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Genotoxicity of waterpipe smoking in young adults from Sarajevo, Bosnia & Herzegovina

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ABSTRACT

Background: Waterpipe, also known as a hookah or narghile, is a type of tobacco products consumption device. Recently it has been increasingly popular in Bosnia and Herzegovina and the region. Waterpipe consumers are predominantly adolescents and young adults. Many of them believe in slighter harmful effects of waterpipes, compared to cigarettes. We aimed to determine the DNA damage in oral leukocytes and buccal cells of young individuals who have smoked a waterpipe for more than one year.

Methods: The study group consisted of 40 cigarette non-smokers who regularly smoked a waterpipe on average of once per week. As a control, 40 non-smoking individuals were selected to match smokers for age. All participants in the study were healthy male and female adults from Bosnia and Herzegovina, 18–30 years of age. Before sampling, detailed survey and informed consent have been provided by each participant. Comet assay in oral leukocytes and buccal micronucleus cytome (BMCyt) assay in exfoliated buccal cells were applied.

Results: Almost half of waterpipe smokers (WPS) tasted waterpipe at 15–16 years of age. Comet assay analysis showed increased tail intensity, tail length, and tail moment values among WPS compared to non-smokers (NS) ($p = 0.0001$, $p = 0.0067$, and $p = 0.0001$, respectively). Frequencies of the micronucleated ($p = 0.0004$), binucleated ($p = 0.01$), karyorrhectic, ($p = 0.0036$), and pycnotic cells ($p = 0.03$) were significantly higher in WPS compared to NS group.

Conclusions: Genotoxicity and DNA damage biomarkers were increased in oral leukocytes and exfoliated buccal cells of young waterpipe smokers from Bosnia and Herzegovina, compared to NS group.

1. Introduction

The most common forms of tobacco consumption are cigarettes and waterpipes [1]. Alternative names for waterpipe are hookah in India and Africa, narghile in East Mediterranean countries including Turkey and Syria, shisha, “boory”, or “goza” in Saudi Arabia,

Abbreviations: WPS, waterpipe smokers; NS, non-smokers; BMCyt, buccal micronucleus cytome; AST, atypically sized tails; LTN, long tailed nuclei; TME, tail moment extremes; MNi, micronuclei; NBUDs, nuclear buds.

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Egypt and North African countries. Its popularity has shortly declined in 1980s, with the re-spread related to the introduction of sweetened flavoured tobacco [2]. With the increasing popularity of waterpipes in the last decades, of particular concern is the fact that hookah users are often adolescents and young adults [3]. According to the fourth Global Tobacco Survey (GYTS) in the Federation of Bosnia and Herzegovina, 44.1% of 6415 participants being 13–15 years old, tasted waterpipe; 16.1% consumed it regularly, and only 5.4% of them refused to consume waterpipe considering themselves to young [4].

Waterpipe smoking, equally popular in both sexes, is generally more positively perceived than cigarette smoking; especially among women [5]. In Eastern Mediterranean Region waterpipe smoking has become a behavioural norm for girls [2,6,7] and women, even during pregnancies [8–10]. Waterpipe smokers (WPS) often believe that this type of consumption is less addictive and dangerous than cigarettes [11]. Nevertheless, the types of Harmful and Potentially Harmful Constituents (HPHCs) in waterpipe tobacco are relatively similar to those in cigarette tobacco, including carbon monoxide (CO), nicotine, particulate matter (PM), volatile organic chemicals (VOCs), acrolein, arsenic and heavy metals [12].

Waterpipe smoke contains 4000 chemical materials, most of which are produced while burning [13]. Compared to cigarette consumption, a waterpipe smoking is less frequent (one to four sessions per day) but sessions are longer (15–90 min) and more intense [14]. The uptake of nicotine is equivalent to 2–12 cigarettes per portion of smoking mixture. A regular WPS usually smokes several portions per session and, on average, 2–3 sessions per day [15]. Therefore, intake of nicotine, for most of WPS, is equivalent to more than one pack of cigarettes per session. During one session, WPS inhale as much smoke as a cigarette consumer would inhale consuming 100 or more cigarettes [16]. Exposure to waterpipe smoke constituents can affect health and lead to a variety of adverse health effects, including cancer, cardiovascular disease and addiction [2,17–20].

