Mutagenesis vol. 18 no. 5 pp. 417–421, 2003 DOI: 10.1093/mutage/geg022

Cytogenetic biomonitoring in petrol station attendants: micronucleus test in exfoliated buccal cells

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To study the effects of occupational exposure to petroleum derivates such as benzene, exfoliated buccal cells from 50 petrol station attendants and 50 age- and sex-matched control subjects were examined for micronucleus (MN) frequency. Frequencies of nuclear abnormalities (NA) other than micronuclei, such as binucleates, karyorrhexis and karyolysis, were also evaluated. Benzene exposure was ascertained by measuring urinary phenol levels. The mean urinary phenol level of station workers was found to be significantly higher than that of control subjects ($P < 0.05$). Analysis of buccal cells revealed that MN and NA frequencies in petrol station workers were significantly higher than in control subjects ($P < 0.01$) and also significantly related to smoking habit ($P < 0.01$). Our findings indicate that the petrol station workers are under risk of significant cytogenetic damage.

Introduction

Exposure to gasoline vapours is classified by the International Agency for Research on Cancer (IARC) as possibly carcinogenic to humans, mainly on the basis of the well-established carcinogenicity of some components such as benzene (IARC, 1989). The association between exposure to benzene or benzene-containing mixtures and certain types of leukaemia has been shown in epidemiological studies in different countries (Aksoy, 1985; Ireland *et al*., 1997; Carletti *et al*., 2002).

Furthermore, an increased level of cytogenetic damage in peripheral blood lymphocytes of workers occupationally exposed to petroleum and petroleum derivates has been demonstrated using different genetic end-points, such as sister chromatid exchange (SCE), DNA strand breaks and micronuclei (Högsted *et al*., 1991; Oesch *et al*., 1995; Bukvic *et al*., 1998). Nevertheless, about 92% of human cancers are derived from the external and internal epithelium. However, effective techniques have not yet been developed for direct chromosome preparations from epithelial tissue. On the other hand, the micronucleus test in exfloliated epithelial cells has been shown to be an effective method to detect unstable chromosomal aberrations (Tolbert *et al*., 1992; Kayal *et al*., 1993; Pastor *et al*., 2001; Huvinen *et al*., 2002).

The buccal epithelium is composed of four strata including the basal cell layer, prickle cell layer, intermediate and superficial layers. The oral epithelium maintains itself by a system of continuous cell renewal in which new cells produced by mitosis in the basal layer migrate to the surface to replace those that are shed. Thus, the mucosa is composed of progenitor and maturing cell populations (Ten Cate *et al*., 1998). Micronuclei are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome fragments or intact whole chromosomes lagging behind in the anaphase stage of cell division. Their presence in cells is a reflection of structural and/or numerical chromosomal aberrations arising during mitosis (Heddle *et al*., 1991; Fenech *et al*., 1999; Norppa and Falck, 2003).

Petrol station attendants are workers chronically exposed to petroleum derivatives primarily through inhalation of the volatile fraction of petrol during vehicle refuelling. Taking into account that occupational exposure to such derivatives may possess genotoxic risk, we aimed to investigate the cytogenotoxic damage in exfoliated buccal cells obtained from petrol station workers and control subjects, using the micronucleus test. Several degenerative nuclear changes, such as binucleates, karyorrhexis and karyolysis, were also analysed in addition to micronuclei. Frequencies of micronuclei and other nuclear abnormalities were compared in both smokers and non-smokers in order to examine the possible effects of smoking.

Materials and methods

Subjects

The study was carried out on 50 petrol station attendants (25 smokers and 25 non-smokers) working at 15 petrol stations located in the urban area of Mersin city. The control group consisted of 50 healthy men (25 smokers and 25 non-smokers) working on the Mersin University campus, without indication of any exposure to petroleum derivates or other potential genotoxic substances. Participants were informed about the study and asked to sign an informed consent form and to complete a standardized questionnaire to obtain necessary data on lifestyles and personal factors (age, working period, health, etc.). None of these study groups showed significant differences with regard to lifestyle and personal factors.

