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Occupational genotoxicity risk evaluation through the comet assay and the micronucleus test

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ABSTRACT. The micronucleus (MN) test and the alkaline single cell gel or comet assay were applied to exfoliated cells of the buccal mucous in order to evaluate the genotoxic risk associated with occupational exposure of 10 storage battery renovation workers, and 10 car painters, with age matched controls, in Pelotas, RS, in southern Brazil. In the MN test, 2000 exfoliated buccal cells were analyzed for each individual, while 100 cells were examined in the comet assay. In the comet test, both comet tail length and a damage index were calculated. Highly significant effects of occupational exposure were found with both the MN test and the comet assay ($P < 0.001$). The comet assay was found to be rapid, of simple visualization, and it is a sensitive technique for measuring and analyzing DNA damage in human cells.

Key words: Micronucleus test, Occupational exposure, Comet assay

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INTRODUCTION

Mutagenesis is involved in the pathogenesis of many neoplasias. Occupational exposure may contribute to the development of pernicious illnesses, many times through mechanisms that involve chromosomal changes. In order to evaluate the possible impact of environmental and occupational exposition on health, it is essential to identify the effects of exposure through epidemiological studies, which also constitute a challenge. Continuous efforts have been made to identify genotoxic agents, to determine conditions of harmful exposition and to monitor populations that are excessively exposed (Maluf and Erdtmann, 2000).

Micronucleus (MN) tests of exfoliated cells in epithelial tissue have been used to evaluate the genotoxic effects produced by low doses of carcinogenic substances or carcinogenic mixtures, to which human populations are exposed (Keshava et al., 1998; Maluf and Erdtmann, 2000). The frequency of MN in human exfoliated cells can be used as an "endogenous dosimeter" in tissues that are specific targets of genotoxic and carcinogenic agents, where carcinomas will develop (Rabello-Gay et al., 1991).

During the last few years, there has been a great interest in developing rapid and simple tests to identify the effects of exposure to environmental agents that can affect the health of individuals due to DNA damage. One of these methods is the comet assay, which is a rapid and sensitive technique to measure sites sensitive to basic pH (alkali-label) and DNA breaks in individual cells. This method was described for the first time by Östling and Johanson in 1984, and in 1988, Singh et al. introduced alkaline conditions to this technique (Wojewódzka et al., 1998).

The assay technique consists of evaluating cells kept in agarose, on a microscope slide, submitted to electrophoresis and dyed with ethidium bromide. Cells with damaged DNA form a comet, consisting of a head (nuclear matrix), and a tail, formed by DNA fragments. The amount of DNA that has migrated is correlated with the damage (Singh et al., 1988; Fairbain et al., 1995; Speit and Hartmann, 1995; Morillas et al., 2002). This assay is extremely versatile, and is used extensively in Biology, Medicine and Toxicology, due to its capacity and sensitivity in demonstrating DNA breaks, both single and double breaks, and alkali-label sites (Fairbain et al., 1995; Miyamae et al., 1998; Bauer et al., 1998; Sardas et al., 1998; Stavreva et al., 1998). The alkaline conditions cause the separation of the paired bases, enabling the detection of simple chain ruptures (McKelvey-Martin et al., 1993; Albertini and Kirsch-Volders, 1997).

Positive results in the comet assay do not always correspond to positive results in the MN tests, especially when the exposure to genotoxic agents is small. The comet assay usually detects more defects than the MN test (Goethem, 1997). The positives in the comet and MN tests are due to different mechanisms; the MN test detects injuries that survive at least one mitotic cycle, while the comet assay identifies reparable injuries or alkali-label sites (Goethem, 1997; Vrzoc and Petras, 1997). Consequently, Goethem (1997) suggests the use of both MN and comet tests.

Wojewódzka et al. (1998) consider interindividual variability important; it can be detected by the analysis of parameters in the comet assay. They found considerable intra-individual homogeneity, and high interindividual variability, suggesting that the extent of the damage, as well as the decrease in the capacity of DNA damage repair, constantly induced by endogenous or exogenous factors, may be involved in the variability of the individual responses found.

Srám et al. (1998) evaluated workers of petrochemical companies, using many tests: changes among chromatid sisters, chromosomal aberrations, MN and comet assays. They clas-

sified the tests in terms of sensitivity. They considered the test of changes among chromatid sisters most sensitive, followed by the test for chromosomal aberrations and then the MN and comet tests, the latter two being similar.

We investigated occupational genotoxic effects in workers who did storage battery renovation or who painted cars, in the city of Pelotas, Rio Grande do Sul, Brazil.

MATERIAL AND METHODS

Ten car painters exposed to lead paint, solvents and benzene, and 10 storage battery reconditioners were included in the study. The respective control groups were matched for age, and had no occupational exposition to toxic agents.

