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Alkaline comet assay as a biomarker of DNA-damage encountered in workers engaged in cigarette manufacturing

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Abstract

Background and Purpose: *The aim of this pilot study was to determine possible genotoxic effects of occupational exposure to tobacco dust between workers employed in tobbaco industry and non-exposed population.*

Subjects and Method: *The exposed group consisted of 20 subjects with mena age of 43.4 years. The average duration of their occupational exposure was 19.95 years. The control group consisted of 40 subjects, mean age 40.18 years and was choosen from general Croatian population. The assessment of primary DNA damage hosted by peripheral blood leukocytes was performed using the alkaline comet assay, the tail length and long-tailed nuclei thereby being the primary outcome of the measure.*

Results and Conclusions: *The results showed that group comet tail length mean measured in the exposed workers was 14.39±1.02 μm whereas mean percentage of long-tailed nuclei was 8.20. In the control group, the mean tail length was 13.91±0.66 μm and long-tailed nucleus percentage was 1.88. Mean values for the tail length measured and the percentage of long-tailed nuclei were significantly higher in the exposed group compared to the control one. Within the exposed population, significant inter-individual differences in DNA damage were found ($P<0.05$). The observed DNA damage frequency characterized the tobacco dust as an undoubted genotoxicant, and the outcome of the alkaline comet assay stressed the importance of biomonitoring of the exposed individuals.*

INTRODUCTION

Throughout the years, cigarette manufacturer have acknowledged both medical and scientific consensus that smoking poses a serious health hazard causing a number of diseases, such as respiratory disease, heart disease and lung cancer. Specific chemicals including nitrosamines, formaldehyde, hydrazine, arsenic, nickel, cadmium, benzapyren, and potassium capable of causing cancer, are mainly contained in tobacco leaves. The processing of tobacco leaves generates a lot of dust and facilitates the release of numerous tobacco components into ambient air. Tobacco also contains nicotine which is readily absorbed in body tissues, including skin, respiratory epithelium, and mucous membranes of the mouth, the nose and the intestines *(1)*. It has been reported previously that hypersensitivity to allergens contained by tobacco leaves causes occupational asthma *(2)*. Tobacco dust is known to

affect the respiratory tract and was also reported to cause allergies, skin rashes, wheezing, as well as dyspnea, rhinitis, nausea, dizziness and vomiting *(3, 4)*.

Certain studies on tobacco workers have revealed this professional category to be prone to pronounced immunological responses and respiratory system alterations *(5, 6, 7, 8)*. The epidemiological study performed by Kjaergaard and Pedersen *(9)* pointed out that tobacco workers exposed to a substantial amount of tobacco dust during their working shifts suffer from eye irritation, typically presenting in morning hours. In addition, objective changes in eye mucous membranes were detected *(9)*. The study by Setimi *et al. (10)* reported a high mortality rate in tobacco processors, mostly due to non-Hodgkin's lymphomas, and put upfront the issue of statistically significant death rate encountered among female workers.

There exists a wide range of cytogenetic endpoints presently employed in biomonitoring of populations exposed to physical and chemical DNA-damaging agents. These methods include chromosome aberration and sister-chromatid exchange analysis, micronucleus test, DNA elution test, diffusion assay, comet assay and its modifications *(11, 12, 13, 14, 15, 16, 17, 18, 19)*. As a rapid and sensitive technique, the comet assay (single-cell gel electrophoresis – SCGE) has gained a widespread acceptance in molecular epidemiology *(20)*. DNA damage evaluated by the comet assay is utilized as a biomarker of exposure *(21, 22, 25, 26)*. Comet assay permits the detection of primary DNA damage and the study of repair kinetics at the single cell level *(23, 24, 27)*. When it comes to measuring the DNA damage by virtue of a comet assay, studies of that type, performed in tobacco workers, are scarce. Zhu *et al. (28)* reported interesting data on tobacco workers, gathered on the basis of comet assay results, revealing age and gender to be only insignificant confounders to the primary outcome. Nevertheless, it can be assumed that smoking and tobacco dust exposure combined facilitate and aggravate the lymphocyte DNA damage.

The aim of our study was to employ the alkaline comet assay to the goal of determining possible occupational risks conferred to subjects engaged in tobacco industry, and exposed to tobacco dust and ^{90}strontium on a daily basis. Within this context, peripheral blood leukocytes served as a biological material.