Urine collection and phenol measurement

Exposure monitoring was performed by the detection of phenol excreted in the urine. Samples were obtained from workers immediately after their 8 h work shift. Urinary phenol measurements were performed following the colorimetric quantitative determination method of Yamaguchi and Hayashi (1977). The reported urinary phenol values were corrected for creatinine content.

Evaluation of micronuclei and nuclear abnormalities in buccal cells

Buccal cell samples were collected at the end of the work shift. Subjects were asked to rinse their mouth with water before sampling. A wooden spatula was used to obtain cell samples from buccal mucosa. The samples were then applied to clean microscope slides. Smears were air dried and fixed in methanol:acetic acid (3:1). Slides were stained by the Feulgen reaction technique according to Stich and Rosin (1984). Three slides were prepared for each subject and 1000 cells were evaluated per slide to determine the micronucleus (MN) frequencies.

Nuclear abnormalities were classified according to Tolbert *et al.* (1992). Briefly, cells with two nuclei were considered as binucleates. Nuclei fragmented into irregular pieces were scored as karyorrhexis. Nuclear dissolution, in which a Feulgen-negative, ghost-like image of the nucleus remains, was evaluated as karyolysis. The following criteria for MN analyses were used in oral epithelial cells. A MN must: (i) be less than one-third the diameter of the main nucleus; (ii) be on the same plane of focus; (iii) have the same colour, texture and refraction as the main nucleus; (iv) have a smooth, oval or round shape; (v) be clearly separated from the main nucleus.

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Table I. General characteristics of the groups studied

Study group		*n*	Age (years)	Average no. of cigarettes/day	Duration of employment (years)	Urinary phenol (mg/g/l creatinine)	
						Mean ± SE	Range
Controls	Smokers	25	30.12 ± 2.54	25		9.03 ± 1.90	5.25–16.10
	Non-smokers	25	36.47 ± 3.11			5.46 ± 1.76	1.93–12.10
	Total	50	33.71 ± 2.25	25		7.25 ± 1.36	1.93–16.10
Workers	Smokers	25	30.79 ± 1.78	20	5.3 ± 0.86	24.50 ± 7.77	8.62–52.82
	Non-smokers	25	32.15 ± 2.72		5.7 ± 0.75	15.19 ± 4.43	4.58–31.10
	Total	50	31.47 ± 1.71	20	5.5 ± 0.91	19.85 ± 4.49[a]	4.58–52.82

[a]$P < 0.01$.

Statistical analysis

All the data are expressed as the mean ± standard error of the mean. Data were tested for normality by the Kolmogorov–Smirnov test. Interactions between smoking and exposure were tested with a two-way analysis of variance. Multiple comparisons were made by using a least significant difference (LSD) test. A *t*-test was used for comparison of urinary phenol levels. The type I error rate was accepted as 0.05. All statistical analyses were performed using the program SPSS 9 for the PC.

Results

Demographic characteristics of the groups studied are summarized in Table I. None of the groups studied showed significant differences in age, sex, smoking habit (cigarettes/day) or duration of employment. As shown in Table I, the mean urinary phenol level of petrol station workers (19.85 ± 4.49 mg/g/l) was found to be significantly higher ($P < 0.01$) than that of control subjects (7.25 ± 1.36 mg/g/l). Analysis of urine samples also revealed higher phenol values in smokers than non-smokers in both controls and exposed subjects, but these differences were not statistically significant ($P > 0.05$) and no interaction was found between smoking and petroleum exposure. Data on the urinary phenol measurements in controls and station workers are shown graphically in Figure 1.

Results for micronuclei and nuclear abnormalities are given in Table II and Figures 2 and 3. Assessment of MN frequencies in exfoliated buccal cells revealed a significant difference ($P < 0.01$) between exposed workers (1.34 ± 0.80) and control subjects (0.47 ± 0.03) and smoking also had a significant effect on MN frequency in both exposed and control groups ($P < 0.01$). However, no interaction was observed between smoking and petroleum exposure.

