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Low air levels of benzene: Correlation between biomarkers of exposure and genotoxic effects

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ABSTRACT

This study was aimed to identify useful biomarkers of exposure and effect in workers exposed to low levels of benzene, and to evaluate any correlations existing between these parameters.

Benzene exposure was measured in 33 petrochemical industry operators (PIO), 28 service station attendants (SSA), 21 gasoline pump maintenance workers (GPMW) and 51 non-exposed controls by GC-FID analysis. Samples were collected with personal passive samplers (Radiello®). End-shift urine samples were collected for *t*,*t*-muconic acid (*t*,*t*-MA) determination by HPLC and for S-phenylmercapturic acid (*S*-PMA) measurement by HPLC–MS/MS. The alkaline version of the comet assay and, in a subgroup of 19 SSA and 16 control subjects, chromosomal aberrations (CA) and glutathione (GSH) levels were measured in peripheral blood lymphocytes.

Personal benzene exposure was significantly higher in PIO, SSA and GPMW as compared to controls. The urinary excretion of the two metabolites showed a significant increase in SSA ($p = 0.0258$ and $p = 0.0001$, for *t*,*t*-MA and *S*-PMA, respectively) and in PIO ($p = 0.0013$ and $p = 0.0001$, for *t*,*t*-MA and *S*-PMA, respectively) as compared with the control group, while no such increase was observed for GPMW, for whom occupational exposure was not continuous and occurred on specific working days only. Significant increases of DNA damage were found by the comet assay for tail moment (TM) and tail length (TL) in SSA ($p < 0.0001$ and $p = 0.008$, for TM and TL, respectively) and PIO ($p < 0.0001$ and $p < 0.0001$, for TM and TL, respectively) when compared with controls. The PIO group also displayed a significant increase in the number of cells with comet ($p < 0.0001$). Smoking habits did not appear to interfere with these results in any of the groups. No difference was found in percentage of CA between exposed workers and controls. Significant correlations were found, in all groups, between benzene exposure and the more representative comet parameter TM ($r = 0.509$, $p = 0.007$; $r = 0.525$, $p = 0.017$ and $r = 0.420$, $p = 0.046$ in SSA, GPMW, and PIO, respectively). A trend of negative correlation was observed between DNA damage and either GSH or urine *S*-PMA for exposed workers. In summary, in present study urinary *S*-PMA and DNA damage by the comet assay were both sensitive to exposure to low levels of benzene, and GSH seems to play an important defence role against benzene-dependent DNA damage.

1. Introduction

Benzene is a volatile aromatic hydrocarbon solvent, which has been widely used in the past in industry, while now is still present in some occupational settings mainly at low concentrations. It is also a ubiquitous environmental contaminant, and a component of cigarette smoke, gasoline and automobile emissions (Duarte-Davidson et al., 2001). Benzene has long been known to cause leukaemia, but there are still uncertainties concerning its mechanism of action and concentration–response relationship. The chronic exposure of humans to low levels of benzene in workplaces has been associated with blood disorders, including aplastic anemia and leukaemia (Snyder et al., 1977). Accordingly, benzene has undergone and continues to undergo regulatory scrutiny to ensure reduced occupational exposures and improved workers' health. The progressive reduction of the levels of exposure in most workplaces, in fact, has shifted the attention from the health effects observed at high doses of benzene to the health risks expected

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from occupational or environmental exposure to low levels of exposure.

Benzene exposure is primarily via benzene inhalation. Following rapid absorption through the lungs, benzene undergoes both phase I and II biotrasformation in the liver. The primary metabolite is benzene oxide, produced by CYP2E1 oxidation, which is rearranged to form phenol or reacts with glutathione to form less toxic or non-toxic derivates via glutathione-S-transferases (GSTs) such as S-phenylmercapturic acid (*S*-PMA), both of which are excreted in the urine. Phenol is also converted to potentially toxic metabolites catechols and hydroquinones. These can be further oxidized in the bone marrow by myeloperossidase (MPO) to benzoquinones, which are detoxified by NAD(P)H:quinoneoxidoreductase 1 (NQO1) to less toxic hydroquinones (Ross, 2000). Quinones, benzoquinones and other benzene metabolites can produce reactive oxygen species (ROS), which can damage critical macromolecular targets including DNA, proteins and lipids. Alternatively, the reactive products can bind covalently to cellular molecules, proteins and DNA inducing DNA strand breaks, sister chromatide exchange (SCE), micronuclei and chromosomal aberrations (CA). An increased number of single strand breaks (SSB_s) in DNA of leukocytes, and of SCE, micronuclei and CA has been reported in workers occupationally exposed to benzene, even if results from cytogenetic surveys are at variance (Anwar, 1994; Whysner et al., 2004). In addition, benzene oxide can be hydrolyzed by microsomal epoxide hydrolase (mEH) to benzene dihydrol that is then converted to catechol or can undergo ring opening to produce tran-trans-muconaldehyde, that is another potentially toxic metabolite, which may ultimately lead to *t,t*-muconic acid (*t,t*-MA), one of the urinary end products of benzene (Bruckner and Warren, 2001).