SUBJECTS AND METHODS

Subjects

Peripheral blood samples intended for alkaline comet assay were taken from healthy volunteers. Altogether 60 subjects (20 exposed and 40 controls) participated in the study. The mean age of the exposed group was 43.4 years (range 22-57 years). The exposed group comprised 8 women (two non-smokers and six smokers) and 12 men (seven non-smokers and five smokers). All subjects were employed in cigarette processing and had been occupationally exposed to tobacco dust for 19.95 years in average (1-38 years), with the average daily exposure of 8 hours.

The control group included 18 women (8 non-smokers and 10 smokers) and 22 men (13 non-smokers and 9 smokers). The mean age of the control subjects was 40.18 years (21-58 years). All of them were recruited from the administrative staff that has never been occupationally exposed to any type of radiation or chemicals, nor subjected to any of long-term pharmacotherapy and/or radio-diagnostics and radiotherapy within 12 months prior to blood sampling. Both the exposed and the control subjects were previously interviewed about recent viral infections, accinations, previous exposures to electromagnetic radiation or chemicals, drug intake and alcohol consumption. The two groups showed no differences in this respect.

Peripheral blood samples of the exposed and control subjects were collected by venipucture and contained into heparinized tubes. All blood samples were coded, cooled and processed within a maximum of 2 h period after sampling. Alkaline comet assay utilizing whole blood samples was performed immediately after sample delivery.

Comet assay

Comet assay was carried out under alkaline conditions, basically as described by Singh *et al. (29)* and Tice *(30)*. Fully frosted slides were covered with 1% normal melting point (NMP) agarose (Sigma). After solidification, the gel was scraped off from the slide. It was then coated with 0.6% NMP agarose. When this layer had solidified, a second layer containing whole blood samples mixed with 0.5% low melting point (LMP) agarose (Sigma) was placed on the slides. After 10 minutes of solidification on ice, the slides were covered with 0.5% LMP agarose. They were then immersed for 1 h in ice-cold freshly prepared lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM tris- HCl, 1%Na-sarcosinate (Sigma), pH 10) with 1% Triton X-100 (Sigma) and 10% dimethyl sulfoxide (Kemika) was added fresh to lyse cells to allow DNA unfolding. The slides were then placed on a horizontal gel-electrophoresis tank, facing the anode. The unit was filled with fresh electrophoretic buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13) in which the slides were put for 20 min to allow DNA unwinding and the expression of alkali-labile sites. Denaturation and electrophoresis were performed at 4 °C under dim light. Electrophoresis was carried out for 20 min at 25 V (300 mA). Subsequently, the slides were rinsed gently three times with a neutralisation buffer (0.4 M Tris-HCl, pH 7.5) in order to remove excess alkali and detergents. Each slide was stained with ethidium bromide (20mg/ml) covered with a cover-slip. The slides were stored at 4 °C in sealed boxes until the time of analysis.

Comet capture and analysis

One hundred randomly selected cells were analyzed per sample. The slides were examined, in a blind study, at 250 × magnifications with a Zeiss fluorescence microscope equipped with a 515–560 nm excitation filter and a 590 nm barrier filter, and images of cells were analyzed

TABLE 1

Results of the alkaline comet assay: peripheral blood leukocytes of non-exposed control subjects.