Genotoxic effects of waterpipe smoke have been studied in human, animal and cell culture models [21–26]. The human buccal mucosal cells and salivary leukocytes have been extensively used for estimation of genotoxic exposure, DNA damage, and impact of nutrition and lifestyle factors [27–29]. Buccal cells are the first barrier to various agents and pathogens in their infiltration into the respiratory and digestive tract. Buccal micronucleus cytome assay (BMCyt) and comet assay on oral leukocytes are non-invasive methods for cell collection and are frequently used in human epidemiological studies [16,30,31].

Regardless of the numerous studies addressing frequency of waterpipe smoking and its genotoxic effects, there is a lack of data related to waterpipe smoking in Bosnia and Herzegovina (BiH). Therefore, this study aimed to determine the genotoxic effects in young adults from BiH who have smoked a waterpipe for more than one year.

2. Material and methods

2.1. Participants

The study group consisted of 40 individuals (17 females; 23 males), cigarette non-smokers who regularly smoke a waterpipe on average once per week. As a control, samples were collected from 40 non-smokers (NS) (33 females; 7 males) matching smokers for age and living area (Sarajevo, the capital of Bosnia and Herzegovina). All participants were healthy adults aged between 18 and 30 years, with average for smokers and non-smokers of 23.95 ± 2.64 and 23.39 ± 2.46, respectively.

Written informed consent was obtained from all participants before inclusion in the study. The questionnaire included socio-demographic data (age and gender), age of initiation and frequency of smoking at the sampling time, the context of use (e.g. home, alone, with friends), quitting willingness and participant's opinion on the harmfulness of waterpipe use compared to cigarettes. Frequency of waterpipe smoking was self-reported and presented as number of sessions per period of time (yearly, monthly, weekly, daily), and in the last month prior sampling, that was used for exposure assessment of WPS. All WPS were accordingly divided into low (<5 sessions), moderate (5–15 sessions) and high (>15 sessions) exposure groups.

Ethics Committee of the University of Sarajevo - Institute for Genetic Engineering and Biotechnology had approved research and experimental procedures (Approval No. 566/20 dated December 18, 2020).

2.2. Chemicals

If not otherwise written, all chemicals used in this study were purchased from the Sigma-Aldrich, St. Louis, MO, USA.

2.3. Comet assay

Saliva samples of each participant were collected in 50 ml tubes after two cycles of 60 s mouth rinsing with saline solution. Cell aggregates were broken and oral leukocytes isolated using density gradient medium Histopaque® 1077. Comet assay was conducted under the alkaline conditions (pH > 13) in order to evaluate primary DNA damage in salivary leukocytes [32,33].

Cell samples in 0.7% low melting point agarose were spread on pre-coated (1% normal melting agarose) duplicate microscope slides per each subject. Slides were incubated overnight in cold lysis solution at +4 °C. Prior to unwinding in a fresh electrophoresis solution (20 min at +4 °C), slides were washed with distilled water. Slides with prepared gels were then subjected to electrophoresis (1 V/cm) for 20 min in the same electrophoresis solution. After electrophoresis, slides were rinsed with PBS (phosphate buffer saline), fixed for 5 min with 70% ethanol and 15 min with absolute ethanol. Prior to analysis, slides were pre-washed with PBS and stained with DAPI (1 μg/ml). DNA damage was observed using a fluorescence Olympus microscope (Olympus BX51, U-MNU2 filter; Tokyo, Japan) at 40 × magnification. Tail intensity – TI (% of DNA in the tail of comets), tail length - TL (length of DNA migration in μm), and tail moment - TM (TL × TI/100) of 200 comets per sample, as descriptors of primary DNA damage, were analyzed using Comet Assay IV

scoring system (Instem, UK) [33]. Results were reported according to MIRCA protocol [34].

Furthermore, based on analyzed parameters (TI, TL, and TM), highly damaged cells (atypically sized tails – AST, long tailed nuclei – LTN, and tail moment extremes – TME) were recorded. Cut–off values were determined as 95th percentile of all nuclei scored [33, 35–37]. AST95, LTN95, and TME95 in the control group (NS) were calculated, and set as threshold levels. Absolute frequencies of highly damaged cells were recorded per each individual in the WPS and NS group.

2.4. Buccal micronucleus cytome assay

Buccal cells were collected by gently scraping buccal mucosa of both cheeks with small-headed plastic collectors that were immediately immersed in buccal cell buffer. After 10 min incubation at room temperature, cells were centrifuged, washed, and fixed in cold fresh solution (ethanol and glacial acetic acid 3:1) for 20 min at +4 °C. Four slides were prepared for each individual by smearing 3–4 drops of cell suspension on the glass surface. Air dried slides were fixed for 1 min in each of 50% (vol/vol) and 20% (vol/vol) ethanol, and washed in dH_2O. After immersion in 5 M HCl for 30 min at room temperature, slides were washed in tap water and treated with Schiff's reagent for 90 min in the dark. Slides were stained with 0.2% Fast Green solution for 2 s, rinsed in dH_2O and air dried overnight [33,38].

