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Effect of cigarette smoking on DNA damage according to nine comet assay parameters in female and male groups*

Dokuz comet parametresine göre kadın ve erkek gruplarda sigara içiminin DNA hasarına etkisi

Esma Söylemez, Zeliha Kayaaltı, Vugar Aliyev, Tülin Söylemezoğlu

Ankara Üniversitesi Adli Bilimler Enstitüsü

**This study is presented as poster in 47th Congress of the European Societies of Toxicology, 2011, Paris, France.*

Aim: Smoking poses a serious threat to public health. The aim of this study was to investigate the relationship between the smoking and DNA damage in lymphocytes. A potential genotoxic effect of cigarette smoking was analyzed with the nine comet assay parameters including comet length (CL), comet intensity (CI), head length (HL), head intensity (HI), tail length (TL), tail intensity (TI), DNA tail (DNAt), tail moment (TM) and olive tail moment (OTM). For the first time in this study, smokers were grouped as female and male, and nine comet parameters were used.

Material and Method: 120 volunteers (60 non-smokers, 60 smokers) were monitored in the way of DNA damage in blood lymphocytes. The levels of DNA damage was measured by BAB Bs Comet Assay system.

Results: The highly significant associations were found between the non-smoker and smoker groups for CI, TL and OTM comet parameters($p<0.01$). Smoker female group had higher CL, CI, HL, HI, TL, TI ($p<0.01$) and TM ($p<0.05$) with regards to DNA damages than non-smoker female group. In contrast, only DNAt, and OTM comet parameters were statistically differences between the smoker male and non-smoker male groups ($p<0.05$). When the smoking index (SI) of all the blood samples from females were compared based on all studied comet parameters, statistically significant association was found except for TM. On the other hand, the blood samples taken from male were significant in terms of CL, HL, HI, TI and OTM parameters ($p<0.05$).

Conclusion: Consequently, it can be said that, smoking cause the DNA damages and females are more sensitive than males against to effect of the smoking.

Keywords: ***Comet assay, DNA damage, smoking.***

Amaç: Sigara kullanımı, halk sağlığı için ciddi bir tehdit oluşturmaktadır. Çalışmamızın amacı, sigara içimi ile lenfosit hücrelerinde DNA hasarı arasındaki ilişkiyi araştırmaktır. Sigaranın potansiyel genotoksik etkisi, comet length (CL), comet intensity (CI), head length (HL), head intensity (HI), tail length (TL), tail intensity (TI), DNA tail (DNAt), tail moment (TM) and olive tail moment (OTM) gibi dokuz comet assay parametresi ile analiz edilmiştir. İlk kez bu çalışmada sigara içenler kadın ve erkek olarak gruplara ayrılmış ve 9 comet parametresine göre değerlendirilme yapılmıştır.

Materyal ve Metod: 120 gönüllü birey (60 sigara içmeyen, 60 sigara içen) kan lenfosit hücrelerindeki DNA hasarları izlenmiştir. DNA hasar dereceleri BAB Bs Comet Assay sistemi ile ölçülmüştür.

Bulgular: Sigara içen ve içmeyen gruplar arasında CI, TL ve OTM comet parametreleri açısından yüksek derecede anlamlılık bulundu ($p<0.01$). Sigara içen kadınların içmeyenlere oranla DNA hasarı CL, CI, HL, HI, TL, TI ($p<0.01$) ve TM ($p<0.05$) parametrelerine göre daha fazladır. Buna karşın, sigara içen ve içmeyen erkek grupları arasında DNAt ve OTM parametreleri açısından anlamlılık gözlenmiştir ($p<0.05$). Sigara indeksi açısından kadınlarda tüm kan örnekleri çalışılan comet parametreleri ile karşılaştırıldığında, TM dışındaki bütün parametreler ile istatistiksel olarak anlamlılık bulunmuştur ($p<0.05$). Diğer taraftan, erkek grubundaki kan örneklerinde CL, HL, HI, TI ve OTM'de anlamlılık gözlenmiştir ($p<0.05$).