No.	Sex/Age	Smoking habit	DNA migration-tail length(μm)				
			Mean±S.D.	Median	Min.	Max.	% of LTN
1	M / 21	NS	12.65±0.92	12.97	11.02	15.56	0
2	M / 21	S	14.53±0.75	14.26	12.97	16.21	0
3	M / 21	S	14.05±1.15	14.26	11.02	15.66	0
4	M / 22	NS	13.89±1.34	14.26	9.08	16.21	0
5	M / 23	NS	13.02±0.94	12.97	11.02	14.91	0
6	M / 23	NS	13.78±.079	13.61	12.32	14.91	0
7	M / 23	NS	13.51±1.49	13.46	10.26	17.95	1
8	M / 24	NS	14.15±0.92	14.26	12.32	17.50	2
9	M / 25	NS	12.54±1.13	13.32	10.37	14.26	0
10	M / 28	S	13.81±0.84	13.94	11.67	14.91	0
11	M / 29	NS	14.23±1.29	14.10	10.90	17.31	1
12	M / 32	NS	13.83±1.35	13.46	10.90	17.31	1
13	M / 32	NS	13.08±1.48	12.82	9.62	17.31	2
14	M / 42	S	14.65±1.62	14.74	10.90	17.95	1
15	M / 43	S	13.09±1.39	12.82	10.26	17.31	1
16	F / 43	S	14.67±1.46	14.74	10.90	17.95	4
17	F / 44	S	14.72±1.64	14.74	10.90	19.87	8
18	M / 44	S	14.90±1.72	14.74	10.26	21.15	13
19	F / 45	S	13.37±1.19	13.46	10.26	17.31	1
20	F / 45	NS	14.54±1.65	14.74	10.26	17.31	7
21	F / 45	NS	15.04±1.40	15.38	11.54	17.95	6
22	F / 45	S	14.09±1.39	14.10	10.26	17.31	1
23	F / 45	NS	14.22±1.29	14.10	10.90	17.95	1
24	M / 45	S	14.88±1.72	14.74	10.26	19.87	9
25	F / 46	S	14.06±1.51	14.10	10.26	17.31	1
26	M / 46	S	14.24±1.36	14.10	11.54	17.95	5
27	M / 46	S	14.57±0.74	14.91	11.67	15.56	0
28	F / 47	S	13.94±1.38	14.10	10.90	16.67	0
29	F / 47	NS	12.83±1.32	12.82	9.62	16.03	0
30	F / 47	NS	14.73±1.39	14.74	10.90	17.31	2
31	F / 48	S	13.81±1.41	13.78	10.90	16.67	0
32	F / 49	NS	13.94±1.33	14.74	10.90	17.31	2
33	F / 49	S	13.81±1.41	13.78	10.90	16.67	0
34	M / 50	NS	12.52±0.91	12.32	9.72	13.61	0
35	F / 51	S	14.04±1.39	14.10	10.90	17.95	2
36	M / 51	NS	14.00±1.47	14.10	10.90	16.67	0
37	F / 52	NS	13.22±1.40	13.14	10.26	16.67	0
38	F / 54	S	14.17±1.31	14.10	11.54	17.31	1
39	F / 56	S	13.61±1.54	13.46	10.26	17.31	1
40	M / 58	NS	13.81±1.51	14.10	10.90	17.31	2

Abbreviations: F-female subject, M-male subject, NS-non-smoker, S-smoker, LTN-long-tailed nuclei

TABLE 2

Results of the alkaline comet assay: peripheral blood leukocytes of workers occupationally exposed to tobacco dust.

No.	Sex/Age	Smoking habit	Exposure (year) / Daily exposure (hour)	DNA migration -tail length (μm)				
				Mean±S.D.	Median	Min.	Max.	% of LTN
1	M / 22	S	3/8	13.12±1.29	13.46	10.26	15.38	0
2	M / 23	NS	3/8	13.24±1.22	13.46	10.26	15.38	0
3	M / 24	NS	6/8	14.42±1.37	14.10	10.26	17.31	5
4	M / 27	S	6/8	12.19±1.43	12.18	8.94	14.74	0
5	M / 32	NS	1/8	15.22±2.40	14.74	10.90	21.79	18
6	M / 42	S	8/8	15.85±2.04	16.03	10.90	20.51	27
7	F / 45	NS	27/8	14.58±1.25	14.74	11.54	17.31	1
8	F / 47	NS	27/8	14.63±1.81	14.10	10.90	21.79	8
9	F / 47	S	26/8	14.35±1.54	14.10	11.54	18.59	7
10	F / 47	S	26/8	14.35±1.37	14.42	10.26	17.31	1
11	M / 47	S	25/8	12.77±1.53	12.82	8.33	15.38	0
12	F / 48	S	30/8	14.89±1.75	14.74	10.90	20.51	12
13	F / 50	S	20/8	15.29±1.97	15.06	10.26	20.51	20
14	F / 50	S	32/8	15.54±1.43	15.38	11.54	18.59	13
15	M / 50	NS	28/8	14.34±1.24	14.10	11.54	16.67	0
16	M / 50	S	29/8	15.29±1.74	15.06	11.54	19.23	20
17	F / 53	S	38/8	15.11±1.18	15.38	12.18	17.95	6
18	M / 53	NS	29/8	13.14±1.52	12.82	10.26	16.03	0
19	M / 54	NS	28/8	15.41±3.19	14.74	10.90	36.54	19
20	M / 57	NS	7/8	14.05±1.79	14.10	10.26	17.31	7