Prepared slides were examined under the fluorescence microscope (Olympus BX51, U-MNU2 filter; Tokyo, Japan) at × 1000 magnification. The frequency of differentiated and pyknotic cells, condensed chromatin, karyorrhectic, karyolytic and binucleated cells were determined upon analysis of 1000 cells. The frequency of genotoxicity biomarkers (MNi – micronuclei and NBUDs – nuclear buds) were scored in a minimum of 2000 differentiated cells and reported per 1000 differentiated cells [38,39]. Analysis was done according to scoring criteria proposed by Thomas et al. (2009) [27].

3. Statistical analysis

Independent sample t-test was used to analyse the differences between log– transformed data of the WPS (study) and NS (control) groups, for each of comet assay parameter (TI, TL, and TM), and between absolute frequencies of observed BMCyt assay parameters in both groups. After Kolmogorov-Smirnov distribution assessment, Mann-Whitney test was applied to compare sex subgroups, while differences between age subgroups were compared by Kruskal-Wallis test. Absolute frequencies of highly damaged cells (AST95, LTN95, and TME95) in both groups were tested for significant differences using Mann-Whitney test. Association between estimated exposure of WPS and observed genotoxic effect was calculated by Kendall's tau rank correlation coefficient (τ). Differences between groups are considered significant at $p < 0.05$.

4. Results

Among 80 participants in the study, 29 (36%) were males and 51 (64%) females. The average age ± SD of WPS and NS were 23.95 ± 2.64 and 23.39 ± 2.46, respectively. Results obtained after processing the data collected through the survey conducted among WPS are presented in Table 1. The highest percentage (55%) of participants consumed waterpipe once a week. Many of them (43%) used waterpipe for the first time at the age of 15–16. According to the survey, the highest percentage of participants considers waterpipe smoking less or equally harmful than cigarette smoking. The survey results about the differences between waterpipe and cigarette consumption are provided in Table 2.

Table 1
Survey responses on waterpipe consumption habits of WPS.

N (%)							
Gender	Male 23 (57,5%)		Female 17 (42,5%)				
Age	Mean 23,95		Median 24		Range 18–30		
Frequency of consumption per period of time	Yearly: 2 (5%)		Monthly: 4 (10%)		Weekly: 22 (55%)		Daily: 12 (30%)
Frequency of sessions in the last month prior sampling	0: 3 (8%)	1-2: 4 (10%)	3-5: 5 (13%)	6-9: 7 (18%)	10-15: 3 (8%)	16-20: 5 (13%)	21+: 13 (33%)
WPS exposure (per no of sessions in the last month prior sampling)	Low (<5): 12 (30%)		Moderate (5–15): 10 (25%)			High (>15): 18 (45%)	
Age groups in which respondents tried waterpipe for the first time	14 or less: 3 (8%)	15-16: 17 (43%)	17-18: 11 (28%)		19-20: 4 (10%)	21-22: 1 (3%)	23 or more: 4 (10%)
Cessation of waterpipe consumption	No: 9 (24%)	Next month: 2 (5%)	In the next 6 months: 5 (13%)			In the future: 22 (58%)	
Consuming alcohol with a waterpipe	Never: 33 (83%)	Rarely: 1 (3%)	Sometimes: 1 (3%)		Always: 0	Never tried: 5 (13%)	
Inhaling smoke while waterpipe consumption	Never: 26 (65%)	Rarely: 7 (18%)	Sometimes: 5 (13%)		Always: 2 (5%)	Never tried:0	

