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Determination of Genotoxic Damage by Comet Assay in Smokers

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ABSTRACT The clinical course of most diseases related to smoking has a strong relationship with genotoxicity. In this study, the researchers aimed to compare DNA damage of smokers and non-smokers to determine the genotoxic risk. In total, 50 volunteers were included in this study; 30 of them smokers and 20 of them forming the non-smoker control group. Peripheral blood samples taken from the volunteers were determined with comet assay. The researchers determined the DNA damage ratio as 12, 75 (± 7. 14) from smokers 10, 41 (± 3.41) from non-smokers (p> 0.05), and also higher DNA damage in male smokers than female ones (p<0. 05). There was no correlation between age and DNA damage. In conclusion, there was no significant difference between smokers and non-smokers in terms of DNA damage, but there were some important changes in the DNA of smokers and genomic instability was adversely affected.

Abbreviations: SCGE: Single Cell Gel Electrophoresis; DNA: Deoxyribonucleic Acid; WHO**:** World Health Organization; HMA: High Melting Agarose; LMA: Low Melting Agarose

INTRODUCTION

Comet assay, in other words *single cell gel electrophoresis or SCGE*, is a widely used, quick, simple and sensitive technique used to analyze DNA (Deoxyribonucleic Acid) damage. The technique, which was established by Rydberg and Johanson (1978) for measurement of DNA single strand breaks and later developed by Östling and Johanson (1984), was used to determine DNA double strand breaks by application under lysis conditions and neutral pH. Today, the comet method developed by Singh et al. (1988) including small changes is the most prevalent method throughout the world. In the comet method, after cells are isolated they are buried in agarose and spread on microscopic slides. After the lysis stage they are left in electrophoresis and validated by being painted with fluorescent paint. In determination of DNA damage quantitatively by the comet method, tail length, tail moment and DNA percentage in the tail are the most widely used parameters in addition to visual evaluation (Dinçer and Kankaya 2010; Lovreglio et al. 2016; Naidoo et al. 2016). Determination of DNA percentage in the tail and visual evaluation of results are preferred as they better reflect the dose response relationship compared to other parameters (Dinçer and Kankaya 2010). One of the most important advantages of the method is the opportunity to work with various cell types (Tice et al. 2000; Collins 2004; Zalata et al. 2007; Basu et al. 2013; Nemmar et al. 2017).

It is assumed that four million people die due to smoking every year and this number is ex-

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pected to reach to 10 million within 20 years. Also, it is known that eleven thousand people die everyday due to smoking related illnesses (Ezzati and Lopez 2003). In the global smoking epidemic 2008 report issued by the World Health Organisation (WHO), Turkey was one of the 10 countries that consumed the most cigarettes. The smoking habit is assumed to be the most prevalent toxicological problem causing death in many countries. (Phillips 2002; Ezzati and Lopez 2003; Karlikaya 2004; Vineis et al. 2007). Smoking is also stated to have a role in lung cancer as well as oral and nasal cavity, oesophagus, larynx, pharynx, liver, kidney, stomach, urinary system and cervix cancers (Philips 2002). When smokers diagnosed with lung cancer were compared to cases with no cancer history including former smokers and non-smokers, it was found that lung cancer cases had a six time increase in DNA damage compared to non-smokers, a three time increase compared to former smokers and a one time increase compared to smokers (El-Zein et al. 2010). Also, in the tissues of smokers it was shown that levels of carcinogen DNA adducts were higher and it was claimed that electrophilic materials in tobacco cause DNA breaks (Nakayama et al. 1985). In recent years, a strong relationship between genotoxicity and the formation and prognosis of many smoking related problems has been emphasised (Akbas et al. 2001; Karlikaya 2004; Kayaalti et al. 2015**)**. Various chemical materials around us spoil the cell DNA of living things and cause mutation-originated carcinogenic effects. In particular, the cancer-causing roles of mutations in somatic cells increase the clinical importance of genotoxicity.