All the individuals were males. They were about 20 to 50 years old (Table 1). All the individuals who agreed to participate in the study were healthy, and they answered a detailed questionnaire according to the protocol published by the International Commission for Protection Against Environmental Mutagens and Carcinogens (Carrano, 1988), which included items about occupational exposure, smoking habit, use of drugs, such as alcohol, virus illnesses, recent vaccinations, and radiological exams.

Table 1. Characteristics of individuals exposed or not to petroleum and lead sub-products (N = 10 in all groups).

Characteristics	Battery renovators (BR)	Controls (BR)	Car painters (CP)	Controls (CP)
Average age (in years)	33.3	33.9	39.2	39.7
Range	23-50	20-48	22-51	24-56
Caucasian	9	10	8	8
Negroid	1	0	2	2
Smokers	4	4	5	3
Non-smokers	6	6	5	7
Consumers of alcohol	8	7	8	8
Abstinent	2	3	2	2
Average working time (years)	10.2	8.4	12.3	10.0
Range	3-38	2-23	4-38	2-27

The exfoliated cells of the buccal mucosa were obtained by scraping the oral cavity with a tongue depressor. Two slides were prepared by smearing the cells onto pre-cleaned slides. Later, the slides were air-dried and fixed with methanol. Staining was done with the Feulgen reaction for the identification of the DNA in the nucleus and MN, followed by counter-staining with fast green to delineate cell cytoplasm.

The MN analysis was done with a light microscope, at 1000X magnification, using coded slides. Two thousand cells from each individual were examined. Only unfragmented cells that were not smeared, clumped or overlapped and that contained intact nuclei, were included in the analysis. Cells undergoing degenerative processes, such as karyorrhexis, karyolysis, fragmentation of the nucleus, broken egg, or pycnosis were recorded separately, according to Tolbert et al. (1992) and Titenko-Holand et al. (1998). Micronuclei had to: a) be less than 1/3 in diameter of the main nucleus, b) be on the same plane of focus, c) have the same color, texture and

refraction as the main nucleus, d) have a smooth oval or round shape, and e) be clearly separated from the main nucleus. Questionable micronuclei were disregarded. The statistical analysis was performed using a two-tailed Student *t*-test. A difference at $P < 0.05$ was considered significant.

Alkaline electrophoresis was performed according to Singh et al. (1988), with modifications described in Silva et al. (2000). An aliquot of blood (5 µl) was mixed with 95 µl of 1% low melting point agarose and spread on two slides previously coated with normal 1.5% agarose. After solidification, the slides were immersed in fresh lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10-10.5, with the addition, at the time of usage, of 1% Triton X-100 and 10% DMSO) for at least 1 h and for up to 2 weeks.

The slides were incubated in alkaline buffer solution, prepared at the time of use (300 mM NaOH and 1 mM EDTA, pH 12.6) for 25 min. The cells were submitted to electrophoresis for 25 min, at 300 mA and 25 V, and then neutralized with 0.4 M Tris, pH 7.5, in three successive washes of 5 min each. The DNA was then dyed with ethidium bromide (2 µg/ml).

Negative and positive controls were used to test the efficiency and electrophoresis conditions. In the positive control, 200 µl of whole blood was incubated for 2 h at 37°C with 50 µl methyl methanesulfonate (MMS; final concentrations of 8×10^{-5} M and 4×10^{-5} M).

Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed from each individual. Comet tail lengths (nuclear region + tail) were measured in arbitrary units. One unit was approximately 5 µm at 200X magnification. The fluorescence microscope was equipped with a BP546/12-nm excitation filter and a 590-nm barrier filter. Cells were also scored visually into five classes, according to tail size (from undamaged - 0, to maximally damaged - 4) and a value was assigned to each comet according to its class. The final overall rating for 100 cells, DNA damage score, between 0 (completely undamaged) and 400 (maximum damage), was obtained by summation (Collins et al., 1995).

The statistical evaluation was performed using a two-tailed Student *t*-test. A difference at $P < 0.05$ was considered significant.

Table 2. Number of cells with micronuclei (among 2000 cells analyzed for each individual) of the individuals exposed to sub-products of petroleum and lead, and their controls.

Individual	Battery renovators (BR)	Controls (BR)	Car painters (CP)	Controls (CP)
1	6	2	8	1
2	10	3	14	3
3	12	4	8	2
4	7	1	5	4
5	5	2	5	0
6	0	1	5	4
7	8	1	4	2
8	8	2	5	5
9	5	1	7	1
10	5	3	8	0
Mean	6.6*	2.0	6.9*	2.2
SD	3.27	1.05	2.92	1.75

*Significantly different from the respective control, $P < 0.001$ (two-tailed Student *t*-test).
SD: standard error.

RESULTS

The main characteristics of the exposed and control workers were recorded (Table 1). The individuals were identified in terms of age, years they had worked, race, and smoking and alcoholic habits. The mean number of MN was significantly greater in both the storage battery renovation workers and the car painters, than in the respective controls (Table 2; P < 0.001), though there was no significant difference between these two occupational risk groups (P > 0.5).