A significant difference in binucleus frequency was observed between controls (0.76 ± 0.05) and exposed (2.05 ± 0.36) workers ($P < 0.01$). The difference between smokers and non-smokers was found to be significant only in the exposed subjects ($P < 0.01$). A significant interaction between smoking and petroleum exposure was also determined ($P < 0.05$).

Karyorrhexis frequency was found to be higher ($P < 0.01$) in exposed individuals (1.62 ± 0.09) than controls (0.44 ± 0.09). Similarly, karyolysis frequency was found to be significantly higher ($P < 0.01$) in exposed subjects (1.65 ± 0.19) than in the controls (0.44 ± 0.07). Once again, the effects of smoking on the frequencies of karyorrhexis ($P < 0.01$) and karyolysis ($P < 0.05$) were found to be significant in both the control and exposed groups. However, the interaction between smoking and petroleum exposure was not significant ($P > 0.05$).

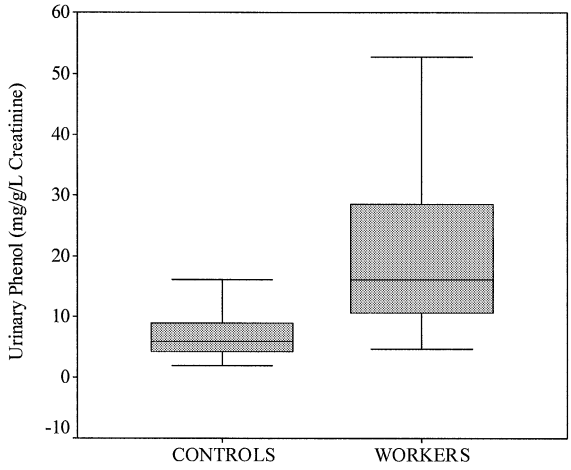

Fig. 1. Box plot diagram showing the distribution of urinary phenol values in petrol station workers and control subjects. Line within the box, median; bars, range.

Discussion

Benzene, an important component of petrol, is a widely distributed environmental contaminant. Today, about 98% of the benzene is derived from the petrochemical and petroleum refining industries. Therefore, occupational exposure to benzene in humans generally takes place in factories, refineries and other industrial settings. Moreover, the general population is exposed to benzene contained in petrol, vehicle exhaust, diesel fuel and cigarette smoke (Zhang *et al*., 2002). Occupational exposure to benzene has mainly been associated with increased incidences of acute myeloid leukemias, but also with chronic myeloid and acute lymphoid leukemias and non-Hodgkin's lymphomas (Aksoy, 1985; Hayes *et al*., 2000). Epidemiological studies also showed that there is a clear relationship between the increase in MN frequency and exposure to benzene and benzene metabolities (Yager *et al*., 1990; Tompa *et al*., 1994; Turkel and Egeli, 1994).

However, the data on cytogenetic damage in petrol station attendants are rather controversial. Evaluation of SCE frequencies in petrol station attendants showed that there was no significant difference between controls and experimental subjects (Carere *et al*., 1995; Pitarque *et al*., 1997). Bukvic *et al*. (1998) also analysed MN and SCE frequencies in peripheral lymphocytes of petrol station attendants and failed to reveal significant differences for SCE, although they found significant differences for MN. Nevertheless, in another study,

Table II. The frequencies (‰) of micronuclei and other nuclear abnormalities in exfoliated buccal cells of controls and exposed subjects