The risk of the genotoxic effect of smoking leading to carcinogenic effects (de Assis et al. 2009; Kocyigit et al. 2011; Chandirasekar et al. 2014) in the future increases the clinical importance of the subject and research related to the carcinogenic effect by a sensitive bioindicator that can be used routinely continues (Kadioglu et al. 2012; Ginzkey et al. 2013; Sobkowiak et al. 2014) In the present study, the aim was to evaluate smokers in terms of basal DNA damage and H_2O_2 induced DNA damage by the comet examination technique, which gives a quick response and is assumed to be the sensitive bioindicator of even minor DNA damage related to the determination of the aforementioned genotoxic risk.

MATERIAL AND METHODS

Subjects

A total of 50 subjects, at least 20 years old, were recruited for the studies. Thirty subjects were included as smokers that did not have chronic disease, did not use any drugs, smoke at least 10 cigarettes per day and were referred from Pamukkale University Hospital, Department of Chest Diseases. Twenty subjects as the control group were non-smokers who were not passive smokers, did not have chronic disease and did not use any drugs. People with a hereditary disease were not included in the present study. The project was approved by the Non-Invasive Clinical Research Ethics Committee (Date: 08.11.2013 Number: 44573).

Sample Collection and Lymphocyte Preparation for Comet Assay

Five (5) mL of peripheral blood samples were collected from volunteers in sterile disposable syringes and transferred into heparinized tubes. Unstimulated lymphocytes were isolated by Histopaque 1077 (Sigma, USA), centrifuged, washed in phosphate buffered saline (PBS) (Lonza, Switzerland) and then re-suspended in ice-cold PBS.

Slide Preparation

Alkaline comet assay was adapted from Singh et al. (1988). The slides were layered with 1.8 percent high melting agarose (HMA) (Lonza, Switzerland). Three (3) mL histopaque solution was added slowly into 3 mL fresh blood samples and centrifuged for 30 minutes at 2100 rpm at +4^{o}C. Lymphocytes were removed to a new tube and washed with PBS. The cells in PBS were mixed with one percent low melting agarose (LMA) (Lonza, Switzerland) at 37^{o}C and 40 il was layerd over agarose coated slides. Four slides were prepared for each sample. Two of the slides were analyzed for basal DNA damage. Hydrogen peroxide (H_2O_2) is known to be a natural source of DNA damage in cells causing a spectrum of DNA lesions, including single and double strand breaks (Collins 1999). So, in the present study, H_2O_2 was used as a positive control in both groups. The slides were covered by a coverslip and placed at 4^{o}C for 40 minutes to solidify. The cover slip was removed and two

out of four slides were treated with 100 μM H_2O_2 for five minutes. Slides were immersed in cold lysis solution (2.5M NaCl, 100mM NA2EDTA, 10mM Tris, pH 10) with one percent Triton X-100 and ten percent DMSO added just before use, for 1 h 15 minutes.

Electrophoresis

The slides were washed with distilled water and immersed in an electrophoresis tank in the presence of freshly prepared alkaline buffer (0.3M NaOH, 1 mM EDTA, pH 13) at room temperature. Before electrophoresis, the slides were left in the solution for 20 minutes to allow the unwinding of the DNA and the expression of alkali-labile damage. Electrophoresis was performed at suitable voltage (1V/cm, 300mA) for 20 minutes at 4ºC. The slides were neutralized using neutralization solution (0.4M Tris, pH 7.5) for 15 minutes. Finally, slides were fixed using cold methanol for five minutes and stored at 4ºC before analysis. For analysis, slides were stained with 45 μL ethidium bromide.

The DNA damage is quantified by measuring the displacement between the genetic material of the nucleus 'comet head' and the resulting 'tail'. Tail Moment and Tail DNA percentage are the two most commonly used parameters to analyze Comet assay results (Recio et al. 2010). In the present study, three different parameters (tail length, tail moment and DNA percentage in the tail) were evaluated for each comet. Tail length is measured from the center of the head to the center of the tail. At least 50-100 cells should be analyzed per sample. Hundred (100) comets/per slide were captured by CometScore 15, Tritek Corporation Image analysis system program by imaging microscope with a 40X objected lens.