Sonuç: Netice olarak, sigaranın DNA hasarlarına sebep olduğu ve kadınların, sigaranın zararlı etkilerine karşı daha duyarlı olduğu söylenilebilir.

Anahtar Sözcükler: ***Comet Assay, DNA hasarı, Sigara içimi.***

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Corresponding Author

Zeliha Kayaaltı
Institute of Forensic Sciences, Ankara University, Ankara, Turkey
Phone : 0 312 319 27 34
GSM : 0 505 366 38 48
Fax : 0 312 319 20 77
E-mail : kayaalti@ankara.edu.tr

Cigarette is a complex mixture of over 4800 chemical compounds, including a high concentration of oxidants, heavy metals, and carcinogens (1, 2). Smoking poses a serious threat to public health (3). Smoke induced-lung tumor has become one of the malignancies with highest incidence and mortality worldwide (4). Extrapolating from the mortality due to smoking rates in 1985, and taking into account population growth, approximately 3-4 million deaths in developed countries from cigarette is anticipated in 2025 (5).

The mechanism by which smoking induces damage is not known for all diseases. One mechanism believed to play a role is oxidative stress. Oxidative stress leads to cellular damage including DNA damage. The term oxidative stress is widely used in the literature, but not very well defined. Oxidative stress occurs when the amount of reactive oxygen species (ROS) generated in cells exceeds the capacity of normal detoxification systems (6,7). The importance of DNA oxidations is emphasized by their mutagenic potential, although there are multiple additional roles in aging and cancer, including, e.g., mitochondrial function, microsatellite instability and telomere shortening (8). Cigarette smoking has been investigated as a major risk factor for renal cell carcinoma (RCC) and squamous cell carcinoma of the head and neck (9). According to a meta-analysis conducted by Hunt and co-workers, ever smokers were increased risk of RCC compared with lifetime never smokers (10).

The alkaline single cell gel electrophoresis (SCGE) technique is highly effective in revealing the association between DNA damage and environmental, genetic, and acquired factors, providing further data on the possible applicability of this assay in genotoxic human surveillance in addition to established tests (11). SCGE, also known as comet assay, is now a well-established genotoxicity test (12).

The comet assay is based on the ability of negatively charged fragments of DNA to be drawn through an agarose gel in response to an electric field. The extent of DNA migration depends directly on the DNA damage present in the cells (13). In order to measure DNA single-strand breaks (14), alkali-labile sites and DNA cross-linking in individual cells, this assay is used. It is applied to both in vivo and in vitro studies for many cells (15). The assay works on the principle that free radicals such as ROS cause breaks in the DNA (16,17). Using this assay we could potentially identify individuals with high levels of residual damage (18). To better characterize the suitability of the comet assay for biomonitoring, we are performing an extensive investigation on blood samples from smokers and non-smokers. Because tobacco smoke is a well-documented source of a variety of potentially mutagenic and carcinogenic compounds (19). In the literature, there are many studies investigating the relationship between smoking and DNA damage. But, our study is the first to investigate the relationship between the smoking and DNA damage separately in lymphocytes for smoker female and male groups according to nine comet assay parameters such as comet length (CL), comet intensity (CI), head length (HL), head intensity (HI), tail length (TL), tail intensity (TI), DNA tail (DNAt), tail moment (TM) and olive tail moment (OTM).