Group		*n*	Micronuclei	Nuclear abnormality		
				Binucleates	Karyorrhexis	Karyolysis
Controls	Smokers	25	0.65 ± 0.03	0.84 ± 0.07	0.52 ± 0.09	0.48 ± 0.10
	Non-smokers	25	0.29 ± 0.02	0.67 ± 0.03	0.36 ± 0.09	0.39 ± 0.06
	Total	50	0.47 ± 0.03	0.76 ± 0.05	0.44 ± 0.09	0.44 ± 0.07
Workers	Smokers	25	1.63 ± 0.08	2.35 ± 0.41	1.85 ± 0.11	1.73 ± 0.12
	Non-smokers	25	1.14 ± 0.06	1.75 ± 0.05	1.39 ± 0.10	1.56 ± 0.11
	Total	50	1.34 ± 0.8[a]	2.05 ± 0.36[a]	1.62 ± 0.09[a]	1.65 ± 0.19[a]

All values are given as mean ± SE.
[a]$P < 0.01$.

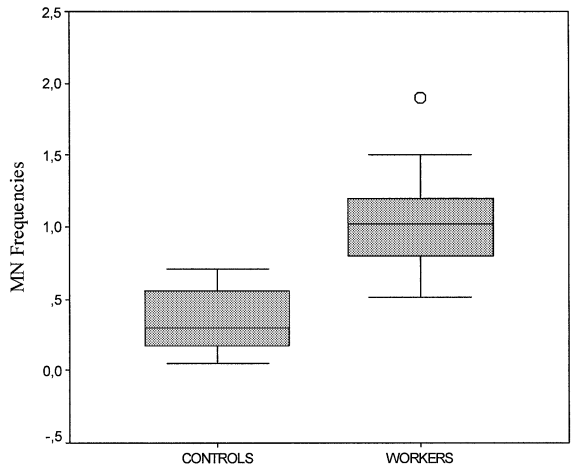

Fig. 2. Box plot diagram showing the distribution of MN frequencies (‰) in petrol station workers and control subjects. Line within the box, median; bars, range; small circle, outlier.

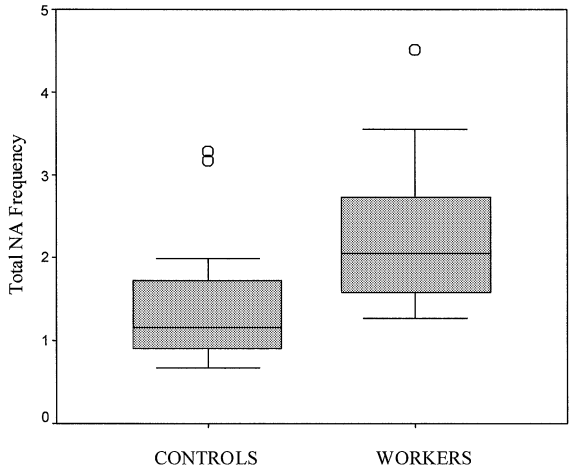

Fig. 3. Box plot diagram showing the distribution of the frequencies of total nuclear abnormalities (NA) (‰) in petrol station workers and control subjects. Line within the box, median; bars, range; small circles, outliers.

no significant increase in MN frequency in cultured blood lymphocytes from a group of filling station attendants was detected (Pitarque *et al*., 1996). Surralles *et al*. (1997) performed a molecular cytogenetic analyis on buccal cells and lymphocytes obtained from benzene-exposed workers employed in a petrochemical complex in Estonia and reported that there was no increase in the frequency of cytogenetic damage with respect to the controls.

In our study, we observed a significant increase in the frequency of micronucleated buccal cells in petrol station workers. Our findings are in agreement with those of Högstedt *et al*. (1991), who detected a significant increase in the frequency of micronuclei in the lymphocytes of petrol station workers. An increased level of chromosomal deletions in Brazilian station workers was also shown by Santos-Mello and Cavalcante (1992). Oesch *et al*. (1995) detected an increase in DNA strand breaks in mononuclear blood cells of non-smoker petrol station attendants. Similarly, Andreoli *et al*. (1997) also found that DNA damage in peripheral lymphocytes of petrol station workers increased, using the single cell gel electrophoresis method.