Abbreviations: F-female subject, M-male subject, NS-non-smoker, S-smoker, LTN-long-tailed nuclei

with the computerized image analysis system (Comet Assay II; Perceptive Instruments Ltd., UK). To quantify DNA damage, the tail length (μm), was evaluated. Tail length (i.e. the length of DNA migration) is related directly to the DNA fragment size and is presented in micrometers. It was calculated from the center of the cell. For each sample the frequency of »damaged« cells, i.e., long-tailed nuclei (LTN) with tail length exceeding 95th percentile for each sample compared to the control, is also reported *(31)*. The analysis did not include the edges and damaged parts of the gel as well as debris, superimposed comets, and comets without distinct head (»clouds«, »hedgehogs«, or »ghost cells«).

Statistical analysis

Statistical analyses were carried out using Statistica 5.0 package (StatSoft, Tulsa, USA). Each sample was characterized for the extent of DNA damage by considering the mean ± SD (standard deviation of the mean), median and range of the comet parameters. In order to normalize distribution and to equalize variances, a logarithmic transformation of data was applied. Multiple comparisons between groups were done by means of ANOVA on log-transformed data. Post-hoc analysis of differences was done by Scheffé test. The level of statistical significance was set at P value of <0.05.

RESULTS

Results of the alkaline comet assay parameters of tail lengths (TLs) and long-tailed nuclei (LTN) for each individual of the exposed and control group are presented in Tables 1 and 2. Results of the mean group values ± SD (standard deviation of the mean) are presented in Figures 1 and 2. In Figure 3 different levels of DNA fragmentation between non-exposed control subject and workers exposed to tobacco dust are presented.

The tail lengths measured in subjects exposed to tobacco dust ranged from 8.33 μm to 36.54 μm (Table 2). In the control subjects, tail lengths ranged from 9.08 μm to 21.15 μm (Table 1). The mean tail length measured in the exposed group was 14.39±1.02 μm and that of the control group was 13.91±0.66 μm. As evident from Figure 1, the mean value of the comet tail lengths docu-

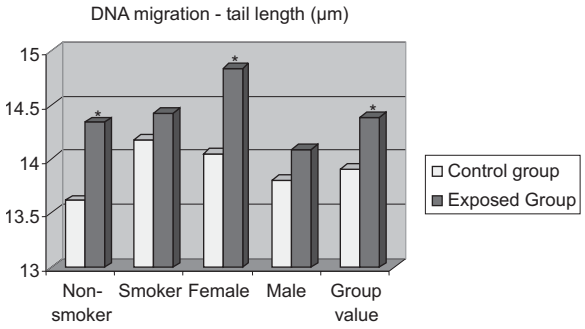

Figure 1. *Mean group values for the tail length parameter (μm) of the alkaline comet assay for the non-exposed control group and workers occupationally exposed to tobacco dust. * Statistically significant increase in comparison to the control group (P<0.05).*

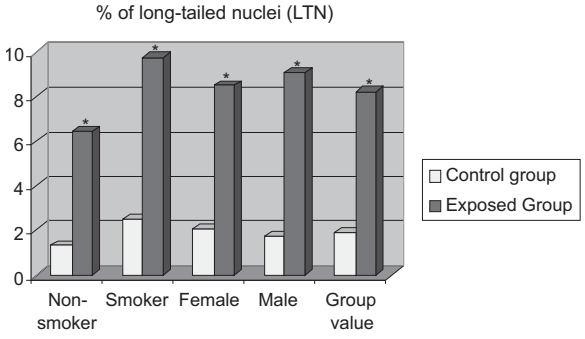

Figure 2. *Mean group values for the percentage of long-tailed nuclei (LTN) of the alkaline comet assay for the non-exposed control group and workers occupationally exposed to tobacco dust. * Statistically significant increase in comparison to the control group (P<0.05).*