MATERIAL AND METHODS

Study subjects

In the study, 60 smokers (30 females and 30 males) and 60 non-smokers (30 females and 30 males), mean ages were 33.32±8.38 years ranging between 21 and 59 years, were monitored in the way of DNA damage in blood lymphocytes. However, all study subjects were grouped as non-smokers (SI=0; n=60), light smokers (SI=1-400; n=50), and heavy smokers (SI=401-800; n=10), mean ages were 33.55±9.60, 31.40±5.73 and 41.60±6.96 years, respectively. Smokers averaged 14.75 cigarettes per day (range 2-50 cigarettes per day) in our study and none of them did use the any cigarette holders. Informed consent was obtained from each individual who were selected randomly as a control group sample from Turkish population. A small questionnaire for gathering the demographic and ethnic information was also given to the individuals, and the individuals stating themselves as Turkish were included in the study. Each subject filled in detailed questionnaires regarding confounding factors for DNA damage such as smoking. The study samples comprise healthy volunteers whose histories revealed non-cancer or **no consumption of alcohol or chronic disease, no diet, no continuous use of drugs, no UV and X-ray exposure,** no occupational exposure to fuels or other chemicals and they were matched for age and gender.

Comet assay

A potential genotoxic effect of cigarette smoking was analyzed with the comet assay. CL, CI, HL, HI, TL, TI, DNAt, TM and OTM defined on comet assay were used. The levels of DNA damage was measured by BAB Bs Comet Assay system.

The comet assay was conducted under alkaline conditions with some modifications, basically as described by Singh et al. (1988). In brief, conventional microscope slides were covered with a first layer of 0.5% normal agarose. Isolated and washed lymphocytes with washing buffer. Then, a 50 µl aliquot of the cell sample was mixed with 100 µl of 0.5% low melting point agarose and added to the slides which were then immediately covered with coverslips. After removing the cover- glass, all slides were immersed for 1h at 4 °C in a lysing solution (2.5M NaCl, 100

mM EDTA, 10 mM Tris, NaOH to pH 10, 1% N-Lauryl Sarcosine, to which 1% Triton X-100 and 10% DMSO were freshly added) in the dark. The slides were placed in an electrophoresis tank containing freshly prepared alkaline buffer (300 mM NaOH, 1 mM EDTA, pH > 13)), and the electrophoresis was conducted at room temperature during 20 min at 300 mA and 25 V. After the stage of electrophoresis, the slides were taken from the tank and washed for three times during 5 min with neutralizing buffer (0.4 M Tris, pH 7.5). Afterwards, each was washed with ethanol for the same time and the period as in the buffer in order to do fixation. Finally, DNAs were stained with ethidium bromide (60 µl of a 20 µl/ml). Two slides were prepared for each sample, and randomly chosen 50 cells were measured by Comet Assay BAB Bs automatic image analysis system fitted with an Olympus BX50 fluorescence microscope (Figure 1). All results were evaluated in terms of nine image-analysis parameters.

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Figure 1. Representative comet assays of cells.

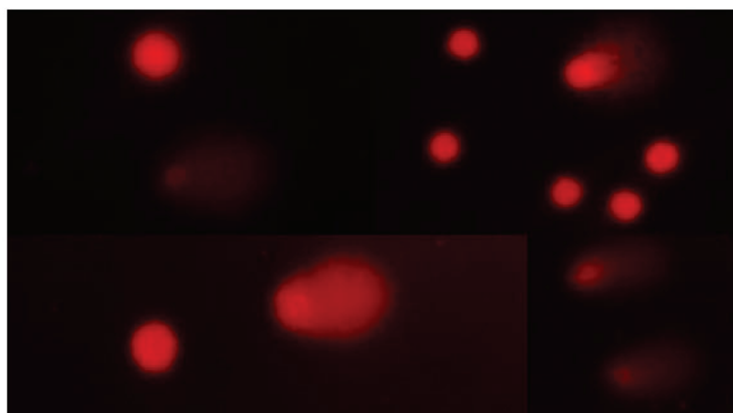

Table 1. The results of the comet parameters in non-smoker and smoker groups.