4.1. DNA damage in oral leukocytes

Comet assay parameters for the evaluation of DNA damage (TI, TL, and TM) were analyzed in 200 comets per each participant using Comet Assay IV software (Instem, UK). Log-transformed values of TI (%), TL (μm), and TM were compared between WPS and NS groups. Results of independent *t*-test showed significantly higher values of these three comet assay parameters in the WPS group compared to NS group ($p = 0.0001$, $p = 0.0067$, $p = 0.0001$) (Fig. 1). The mean and standard error values of log-transformed data of comet assay parameters and highly damaged cells (AST95, LTN95, and TME95) are summarized in Table 3. Mann-Whitney test revealed significantly higher values of TI ($p = 0.0003$), TL ($p = 0.0024$), and TM ($p = 0.0004$) in the 21–25 years old subgroup of WPS compared to the same NS subgroup. Differences for sex subgroups were not compared because of the low statistical power caused by the low number of males in the NS group ($N = 7$). Absolute frequency values of highly damaged cells (AST95, LTN95, and TME95) were statistically higher in the WPS group ($p < 0.0001$, $p = 0.0011$, $p < 0.0001$) as well (Fig. 2). Kendall's tau rank correlation coefficient showed positive but insignificant association for TI ($\tau = 0.164$, $p = 0.1401$), TL ($\tau = 0.0757$, $p = 0,5008$), TM ($\tau = 0.139$, $p = 0.2127$), AST95 ($\tau = 0.130$, $p = 0.2426$), and TME95 ($\tau = 0.154$, $p = 0.1672$) in exposure subgroups (low, moderate and high).

4.2. Genotoxicity in buccal cells

Results of BMCyt assay (Fig. 3) revealed the significant differences of basal MNi frequency between WPS and NS ($p = 0.0004$) (Fig. 3(A)). The frequency of MNi, as biomarkers of genotoxicity, was significantly higher in the WPS compared to the NS group. Statistical significance was particularly noticeable in participants over 20 years of age (age groups 21–25 and 26–30 years of age). Similarly, in the WPS group the frequencies of cytotoxicity biomarkers, namely binucelar cells, karyorrhectic and pyknotic cells ($p = 0.019$, $p = 0.0036$, $p = 0.03$) were also increased in comparison to the NS group (Fig. 3(C) and (D)). Results of BMCyt assay in both groups are presented in Table 4. Kendall's tau rank correlation coefficient was positive and significant for karyorrhectic cells ($\tau = 0.259$, $p = 0.0193$) in all exposure subgroups (low, moderate and high) of WPS. For basal ($\tau = 0.0953$, $p = 0.3958$), condensed chromatin ($\tau = 0.0811$, $p = 0.4707$), and pycnotic cells ($\tau = 0.0543$, $p = 0.6321$) association with exposure was positive and insignificant while negative insignificant correlation was found for BMCyt biomarkers; MNi ($\tau = -0.0385$, $p = 0.7153$), binuclear ($\tau = -0.0647$, $p = 0.5446$) and karyolitic cells ($\tau = -0.0247$, $p = 0.7918$).

5. Discussion

Waterpipe smoking has been extensively practiced for more than 400 years, especially in Arabic countries, Turkey, India and Pakistan [40]. Waterpipe smoking and electronic cigarette consumption has significantly emerged [41,42] with a high prevalence of waterpipe smoking among adolescents, mainly university students. Registered frequencies of adolescent WPS are 36.11% in the Arabian countries (especially Palestine), 20.23% in Turkey and 18% in Siria [43,44]. Very high prevalence (43.8%) has been recorded in Iran young adults, mainly men (18–24 years old) with university education [45]. Recent study, presenting data from 72 countries but not including Bosnia and Herzegovina, showed following waterpipe smoking prevalence among young adolescents, 11–16 years of age: European region (10.9%), Eastern Mediterranean region (10.7%), Western Pacific region (1.9%) [46]. According to the latest available data in the Federation of Bosnia and Herzegovina, conducted in 2019, with the majority of participants being 13–16 years old, 44.1% of them smoked waterpipe [4]. According to the study conducted by Brankovic et al. (2017) 21.8% out of 410 included university students have been consuming tobacco products [47]. In our study, we found that 43% of WPS participants used waterpipe for the first time at the age of 15 or 16. The average initiation age of 12.9 years was reported among Lebanese adolescents [48]. In regards to sex distribution, our study included 36% of male and 64% of female participants, with 42.5% of female smokers. According to Ramic-Catak et al. [4], 39.9% of female school children ever smoked waterpipe, while no differences in frequencies of currently smoking males (17.7%) and females (14.4%) were found.

Many of WPS (55%) included in our study consumed waterpipe at least once a week. Majority of WPS consider harmfulness of waterpipe smoking equal or lower compared to those of cigarette smoking. 50% of participants believed that waterpipe smoking results in lower nicotine intake while 42% and 28% believe in lower tar and carcinogens intake, respectively. However, it has been suggested that WPS daily absorb as much nicotine as a cigarette smoker in 10 days [49]. Waterpipe smoke contains higher level of arsenic, chromium and lead, and 20 times more tar than a single low-tar cigarette [50]. Schubert et al. (2015) reported that

Table 2
Survey responses about the differences between waterpipe and cigarettes consumption (all respondents included).

