ethics committee of Islamic Azad University, Tehran Medical Sciences (IAUTMU) under the number of IR.IAU.TMU.REC.1399.005

Conflict of interests

The authors declare no competing interests.

Author contribution

Negar Bahrami performed all lab experiments as part of her Pharm., D. thesis. She provided the first draft of this manuscript too. Sepideh Arbabi Bidgoli is the principal supervisor of this study who planned, designed the work,

conducted, performed the statistical analysis, and provided the revised manuscript. Ramin Abrishami was the second supervisor of this thesis, who suggested the main topic and contributed to the planning and study design. Masoumeh Heshmati and Aziz Mahmoudzadeh were co-advisors of this study which contributed to all parts of this study. They read and approved the manuscript before submission.

Availability of data and materials

All data and materials are available upon journal request.

Consent to participate

Not applicable.

Consent for publication

The authors ensure that this Journal and the Publisher have the Author's permission to publish the relevant Contribution.

Funding resource

Not applicable.

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