COMET PARAMETERS	Control group n=60 (Mean ± SD)	Smoking group n=60 (Mean ± SD)	p
Comet Length	25.53±1.26	26.60±4.59	>0.05
Comet Intensity	53581.33±3952.60	62457.73±25631.69	**<0.01***
Head Length	16.01±0.74	16.17±2.32	>0.05
Head Intensity	83080.74±5916.58	85652.73±21829.43	>0,05
Tail Length	4.94±0.32	5.47±1.55	**<0.01***
Tail Intensity	37624.85±3779.95	38335.78±10852.39	>0.05
DNA Tail	78.83±7.88	72.02±25.71	>0.05
Tail Moment	3.39±0.29	3.40±0.79	>0.05
Olive Tail Moment	603.83±513.90	387.77±367.54	**<0.01***

Statistical analyses

The Statistical Package for Social Sciences (SPSS) version 16.0 software was used for the statistical analyses. While the mean differences between two groups were compared by using the Student t-test, the Mann Whitney U test was applied for the comparison of median values. The Kruskal-Wallis analysis of variance was utilized for the comparison of more than two groups in terms of metric variables. Apart from all significant tests, Pearson correlation was computed for age and for all comet parameters. Smoking Index (SI) was calculated as cigarettes smoked per day x years of smoking. P values less than 0.05 were considered to be statistically significant.

RESULTS

In this study, 60 smokers and 60 control subjects were determined using a nine comet assay parameters in terms of DNA damage and the results were statistically analyzed according to smoking, age, gender and SI groups. Hereunder, comet assay effects of non-smokers and smokers samples of blood lymphocytes are given in Table 1. The highly significant associations were found between the non-smoker and smoker groups for CI, TL and OTM comet assay parameters (p<0.01), however there is not any statistical significance for the other comet assay parameters.

Table 2. The comet assay parameters for females and males in non-smokers and smoker groups.

	Female			Male		
	Control group (Mean±SD) (n =30)	**Smoking group (Mean±SD) (n=30)**	**p**	**Control group (Mean±SD) (n =30)**	**Smoking group (Mean±SD) (n=30)**	**p**
Comet Length	25.48±1.31	27.66±4.15	**<0.01***	25.58±1.22	25.54±4.83	>0.05
Comet Intensity	53518.84±4453.24	65944.78±24412.60	**<0.01***	53643.82±3456.20	58970.68±26748.63	>0.05
Head Length	15.90±0.70	16.97±2.07	**<0.01***	16.11±0.77	15.36±2.31	>0.05
Head Intensity	81501.92±6123.98	93780.03±21551.04	**<0.01***	84659.56±5344.03	77525.44±19192.99	>0.05
Tail Length	4.97±0.34	5.60±1.40	**<0.01***	4.91±0.29	5.33±1.70	>0.05
Tail Intensity	36483.84±3376.3	41573.08±9035.40	**<0.01***	38765.86±3869.83	35098.48±11674.27	>0.05
DNA Tail	76.59±6.93	75.31±27.10	>0.05	81.08±8.24	68.74±24.24	**<0.05***
Tail Moment	3.31±0.26	3.59±0.70	**<0.05***	3.47±0.30	3.21±0.84	>0.05
Olive Tail Moment	499.46±441.17	348.79±294.10	>0.05	708.21±565.80	426.76±430.33	**<0.05***

Table 3. Pearson's correlation coefficient for all comet assay parameters.

Comet parameters								
Comet Length	**Comet Length**							
Comet Intensity	**p<0.01***	**Comet Intensity**						
Head Length	**p<0.01***	**p<0.01***	**Head Length**					
Head Intensity	**p<0.01***	**p<0.01***	**p<0.01***	**Head Intensity**				
Tail Length	**p<0.01***	**p<0.01***	**p<0.01***	**p<0.01***	**Tail Length**			
Tail Intensity	**p<0.01***	**p<0.01***	**p<0.01***	**p<0.01***	**p<0.01***	**Tail Intensity**		
DNA Tail	**p<0.01***	**p<0.01***	p>0.05	p>0.05	**p<0.01***	**p<0.01***	**DNA Tail**	
Tail Mo-ment	**p<0.01***	p>0.05	**p<0.01***	**p<0.01***	p>0.05	**p<0.01***	**p<0.01***	**Tail Moment**
Olive Tail Moment	**p<0.01***	**p<0.01***	p>0.05	p>0.05	**p<0.01***	p>0.05	**p<0.01***	**p<0.01***