Answers			
Questions	Less:	Equally:	More:
Addictive	33 (41%)	43 (54%)	4 (5%)
Harmfulness of consumption	19 (24%)	42 (53%)	19 (24%)
Nicotine content	40 (50%)	24 (30%)	16 (20%)
Tar content	33 (42%)	25 (32%)	21 (27%)
Carcinogenic exposure	22 (28%)	15 (51%)	17 (21%)
Harmfulness of passive consumption	35 (44%)	36 (45%)	9 (11%)
Harmfulness of consumption to the fetus	14 (18%)	47 (59%)	19 (24%)

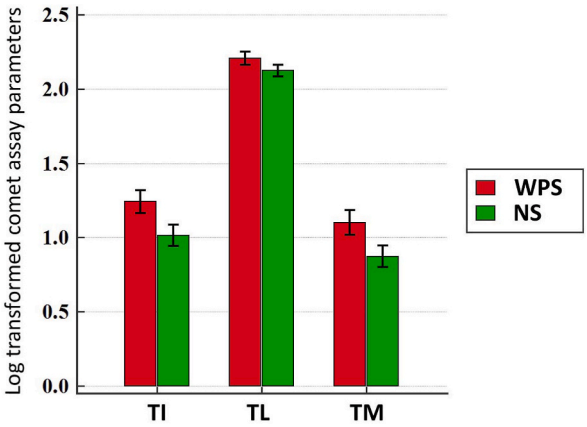

Fig. 1. Log-transformed comet assay values for TI, TL and TM parameters for the WPS and NS groups.

Table 3
Comet assay (tail intensity-TI, tail length-TL, tail moment-TM) and highly damaged cells (atypically sized tails-AST, long tailed nuclei-LTN, tail moment extremes-TME) parameters (log transformed data) in WPS and NS groups (mean ± SD).

Comet assay parameters	All	Male	Female	p	Age groups			
	TI (%)				≤20	21–25	26–30	p
WPS	1.24 ± 0.24	1.26 ± 0.21	1.21 ± 0.27	0.73	1.21 ± 0.16	1.21 ± 0.25	1.31 ± 0.24	0.47
NS	1.01 ± 0.22	1.18 ± 0.34	0.97 ± 0.17	0.15	1.09 ± 0.28	0.96 ± 0.19	1.10 ± 0.25	0.22
p value	0.0001*	0.86	0.0025*		0.48	0.0003*	0.06	
	TL (μm)							
WPS	2.20 ± 0.14	2.21 ± 0.11	2.19 ± 0.17	0.98	2.10 ± 0.13	2.21 ± 0.13	2.22 ± 0.14	0.22
NS	2.12 ± 0.12	2.19 ± 0.14	2.10 ± 0.11	0.13	2.16 ± 0.14	2.10 ± 0.11	2.14 ± 0.13	0.69
p value	0.0067*	0.78	0.058		0.48	0.0024*	0.21	
	TM							
WPS	1.10 ± 0.25	1.12 ± 0.23	1.07 ± 0.29	0.52	1.02 ± 0.20	1.08 ± 0.27	1.16 ± 0.23	0.61
NS	0.87 ± 0.22	1.04 ± 0.34	0.83 ± 0.18	0.18	0.96 ± 0.28	0.82 ± 0.20	0.95 ± 0.26	0.42
p value	0.0001*	0.78	0.0038*		1.00	0.0004*	0.10	
	AST95							
WPS	36.95 ± 30.11	39.39 ± 30.67	33.64 ± 29.94	0.41	35.00 ± 21.00	35.28 ± 32.71	42.20 ± 27.72	0.56
NS	10.00 ± 12.13	15.85 ± 15.48	8.75 ± 11.20	0.28	11.00 ± 6.05	8.76 ± 13.47	12.80 ± 10.40	0.14
p value	<0.0001*	0.0470*	0.0003*		0.11	<0.0001*	0.0091*	
	LTN95							
WPS	26.00 ± 26.41	23.82 ± 23.23	28.94 ± 30.69	0.70	13.50 ± 20.72	26.92 ± 27.17	28.30 ± 26.67	0.51
NS	10.12 ± 15.66	18.14 ± 20.26	8.42 ± 14.31	0.19	13.50 ± 16.34	9.00 ± 16.30	11.70 ± 14.96	0.44
p value	0.0011*	0.47	0.0052*		0.65	0.0019*	0.095	
	TME95							
WPS	36.15 ± 33.26	37.86 ± 28.61	33.82 ± 39.51	0.32	25.50 ± 30.38	36.76 ± 35.75	38.80 ± 29.63	0.68
NS	10.12 ± 14.60	22.42 ± 22.48	7.5 ± 11.18	0.24	16.75 ± 17.46	8.03 ± 14.20	12.90 ± 14.89	0.18
p value	<0.0001*	0.24	0.0004*		0.68	<0.0001*	0.0126*	