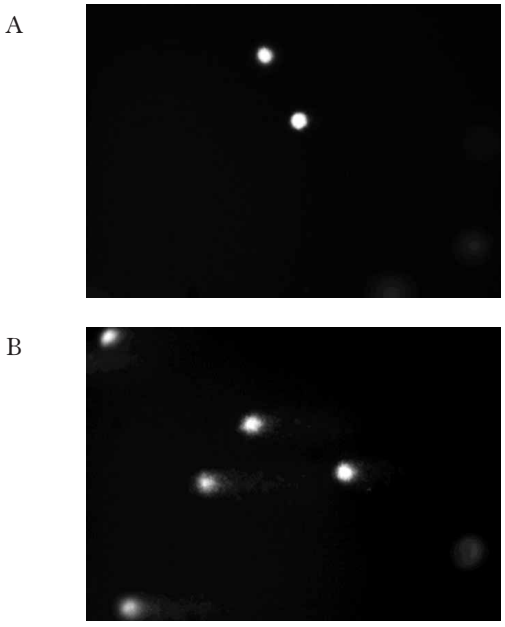

Figure 3. *Undamaged leukocytes from a non-exposed subject (A) and comets with long-tailed nuclei from a worker occupationally exposed to tobacco dust (B).*

mented in the exposed non-smokers was 14.34±0.77 μm, as opposed to that of 14.43±1.22 μm established in the exposed smokers. In the control group, the mean value of the tail length established in non-smokers was 13.62±0.77 μm, as opposed to that of 14.18±0.50 μm found in smokers. In the exposed female population, the mean tail length equalled 14.84±0.44 μm, while in the controls the comet tail measured 14.05±057μm on the average. As for male subjects, the mean tail length found in the exposed and the control group were 14.09±1.19 μm and 13.81±0.73 μm, respectively. In addition, all comets were classified by considering threshold levels indicating the comets with a long-tailed nucleus (LTN). The percentage of LTNs ranged from 0% to 27% in the exposed group compared to the control group where that percentage ranged from 0% to 13% (Figure 2).

The observed data on the level of DNA migration differ in a significant manner ($P<0.05$) between control and the exposed group for all the volunteers taken together. For the non-smokers and females, that difference was also statistically higher whereas for smokers and males there was no statistical difference between workers exposed to tobacco dust and the control group even though the values found for the DNA migration were slightly higher. Cigarette smoking and gender proved to be potent confounders, both in the exposed and in the control group. Results gained for the percentage of LTNs showed significant increase for all the groups evaluated between workers exposed to tobacco dust and the control non-exposed group. In addition, in the the exposed population, there were significant inter-individual differences in the DNA damage and the percentage of LTNs.

DISCUSSION AND CONCLUSION

In the present study, the alkaline comet assay was used to evaluate the baseline DNA damage occurring in white blood cells of workers employed in tobacco industry. Our results showed that the risk of DNA damage, existent among workers occupationally exposed to tobacco dust, is significant. Gender and smoking habit proved to be as potent confounders both in the exposed and in the control group, as demonstrated by statistically significant differences between male and female workers, and smokers vs non-smokers. Zhu *et al. (28)* reported statistically significant positive correlations between the observed DNA damage (in terms of the tail moment and tail length) and smoking status. They even suggested that smoking and tobacco dust combined are capable of inducing lymphocyte DNA damage and could act in synergy to this goal.

Results provided by Umadevi *et al. (1)* have shown the relationhip between the increased number of chromosome aberrations and chronic exposure to tobacco dust. It has been previously shown that respiratory symptoms described by such workers, and their strong correlation with the length of occupational involvement, are highly suggestive of an occupational disease. In support of this, skin test and bronchial provocation carried out in such

workers using the tobacco dust as a challenger yielded positive results *(2)*. Žuskin *et al. (5)* and Yanev *et al. (7)* pointed toward a significant increase in the total amount of IgE, present in 12.7% of tobacco workers, and zero controls. They also reported a reduction in the lung volume, encountered among female tobacco processors, but considered it to be of minor relevance for the symptoms and lung function abnormalities presented by their subjects. Such a standpoint contradicts the results of Mustajbegović *et al. (6)* who reported a high prevalence of chronic respiratory symptoms among tobacco workers, in particular the female ones.

Based on the foregoing, further research on the health effects of tobbaco dust should be carried out, with a special emphasis on the role and strength of potential confounders, such as gender, age, and smoking habit. The period elapsing from blood sampling to the time of DNA damage detection should be cut down to the shortest one possible to avoid further DNA damaging and to minimize health consequences. Therefore, the advantages offered by the alkaline comet assay, in terms of rapid, cost-effective and fairly sensitive detection of genomic damage, should be more wildly put in routine use to the goal of protecting and preserving human health both in occupational and environmental settings.

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