When the nine comet assay parameters were evaluated among the females and males in the non-smoker and smoker groups, smoker female group had higher CL, CI, HL, HI, TL, TI ($p<0.01$) and TM ($p<0.05$) with regards to DNA damages than non-smoker female group. In contrast, only DNAt and OTM comet parameters were statistically differences between the smoker male and non-smoker male groups ($p< 0.05$) (Table 2). Not surprisingly, the nine comet assay parameters were evaluated for females and males in the smoker groups and, the significant associations were found between this gender groups for CL, CI, HL, HI, TI and OTM comet parameters ($p<0.05$).

When the correlation coefficients were calculated with each other all the comet parameters, the statistically significant correlation was found in twenty-nine of thirty six correlations. Only seven correlations (CI and TM; HL and DT; HL and OTM; HI and DT; HI and OTM; TL and TM; TI and OTM) were not statistically significant. The correlation coefficients for all comet assay parameters were presented in Table 3.

All study subjects were grouped according to their smoking habit and smoking levels as non-smokers, light smokers (1-400) and heavy smokers (401-800). Smoking index (SI) of all the blood samples were compared based on all studied comet parameters and it was found statistically significant association between the SI and only OTM comet parameters ($p<0.05$), in addition to SI and age ($p<0.01$) (Table 4). However, it was found statistically significant association between the SI and all studied comet parameters ($p<0.05$) except for TM in females. On the other hand, the blood

Table 4. The results of the comet parameters and age in non-smoker and smoking index groups.

SAMPLES			Age	Comet Length	Comet Intensity	Head Length	Head Intensity	Tail Length	Tail Intensity	DNA Tail	Tail Moment	OliveTail Moment
Non-smokers	(n=60)	Mean	33.55	25.53	53581.33	16.01	83080.74	4.94	37624.85	78.83	3.39	603.83
		S.D.	9.60	1.26	3952.60	0.74	5916.58	0.32	3779.95	7.88	0.29	513.90
		Min.	21.00	23.64	45291.05	14.82	72402.93	4.43	27538.29	57.33	2.52	107.59
		Max.	59.00	30.40	64659.64	19.36	102773.17	6.12	49214.68	100.11	4.26	2591.35
Smoking Index (SI)	Light Smoker 1-400 (n=50)	Mean	31.40	26.54	62514.16	16.11	85170.78	5.47	38691.85	72.99	3.43	412.14
		S.D.	5.73	4.69	26508.64	2.37	22047.31	1.63	11150.09	26.28	0.81	385.32
		Min.	22.00	14.05	33379.08	9.27	39234.02	2.50	15399.27	29.49	1.88	12.01
		Max.	46.00	37.75	148115.68	21.06	129994.58	10.58	68609.70	137.41	5.10	1665.09
	Heavy Smoker 401-800 (n=10)	Mean	41.60	26.91	62175.58	16.42	88062.48	5.45	36555.40	67.18	3.26	265.92
		S.D.	6.96	4.28	21930.12	2.17	21672.09	1.17	9537.98	23.30	0.70	239.59
		Min.	27.00	20.40	32859.13	12.93	55870.73	3.87	22810.29	36.50	2.05	11.70
		Max.	51.00	33.37	99646.48	19.52	120799.07	7.43	51915.20	103.09	4.43	640.10
Total	(n=120)	Mean	33.33	26.06	58019.53	16.09	84366.74	5.20	37980.31	75.43	3.40	495.80
		S.D.	8.38	3.39	18797.35	1.72	15977.59	1.15	8099.62	19.24	0.59	457.91
		Min.	21.00	14.05	32859.13	9.27	39234.02	2.50	15399.27	29.49	1.88	11.70
		Max.	59.00	37.75	148115.68	21.06	129994.58	10.58	68609.70	137.41	5.10	2591.35
		p	**<0.01***	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	**<0.05***

SI= (cigarettes smoked per day) x (years of smoking)

samples taken from male were significant for CL, HL, HI, TI and OTM comet parameters ($p<0.05$).