WPS – waterpipe smokers (N = 40); NS – non-smokers (N = 40); * - significantly different between WPS and NS ($p < 0.05$); ** - significantly different between males and females.

mainstream waterpipe smoke contains 6.2 times higher levels of human carcinogen benzene which presents serious health hazard [51]. Analyses of urine samples of WPS before and after smoking, revealed an average 73-fold increase in nicotine, a 4-fold increase in cotinine, a 2-fold increase in 4-(methylnitrosamino)-1-(3-pyridyl)- 1-butanol (NNAL), and 14%–91% increase in mercapturic acid metabolites of volatile organic compounds immediately following waterpipe smoking [52].

Waterpipe smoke also contains high doses of small particulate matter ($PM_{2.5}$) which play key role in damaging the cardio and respiratory systems [53]. Waterpipe smoking has been significantly associated with lung cancer, respiratory illness, low birth weight, and periodontal disease [11]. Findings of our study indicated that more than half of waterpipe users (57.5%; N = 23) believe that probability to cause addiction is lower compared to cigarette smoking. However, even a light exposure to nicotine has been associated with the addiction development, particularly in young people [54].

In this study, we examined the DNA damage induced by waterpipe smoking in oral leukocytes by comet assay and in buccal exfoliated cells by BMCyt assays. Results of comet assay showed that all observed parameters (TI, TL, TM, AST95, LTN95, and TME95) were significantly higher in WPS, especially in the 21–25 years of age subgroup, compared to the NS group. Majority of WPS belonged to this subgroup (62.5%). The WPS and NS subgroups of participants below 20 years of age consisted of 4 participants each, while the 26–30 years of age subgroups comprise of 10 participants each, therefore the statistical relevance for these subgroups is low.

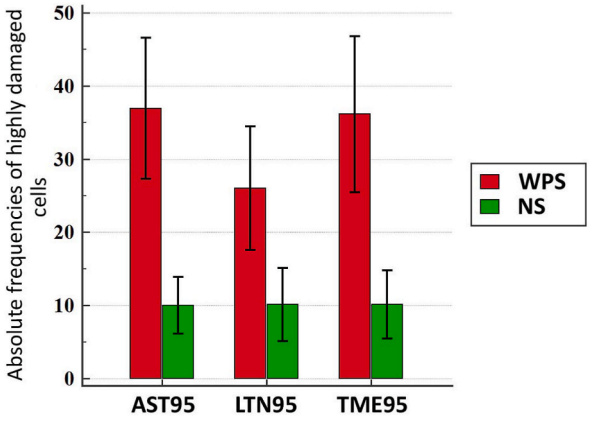

Fig. 2. Mean values of absolute frequencies of highly damaged cells (AST95, LTN95, TME95) for the WPS and NS groups.