Analysis of exfoliated cells of buccal mucosa also provides evidence of other nuclear abnormalities such as binucleates (presence of two nuclei within a cell), karyorrhexis (nuclear fragmentation) and karyolysis (nuclear dissolution) (Tolbert *et al*., 1992). Binucleus formation is considered as indicator of cytotoxicity, while karyorrhexis and karyolysis are considered as indicators of apoptosis. In our study, a comparison of nuclear abnormalities also revealed significant differences between controls and exposed subjects. Similar results were observed for buccal cells of firebreathers exposed to diesel (Torres-Bugarín *et al*., 1998). Revazova *et al*. (2001) reported an increase in nuclear abnormalities in buccal cells of women living in a dioxin contaminated area. Increased frequencies of nuclear abnormalities in buccal cells of smokeless tobacco users was also shown by Livingston *et al*. (1990).

Cigarette smoking is one of the factors that may influence the rate of cytogenetic damage, such as chromosomal aberrations, SCE and micronuclei in humans (Al-Sabti *et al*., 1992; Lakhanisky *et al*., 1993; Pitarque *et al*., 1997). Smoking is also reported to increase the MN frequency in buccal cells (Sarto *et al*., 1987; Piyathilake *et al*., 1995). Our findings indicate that cigarette smoking significantly increases the frequencies of MN and other nuclear abnormalities in both controls and exposed subjects. However, an interaction between smoking and benzene exposure was found to be significant only for binucleus formation.

Phenol is the principal metabolite of benzene. Therefore, phenol concentration in the urine of exposed workers can be used as a biomarker of external exposure. Biological monitoring of petrol station attendants showed substantially higher levels of urinary phenol when workers were compared with subjects with no known exposure to either gasoline or benzene (Pandya *et al*., 1975; Hein *et al*., 1989; Verma and Rana, 2001).

Khoschsorur and Peter (2000) reported that urinary phenol concentration in petrol station workers (17.28 mg/g/l creatinine) was higher than in control subjects (4.4–6.6 mg/g/l creatinine). In this study we also found significantly higher urinary phenol levels in petrol station workers (19.85 mg/g/l creatinine) than in control subjects (7.25 mg/g/l creatinine). The mean urinary phenol level of smokers was also found to be higher than that of non-smokers in both controls and exposed subjects. However, these differences were not statistically significant.

On the other hand, it is known that the urinary phenol level can be affected by demographic factors, such as diet, smoking and ingestion of medicine. Therefore, analysis of other urinary biomarkers such as *trans*,*trans*-muconic acid and *S*-phenylmercapturic acid have also been suggested for bimonitoring of low level benzene exposure (Qu *et al*., 2000). Since none of the subjects in our study group differed in their demographic parameters, the observed differences in urinary phenol levels could be attributed to the occupational exposure to benzene.

Benzene exposure of petrol station attendants can vary widely due to several factors, such as quantity of fuel pumped, type and number of vehicles filled, protective measures and total content of benzene in the petroleum. According to data obtained from the petrol stations included in this study, each worker pumps an average 2000 l of petroleum, containing 5% (v/v) benzene, during their 8 h work shift. On the other hand, it should also be emphasized that petrol station attendants are not only exposed to hydrocarbons present in petrol vapours, but also to the emissions produced by engines during fuel combustion. It was shown that these emissions may also cause cytotoxic and genotoxic effects (Hadnagy and Seemayer, 1988).

In conclusion, our results reveal that petrol station workers could be under risk of significant cytogenetic damage. The micronucleus test in exfoliated epithelial cells seems to be a useful biomarker of occupational exposure to genotoxic chemicals. As demonstrated in this study, other nuclear abnormalities, such as binucleates, karyolysis and karyorrhexis, are also useful indices of chemical exposure and toxic response. Therefore, a combination of micronuclei and nuclear abnormalities may increase the sensitivity of the exfoliated epithelial cell technique in assessment of genotoxicty.

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Received on November 14, 2002; revised on June 12, 2003; accepted on June 30, 2003