In this study, also the correlation analysis between the age and all studied comet parameters were statistically evaluated. However, no significant correlation coefficients were detected ($p>0.05$).

DISCUSSION

Cigarette smoke is known to contain many carcinogens, with polycyclic aromatic hydrocarbons (PAHs), aromatic amines, N-nitrosamines and aldehydes representing the major classes of harmful substances (20,21) and thus DNA damage induced by smoking is caused by free radicals generated (22,23). It is important to know that the basal level of DNA damage, at least in lymphocyte, is also influenced by ***endogenous (aging, cancer, chronic disease, ROS) and exogenous (occupational exposure, smoking-drinking habits, UV and X-ray exposure etc).*** These parameters need to be considered in each biomonitoring study. Therefore, in this study, we used the comet assay to measure DNA damage and analyzed the association between the level of DNA damage in terms of nine comet parameters and smoking.

The comet assay has found wide acceptance in monitoring human genotoxicity caused by lifestyle and occupational and environmental factors (24). Comet assay is based on the assumption that DNA migrating from the nucleus within the gel after electrophoresis is the result of genotoxic damage that is converted to DNA single- or double-strand breaks. Many studies have found that cigarette smoking increased DNA migration (25, 26) and our results are consistent with the findings. According to a previous study, some human biomonitoring studies with the alkaline comet assay have found a significant relationship between DNA damage and smoking habits (15). However, some studies did not show differences in the DNA damage between smokers and non-smokers. These studies, ex-smokers had been referenced as non-smokers or number of subjects had been narrowed relatively (21, 27). Giovannelli and co-workers did not find an effect of smoking on DNA oxidation, possibly because of the small number of current smokers in their sample (16.9%) (28).

Previous studies have offered that DNA migration increase with aging (29). Singh and co-workers observed that although the DNA damage was significantly different with age, the mean levels of DNA damage increased only slightly (30). *The study sample generally consisted of young and middle age individuals. Therefore, the damages that may occur with age (the age effect of DNA damage) and, on the effect of smoking on DNA damage will affect the outcome. Thus, our study did not include the elderly group. Probably, therefore* it was not found statistically significant association differences between the coment parameters and ages in our study ($p>0.05$).

Increases in DNA strand breakages were determined using the comet assay in lymphocytes of smoking by comparison with controls, which might indicate that these cells are handling more oxidative damage.

The nine comet assay parameters were evaluated among the females and males in the non-smoker and smoker groups, and seven of nine comet parameters were found statistically significant between smokers and non-smokers females, only two of them parameters were statistically significant in male smokers and non-smokers. However, we determined that more DNA damages in female smokers than male smokers for comparison with the six parameters of comet. According to these results of the present study, it may be considered that females are more sensitive against the DNA damage by causing the smoking. *Estrogens are converted to catecholestrogens and these produce ROS, which cause many types of DNA damage. 4-Hydroxyequileinin, a metabolite of equine estrogens has been revealed to induce genotoxic and carcinogenic effects (31). Several studies revealed that formation of estrogen induced endogenous DNA adducts in animals and humans (32, 33).* To our knowledge, this is the first result in the literature and the first report on the effect of cigarette smoking in female and male groups separately according to nine comet assay parameters.

Consequently, our study results may provide a framework for future studies regarding the comet assay for the evaluation of DNA damages in cancer and other chronic diseases.

Conflict of interests

The authors declare that they have no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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