Statistical Analysis

Data were analyzed with the SPSS program. Continuous variables were stated as mean± standard deviation, median, minimum-maximum values. Categoricals were stated as number (percentage). In the comparison of independent tests, 'independent samples t test' was used when parametric test assumptions were obtained and 'Mann-Whitney U test' was used when parametric test assumptions were not obtained. In the comparison of dependent tests, 'paired samples t test' was used when parametric test assumptions were obtained and 'Wilcoxon signed rank test' was used when parametric test assumptions were not obtained. 'Shapiro-Wilk test' was used to assess the suitability of variables to normal distribution. 'Chi-square analysis' was used for the comparison of categorical variables. $p<0.05$ was accepted as statistically significant. Spearman correlation analysis was performed to examine the relationship between continuous variables.

RESULTS

Demographic features of smokers (n=30) and non-smokers (n=20) are presented in Table 1. No statistically significant differences were found between the smokers and non-smokers from the point of age and gender ($p>0.05$). The mean age (– SD) of the smokers was 30.57 ± 5.87 (min 21-max 45), and of the non-smokers 29.2 ± 5.3 (min 22-max 44). They were under 45 years of age. The male/female ratio was 22/28.

Comet parameters for basal DNA and H_2O_2 induced DNA damage were investigated in smokers and non-smokers. Smokers showed high levels of basal DNA damage compared to non-smokers The mean tail length (-SD) of the smok-

Table 1: Demographical features of smokers and non-smokers

		Smokers	*Non-smokers*	*p*
Number (N)		30	20	
Gender	Female	15	13	p=0.295
	Male	15	7	
Age	Mean ± SD	30.57 ± 5.87	29.2 ± 5.3	p=0.406
	(min-max)	(21-45)	(22-44)	
Number of Cigarettes per day	Mean ± SD	17.67 ± 7.63		
	(min-max)	(10-40)		
Smoking Duration (year)	Mean ± SD	11.72 ± 7.26		
	(min-max)	(1-35)		

ers was 5.11 ± 3.45 and of the non-smokers 3.59 ± 1.75. The mean DNA percentage in the tail of the smokers was 12.75 ± 7.14 and in nonsmokers 10.41 ± 3.41. The mean tail moment of the smokers was 2.09 ± 2.05 and of non-smokers 1.29 ± 0.69 but no statistically significant differences were observed ($p>0.05$). Based on basal DNA damage there was no statistical difference in tail length, DNA percentage in the tail and tail moment parameters between smokers and non-smokers. However, based on H_2O_2 induced DNA damage there was a statistically significant difference ($p<0.05$) in tail length (the mean of smokers was 25.6±7.92 and of non-smokers 19.68±8.82) and tail moment parameters (the mean of smokers was 16.06±7.27 and of non-smokers 11.73±7.48) (Table 2).

Results related to basal DNA and H_2O_2 induced DNA damage according to gender (see Table 3) in smokers showed a statistically significant difference in the parameters of tail length (the mean of males was 6.33 ± 4.26 and of females 3.89 ± 1.82) and DNA percentage in the tail (the mean of males was 15.68± 8.51and of females 9.81 ± 3.86) among males and females ($p<0.05$). However, in the cells treated with H_2O_2 no statistically significant differences were observed ($p>0.05$). In the non-smokers control group, when we compared basal DNA and H_2O_2 induced DNA damage results with gender we did not find a statistically significant difference ($p>0.05$).

Number of cigarettes smoked per day (– SD) was 17.67 ± 7.63 (min 10-max 40). Smoking dura-

Table 2: Basal and H_2O_2 induced DNA damage (tail length, DNA percent, tail moment) of smokers and non-smokers

Basal DNA damage	*Smokers (n=30)*			*Non-smokers (n=20)*			
	Mean ± SD	*Median*	*Min -Max*	*Mean ± SD*	*Median*	*Min -Max*	*p*
Tail length	5.11 ± 3.45	4.13	1.73–15.99	3.59 ± 1.75	3.28	1.6– 8.14	0.063
DNA percent in tail	12.75 ± 7.14	10.4	3.35–33.77	10.41 ± 3.41	9.89	5.67–18.7	0.452
Tail moment	2.09 ± 2.05	1.44	0.43– 8.85	1.29 ± 0.69	1.05	0.47– 2.99	0.178
H_2O_2 induced DNA Damage	*Mean ± SD*	*Median*	*Min -Max*	*Mean*	*Median ± SD*	*Min -Max*	*p*
Tail length	25.6± 7.92	25.68	11.09–43.94	19.68± 8.82	16.54	7.21–36.19	0.017*
DNA percent in tail	53.07± 13	55.21	24.96–78.49	45.26±15.29	40.64	20.7–73.68	0.058
Tail moment	16.06± 7.27	15.89	4.18–34.84	11.73± 7.48	9.06	2.64–26.93	0.021*

