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Araştırma Makalesi / Research Article

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

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**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 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: 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 regard to DNA damages than the non-smoker female group. In contrast, only DNAt, and OTM comet parameters were statistically significantdifferences 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 males were statistically significant in terms of CL, HL, HI, TI and OTM parameters ($p<0.05$).

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

Keywords: ***Comet assay, DNA damage, cigarette 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) ve 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 dokuz comet parametresine göre değerlendirme yapılmıştır.

Materyal ve Metod: 120 gönüllü birey (60 sigara içmeyen, 60 sigara içen) kan lenfosit hücrelerindeki DNA hasarları açısından 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 anlamlı ilişki bulundu ($p<0.01$). Sigara içen kadın grubunun DNA hasarı açısından CL, CI, HL, HI, TL, TI ($p<0.01$) ve TM ($p<0.05$) parametreleri sigara içmeyen kadın grubuna kıyasla daha yüksektir. Buna karşın, sigara içen ve içmeyen erkek grupları arasında sadece DNAt ve OTM parametreleri açısından istatistiksel anlamlı fark gözlenmiştir ($p<0.05$). Kadınlardan alınan tüm kan örneklerinin sigara indeksi (SI), tüm çalışılan comet parametrelerine dayanarak karşılaştırıldığında, TM dışındaki bütün parametreler ile istatistiksel olarak anlamlı ilişki bulunmuştur. Diğer taraftan, erkek grubundaki kan örneklerinde CL, HL, HI, TI ve OTM parametrelerinde istatistiksel 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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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 the 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(10), ever smokers had an 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), alkaline-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 perform 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 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) whose 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. 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), and their 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 (between 2-50 cigarettes per day) in our study and none of them used cigarette holders. The study design was approved by the institutional ethics committee (Approval number: 147-4532;23.02.2009). Informed consent was obtained from each individual who were selected randomly as a control group sample from the 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 comprised 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. Lymphocytes were isolated and washed 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 was added to the slides which were then immediately covered with coverslips. After removing the cover-

glass, all slides were immersed 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) for one hour at +4° C 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 for 20 min at 300 mA and 25 V. After the stage of electrophoresis, the slides were taken from the tank and washed three times for 5 min with neutralizing buffer (0.4 M Tris, pH 7.5). Afterwards, each slide 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 (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.

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 by using 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.

When the nine comet assay parameters were evaluated for 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 different 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 all the comet parameters, 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 are 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

Figure 1. Representative comet assays of cells.

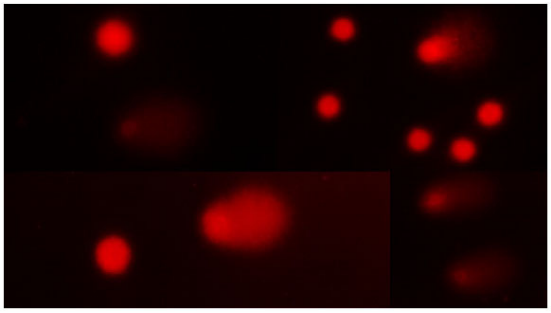

Table 1. The results of the comet parameters in non-smoker and smoker groups.

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***

Table 2. The comet assay parameters for females and males in non-smoker 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 Moment	**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***

parameters and statistically significant association was found only between the SI and OTM comet parameters ($p<0.05$), in addition to SI and age ($p<0.01$) (Table 4). However, statistically significant association was foound between the SI and all studied comet parameters ($p<0.05$) except for TM in females. On the other hand, the blood samples taken from male were significant for CL, HL, HI, TI and OTM comet parameters ($p<0.05$).

In this study, no significant correlation coefficients were detected ($p>0.05$). between age and studied comet parameters.

Table 4. 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)

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). 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 factors ***(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 gained 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. In these studies, ex-smokers had been referenced as non-smokers or number of subjects had been narrowed relatively (21, 27). Giovannelli and co-workers (28) did not find an effect of smoking on DNA oxidation, possibly because of the small number of current smokers in their sample (16.9%) .

Previous studies have offered that DNA migration increase with aging (29). Singh and co-workers (30) observed that although DNA damage significantly differed with age, the mean levels of DNA damage increased only slightly. 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 no statistically significant association was found 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 to be statistically significant between smokers and non-smokers females, only two of the parameters were statistically significant in male smokers and non-smokers. However, we determined more DNA damages in female smokers than male smokers for comparison with the six parameters of comet. According to the results of the present study, it may be considered that females are more sensitive to DNA damage caused by 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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