Table 3. Size of the comet tail in µm of 100 cells analyzed for each individual exposed to petroleum and lead sub-products, and their controls.

Individual	Battery renovators (BR)	Controls (BR)	Car painters (CP)	Controls (CP)
1	33.1 ± 13.83	30.5 ± 5.00	34.7 ± 17.77	31.0 ± 7.03
2	35.3 ± 19.38	31.0 ± 7.03	36.8 ± 21.96	30.5 ± 5.00
3	35.9 ± 19.12	30.9 ± 5.61	36.0 ± 19.54	30.5 ± 5.00
4	33.6 ± 14.73	30.0 ± 1.58	33.0 ± 13.59	30.5 ± 5.00
5	32.3 ± 11.44	30.0 ± 2.24	32.5 ± 13.09	30.6 ± 6.00
6	31.7 ± 9.74	30.9 ± 5.50	32.5 ± 12.74	30.9 ± 5.60
7	36.0 ± 20.76	31.1 ± 7.77	32.1 ± 10.37	31.0 ± 7.06
8	34.3 ± 17.18	30.6 ± 6.00	32.7 ± 13.62	31.9 ± 11.07
9	35.7 ± 19.83	30.5 ± 5.72	34.4 ± 17.88	30.4 ± 4.00
10	33.9 ± 16.01	30.0 ± 2.46	33.8 ± 15.49	30.0 ± 2.25
X	34.18*	30.54	33.85*	30.73
SE	0.484	0.136	0.507	0.162

*Significantly different from the respective control, P < 0.001 (two-tailed Student *t*-test).
SE: standard error of the mean.

Table 4. Damage index, evaluated in 100 cells analyzed for each individual in the comet assay, of individuals exposed to sub-products of petroleum and lead and their respective controls.

Individual	Battery renovators (BR)	Controls (BR)	Car painters (CP)	Controls (CP)
1	11	1	15	2
2	18	2	27	1
3	21	2	19	1
4	9	0	9	1
5	7	0	7	2
6	5	2	8	2
7	22	3	5	2
8	10	1	9	1
9	20	1	13	6
10	13	0	16	0
Mean	13.6*	1.2	12.8*	1.8
SD	6.18	1.03	6.67	1.61

*Significantly different from the respective control, P < 0.001 (two-tailed Student *t*-test).
SD: standard deviation.

The comet assay values were significantly higher in each of the occupational exposure groups (Table 3; P < 0.001). The damage index ratings (based on comet tail size) were also significantly greater in the battery renovator workers and the car painters, than in their respective controls (Table 4; P < 0.001).

DISCUSSION

The comet assay gave a similar sensitivity to the MN test. Maluf and Erdtmann (2000) also found highly significant differences with both techniques in an evaluation of doctors and technicians who work with X-ray machines, in a hospital in the city of Sapucaia, RS (P = 0.005 and P = 0.0078, for the comet and MN tests, respectively).

A greater difference was observed between the averages of the number of MN both in the battery renovation workers as well as in the car painter workers, demonstrating that the agents to which they are exposed to are genotoxic. There was also a big variation in the individual results in each exposed group, while control groups were quite homogeneous. In the comet assay there were also significant differences between the two exposed groups and their controls in terms of the size of the comet. Wojewódzka et al. (1998) found a significantly longer comet tail in a group of workers exposed to low doses of radiation (163.07 ± 7.68 µm), compared to the control group (117.98 ± 5.24 µm).

We found large differences in the damage index in the exposed groups, compared to their controls, approximately 11 times greater in the battery renovator group and 7 times in the car painter group, which was similar to what was found by Collins et al. (1995). The damage index appears to be a very sensitive parameter, as also found by Collins et al. (1995) with HeLa cells (cultivated tumor cells) and human lymphocytes and by Silva et al. (2000), who studied rodents from a carboniferous zone. Baltaci et al. (1998) evaluated, through the comet assay, cells of women with habitual abortions; they obtained a damage index of 87% with no migration, 8.2% with limited migration and 4.5% with extensive migration, while in women of the control group they found 94% with no migration, 4.5% with limited migration and 1.6% with extensive migration; the differences were highly significant (P < 0.001).

There was considerable variation in the damage index in the exposed groups, while it was quite small in the controls (Table 4). The storage battery renovator and painter workers had an increased frequency of MN, due to the genotoxic action of the substances to which they are exposed in their work. The buccal mucous cells express the action of environmental agents with genotoxic potential. Consequently, MN of this type of tissue provide a good evaluation of the degree of occupational exposure.

The study and the standardization of tests for the evaluation of biological damage are essential for public institutions that are concerned with environmental quality and public health. Genotoxic evaluation is necessary to guarantee environmental quality and occupational health, as well as to orient workers to help reduce genetic damage and the risk of serious illness.

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