*Significant difference according to smokers and non-smokers ($p<0.05$)

Table 3: Basal and H_2O_2 induced DNA damage (tail length, DNA percent, tail moment) for male and female in smokers

Basal DNA damage	*Male (n=15)*			*Female (n=15)*			
	Mean ± SD	*Median*	*Min -Max*	*Mean ± SD*	*Median*	*Min -Max*	*p*
Tail length	6.33 ± 4.26	4.67	1.96–15.99	3.89 ± 1.82	3.10	1.73– 7.55	0.045*
DNA percent in the tail	15.68± 8.51	12.58	4.83–33.77	9.81 ± 3.86	8.7	3.35–15.94	0.025*
Tail moment	2.75 ± 2.65	1.47	0.43–8.85	1.44 ± 0.87	1.36	0.46-3	0.174
H_2O_2 induced DNA Damage	*Mean ± SD*	*Median*	*Min -Max*	*Mean ± SD*	*Median*	*Min -Max*	*p*
Tail length	26.43± 8.42	27.2	11.09-43.94	24.77 ± 7.59	23.69	13.39-41.01	0.576
DNA percent in tail	56.12±13.32	58.62	26.71-78.49	50.03 ±12.35	48.48	24.96-73.87	0.205
Tail moment	17.19± 7.53	17.81	4.72-34.84	14.92± 7.07	13.64	4.18-30.74	0.401

*Significant difference regards to basal and H_2O_2 induced DNA damage ($p<0.05$)

tion was 11.72 ± 7.26, (min 1-max 35). Smokers who were using at least 10 cigarettes per day were included in our study. In smokers, the researchers did not observe a statistically significant correlation with the Spearman correlation test between the smoking duration and basal DNA damage (DNA percentage in the tail). Basal DNA damage and number of cigarettes per day were also investigated. Spearman correlation test results demonstrated that there was no relationship between the number of cigarettes per day/basal DNA damage and ages of smokers/non-smokers and basal DNA damage in smokers. There was no correlation between ages of volunteers and basal DNA damage and no statistically significant relationship was observed between the years of smoking and basal DNA damage.

DISCUSSION

In this study, investigation of the relationship between smoking and DNA damage separately (basal and H_2O_2 induced) in lymphocytes for female and male smokers was measured according to three comet assay parameters such as tail length, DNA percentage in the tail and tail moment. The researchers could not observe statistically significant differences in basal DNA damage in comet parameters between smokers and non-smokers. However, they observed a significant difference in H_2O_2 induced basal DNA damage related to tail length and tail moment parameters ($p<0.05$). It was noticeable that this ratio was increased in smokers. Previously, DNA damage in lymphocytes was analyzed by comet assay and it was shown that comet length, tail moment and olive tail moment were found to be highly significant with regards to comet parameters between smokers and non-smokers ($p<0.01$) (Söylemez et al. 2012). In addition, they observed that male subjects were more affected by smoking. In the parameters for tail length and DNA percentage in the tail, they observed that male subjects had far more basal DNA damage compared to females ($p<0.05$). Söylemez et al. (2012) showed that smoking causes DNA damage and that females are more sensitive to the effects of smoking than males. It was also found that DNA percentage in the tail was significantly higher ($p<0.001$) in cases of those who smoke 20 cigarettes per day (Dinçer et al. 2003). The researchers have included smokers who use 10 cigarettes per day in their study. They could not observe any correlation between the number of cigarettes per day and basal DNA damage but discriminately in their study it was shown that DNA damage was significantly increased by the number of cigarettes (Zhu et al. 1999). Lu and Morimoto (2008) have shown that the levels of daily exposure to cigarette tar or nicotine cigarette pack-years and years of smoking correlate significantly with the level of DNA strand breaks as assessed by the alkaline comet assay. In contrast, the numbers of cigarettes smoked per day do not show a statistical relationship with the level of DNA strand breaks.