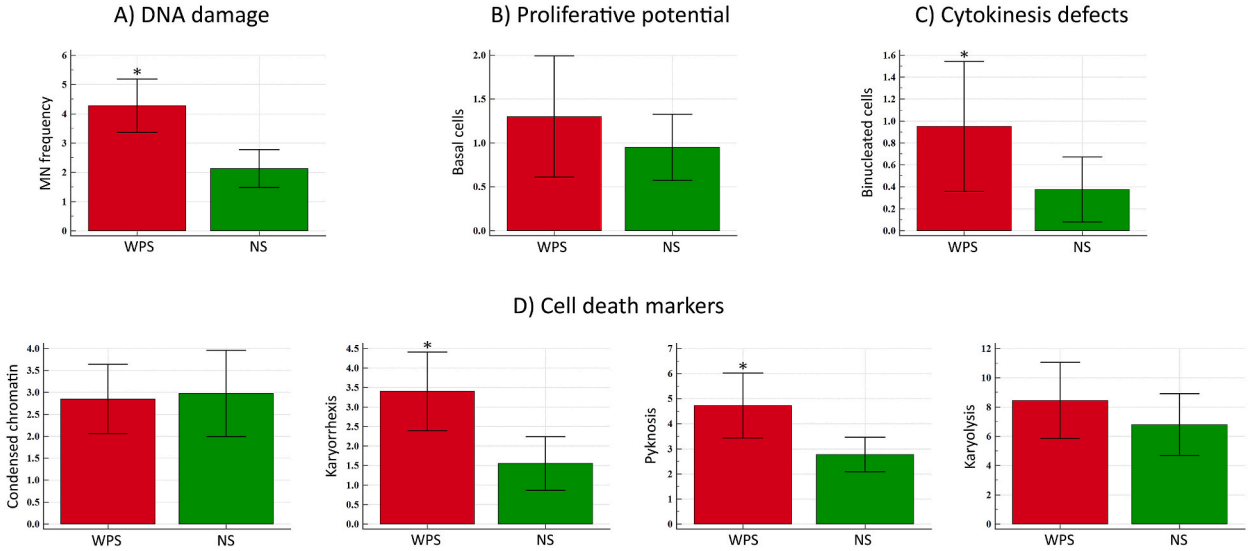

Fig. 3. The median ± SD of BMCyt assay parameters: (A) DNA damage-frequency of MN; (B) proliferative potential-frequency of basal cells; (C) cytokinesis defects-binucleated cells; (D) cell death markers-condensed chromatin, karyorrhexis, pyknosis, and karyolysis. *Significantly different at $p < 0.05$.

Additionally, according to the survey, the majority of participants from the 26–30 years of age subgroup started to consume waterpipes at 23 years of age, while only one of participants in this subgroup started with waterpipe smoking in the ages from 15 to 16.

In accordance with our results, Al-Amrah et al. (2014) also reported significantly higher DNA damage observed by comet assay in buccal cells of WPS. Furthermore, comet assay results on peripheral blood leukocytes *in vitro* showed significant DNA damage after treatment with waterpipe smoke condensate [16]. Comet assay in mice, chronically exposed to waterpipe smoke for six months, also revealed significant lung DNA damage [55]. Waterpipe smoking is also being associated with global epigenetic changes and DNA methylation of tumor-suppressor gene *MHL1* promoter [56].

Higher genotoxic damage in buccal cells of WPS was also recorded in this study by BMCyt assay. Our results are also aligned with previous studies reporting higher MN frequency in buccal mucosa cells of WPS [30,57–61]. Recent study conducted by Salih et al. (2022) confirmed the highest MN frequency association with waterpipe consumption when compared to cigarette smoking and not smoking at all [62]. Previous analysis of MN frequency in peripheral blood lymphocytes and exfoliated cells of cigarette smokers from Bosnia and Herzegovina revealed significant positive correlation, confirming reliability of BMCyt assay use in human monitoring [63]. Study by Khabour et al. (2011) showed a significant increase in sister chromatid exchanges (SCEs) in the lymphocytes of WPS compared with cigarette smokers, indicating that waterpipe smoking is more genotoxic [64]. Derici Eker et al. [57] and Alsatari et al. [65] reported significantly higher frequency of gaps and aberrations in lymphocytes of WPS than cigarette smokers, with DNA damage being dose-dependent [66]. Additionally, we also found increased frequency of binuclear, karyorrhectic and pycnotic cells in WPS compared to NS, indicating cytokinesis defects and cell death process. Frequency of karyorrhectic cells significantly correlated ($\tau = 0.259$, $p = 0.0193$) with waterpipe exposure in the WPS group. Karyorrhexis as the occurrence of nuclear fragmentation, leads to the

Table 4
Results of BMCyt biomarkers in WPS and NS groups (mean ± SD).