The effects of smoking on DNA damage in human cells were examined and it was concluded that the comet assay was quite a practical method to determine the effect of even the smallest quantity of chemicals in samples (Hang et al. 2013). By using comet assay it was also detected that there was high incidence of basal DNA damage in active smokers compared to non-smokers (Fracasso et al. 2006). The results of previous studies on determining DNA damage to smokers are controversial (Hoffmann et al. 2005). Hoffmann et al. (2005) revealed that out of 37 studies to determine the effects of smoking on DNA damage by using comet assay in peripheral blood samples, there were only 14 studies that showed significant differences and statistical data between smokers and non-smokers. In another five studies there were significant differences but statistical data were not shown. Just one study (Piperakis et al. 1998) among the five approached the genotoxic effect of smoking but the other four studies did not observe any differences between smokers and the control group. In some studies weak statistical analysis and bias were noticed. It has also been claimed that smoking does not affect the DNA damage in lymphocytes (Cloos et al. 1996; Ginzkey et al. 2013).

Among comet assays, the results of other studies on determining DNA damage (Nemmar et al. 2017) provided evidence that long-term exposure to Water-pipe tobacco smoking (WPS) is harmful to the cardiovascular system and supported interventions to control the spread of WPS, particularly among youths. WPS exposure significantly increased heart DNA damage assessed by comet assay. They concluded that chronic nose-only exposure to WPS impairs cardiovascular homeostasis. Al-Amrah et al. (2014)

found that waterpipe smoke caused DNA damage in buccal cells. The smoke condensate of both jurak and moassel caused comet formation suggesting DNA damage in peripheral blood leukocytes. Sardas et al. (2009) assessed the possible DNA damaging effects of Maras Powder and cigarette smoking. They evaluated the frequencies of total comet scores (TCS) of peripheral lymphocytes of Maras Powder users, cigarette smokers, and non-smokers. They observed that mean TCS (±SD) frequency in the peripheral lymphocytes was 14.4 (±10.04) for MP users and 8.26 (±5.38), and 5.94 (±3.87) for cigarette smokers ($p<0.05$) and non-smoking control subjects, respectively ($p<0.001$). Their study showed that the oral use of smokeless tobacco represents a genotoxic hazard that was even higher than the DNA damage observed in cigarette smokers. Sobkowiak et al. (2014) suggested that nicotine, at a reasonably low concentration (0.1 mM), comparable to that found in the blood of habitual smokers, may have a protective effect, whereas higher doses of nicotine (1 and 10 mM) are genotoxic. The possible participation of reactive oxygen species in the DNA-damaging potential of nicotine has been discussed. Yu et al. (2016) showed that electronic cigarette vapor, both with and without nicotine, is cytotoxic to epithelial cell lines and is a DNA strand break-inducing agent. Further assessment of the potential carcinogenic effects of electronic cigarette vapor is urgently needed.

CONCLUSION

Although the researchers could not detect statistically significant differences in basal DNA damage with regards to tail length, DNA percentage in the tail and tail moment parameters, they detected a significant increase in DNA damage in smokers. When the three comet assay parameters were evaluated between females and males in the smokers and non-smoker groups, it was observed that males were more affected than females. There was no relationship between DNA damage and the number of cigarettes per day or smoking duration. Also, there was no relationship between DNA damage and age. The contributions of environmental factors such as job and diet were not included in this study. The viability of cells was not tested. Apoptosis process was not evaluated and because the number of former smokers was limited (three people) the researchers could not include them in the present study.

RECOMMENDATIONS

Comet assay, as a sensitive method to evaluate DNA damage caused by smoking, can come to the forefront and new studies can be planned. In these new studies, samples can be collected in particular periods after people stop smoking and DNA damage and repair mechanisms can be evaluated. Apart from lymphocytes, epithelial cells from in-mouth and the respiratory tract can be examined. Also, products that are produced to help stop smoking can be evaluated to see if they cause DNA damage or not and can be compared to the period for smoking. Comparisons can also be performed to establish the genotoxic damage caused by different types of cigarettes. It would also be very useful and to perform a new study to evaluate DNA damage to people consuming these different types of tobacco.

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