Biomarkers of BMCyt	All	Male	Female	p	Age groups			
	Micronuclei				≤20	21–25	26–30	p
WPS	4.27 ± 2.84	4.60 ± 2.75	3.82 ± 2.98	0.27	5.00 ± 4.08	4.00 ± 2.43	4.70 ± 3.52	0.96
NS	2.12 ± 2.02	3.00 ± 2.00	1.93 ± 2.01	0.14	1.50 ± 2.38	2.38 ± 2.04	1.70 ± 1.94	0.52
p value	0.0004*	0.20	0.02*		0.11	0.01*	0.04*	
	Basal cells							
WPS	1.30 ± 2.16	1.00 ± 1.59	1.70 ± 2.75	0.41	1.50 ± 1.19	1.57 ± 2.50	0.50 ± 0.84	0.36
NS	0.95 ± 1.17	0.57 ± 1.13	1.03 ± 1.18	0.24	0.00 ± 0.00	1.15 ± 1.28	0.80 ± 0.91	0.10
p value	0.96	0.49	0.69		0.18	0.94	0.42	
	Binuclear cells							
WPS	0.95 ± 1.85	0.69 ± 1.01	1.29 ± 2.59	0.41	1.00 ± 1.41	1.07 ± 2.20	0.60 ± 0.69	0.94
NS	0.37 ± 0.92	0.14 ± 0.37	0.42 ± 1.00	0.54	0.00 ± 0.00	0.50 ± 1.10	0.20 ± 0.42	0.45
p value	0.019*	0.20	0.0255*		0.18	0.15	0.16	
	Karyolysis							
WPS	8.45 ± 8.14	9.43 ± 8.05	7.11 ± 8.30	0.22	16.25 ± 14.52	7.34 ± 6.22	8.20 ± 8.87	0.53
NS	6.80 ± 6.59	7.20 ± 4.46	6.69 ± 7.01	0.46	4.00 ± 5.41	7.46 ± 6.74	6.20 ± 6.86	0.35
p value	0.35	0.78	0.86		0.31	0.84	0.57	
	Karyorrhexis							
WPS	3.40 ± 3.14	3.08 ± 2.69	3.82 ± 3.71	0.66	1.75 ± 2.87	3.26 ± 2.89	4.40 ± 3.80	0.35
NS	1.55 ± 2.14	1.57 ± 1.51	1.54 ± 2.27	0.49	0.75 ± 0.95	1.57 ± 2.04	1.80 ± 2.78	0.70
p value	0.0036*	0.21	0.0113*		0.88	0.0119*	0.13	
	Condensed chromatin							
WPS	2.85 ± 2.47	3.21 ± 2.61	2.35 ± 2.26	0.27	1.50 ± 1.73	2.88 ± 2.61	3.30 ± 2.35	0.23
NS	2.97 ± 3.05	0.85 ± 1.21	3.42 ± 3.15	0.02*	1.00 ± 0.81	3.42 ± 3.30	2.50 ± 2.75	0.31
p value	0.71	0.016*	0.36		0.88	0.75	0.25	
	Pyknosis							
WPS	4.72 ± 4.03	5.56 ± 4.05	3.58 ± 3.84	0.07	6.50 ± 5.68	4.34 ± 4.05	5.00 ± 3.49	0.57
NS	2.77 ± 2.15	3.14 ± 2.54	2.69 ± 2.09	0.80	3.00 ± 3.16	2.88 ± 1.70	3.00 ± 3.19	0.78
p value	0.0310*	0.11	0.81		0.38	0.35	0.12	

WPS – waterpipe smokers (N = 40); NS – non-smokers (N = 40); * - significantly different between WPS and NS ($p < 0.05$); ** - significantly different between males and females.

eventual disintegration of the nucleus [27] and presents a biomarker often increased in WPS compared to NS [67]. Nonsignificant positive correlation between other comet assay parameters as well as basal, condensed chromatin and pycnotic buccal cells, and WPS exposure indicates increase in DNA damage and cytotoxicity with intensified waterpipe smoking [67]. Statistical significance was not found, probably due to the smaller number of WPS samples. Likewise, nonsignificant negative correlation between WPS exposure groups and some parameters (LTN95, MN, binuclear and karyolytic cells) was probably the result of unequal distribution of participants within low, moderate and high exposure subgroups. Additional limitation of this study is the lack of measurement of the proportions of nicotine, organic compounds and metabolites in the blood, saliva and urine of the participants, which could contribute to a better representation of the genotoxicity and cytotoxicity of waterpipe smoking.

6. Conclusion

DNA damage in oral leukocytes and exfoliated buccal cells of waterpipe smokers is higher compared to those not consuming waterpipes. Because of the frequent waterpipe use in teenagers and young adults, potentially resulting in addiction and adverse health effects, it is important to implement continuous monitoring in order to identify early genotoxic events.

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Ethics

Committee of the University of Sarajevo - Institute for Genetic Engineering and Biotechnology had approved research and experimental procedures (Approval No. 566/20 dated December 18, 2020).

Autors' contribution

Tamara Cetkovic Pecar: Conceived and designed the experiments; performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Anja Haveric: Analyzed and interpreted the data; wrote the paper.

Lejla Caluk Klacar: Analyzed and interpreted the data; performed the experiments.

Sanin Haveric: Analyzed and interpreted the data.

Alen Dzaferspahic, Mahira Mehanovic, Irma Durmisevic, and Selma Behmen: Performed the experiments.
Maida Hadzic Omanovic: Conceived and designed the experiments; contributed reagents, materials, analysis tools or data.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e17073.

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