Benzene has also been suggested to act as a mutagen via an indirect mechanism, which leads to oxidative DNA damage by promoting the formation of hydroxyl radicals from hydrogen peroxide (Andreoli et al., 1997). Since oxidative DNA damage is subjected to a specific repair, the cellular steady-state levels reflect the balance between generation and removal of the lesions. Cell lesion generation is modulated by cellular antioxidant systems, and among these, reduced glutathione (GSH) is an important scavenger of free radicals and regulates the redox status of many other cellular substances, thus playing an essential role for detoxication processes.

This study has been undertaken: (a) to identify useful biomarkers of exposure to low levels of benzene in workers with different occupations, such as gasoline service attendants, pump maintenance workers and petrochemical industry operators; (b) to test in these subjects DNA damage (using the alkaline version of the comet assay), chromosome aberrations and GSH content in lymphocytes as biomarkers of effect; (c) to evaluate any correlations existing between these parameters.

2. Materials and methods

2.1. Study groups

Peripheral blood lymphocytes were obtained from 33 petrochemical industry operators (PIO), 28 service station attendants (SSA), 21 gasoline pump maintenance workers (GPMW) and 51 unexposed workers, working mainly in office jobs (controls), all males, including smokers and non-smokers. The relevant characteristics of all subjects are summarized in Table 1. Lymphocytes were isolated for analysis of CA, DNA damage (alkaline comet assay) and GSH levels.

2.2. Personal exposure

Exposure to benzene during the work shift (approximately 8 h) were measured using personal diffusive samplers containing an active carbon cartridge (Radiello®) and worn by all the subjects at the breathing zone level. Analysis was performed by GC-FID after desorption of benzene from the active carbon with carbon disulfide low in benzene content according to a modified NIOSH method (NIOSH, 2003). The detection limit of the procedure was 3 $\mu g/m^3$.

Urine samples were obtained at the end of personal air sampling when this was performed. 10 ml of each urine sample were collected in a sterile container without the addition of preservatives or stabilizers and were sealed and stored at −20 °C until analysis. In these conditions samples can be stored for 1 month.

Urinary *t,t*-MA analysis was carried out by an HPLC–UV technique with absorbance at 264 nm, after extraction by SPE (SAX column-Varian) and according to the analytical method described elsewhere (Aprea et al., 2008). The detection limit of the procedure was 10 μg/l. For each urine sample, which was collected in a sterile container, 5 ml were acidified with 50 μl of HCl, sealed, and stored at 2–8 °C until analysis. In these conditions, samples can be stored for at least 6 months. Urinary *S*-PMA was determined using an *S*-PMA immunoassay test with chemiluminescent detection using kits supplied by MLT Research (Cardiff, UK) (Carrieri et al., 2006). The detection limit of the procedure was 0.5 μg/l. Values of *t,t*-MA and *S*-PMA were finally corrected for urinary creatinine, as determined by a colorimetric test kit supplied by Sigma Diagnostics Inc. (St. Louis, MO, USA).

2.3. Lymphocyte GSH

Cellular GSH level in lymphocytes was measured by HPLC with ortho-phthalaldehyde (OPA) pre-column derivatization as described by Cereser et al. (2001), with some modification. Glutathione ethyl ester (GEE) was added to all samples as the internal standard. Values were expressed as $\mu mol\ GSH/10^6$ lymphocytes.

2.4. Chromosomal aberrations (CA)

CA analysis was performed on a subgroup of 19 SSA and on 16 controls. In brief, for each subject, 400 μl of whole venous heparinized blood were cultured in 5 ml of RPMI 1640 with 10% fetal calf serum (FCS), 5 μg/ml of phytohemagglutinin (PHA), 100 U/ml of penicillin, 100 μg/ml of streptomycin, 2 mM L-glutamine within 24 h from collection. All cultures were incubated in duplicate, at 37 °C for 48 h, and cell division was stopped by adding 0.2 μg/ml Colcemid 2 h before harvesting. Chromosome preparations were made and stained according to standard procedures and for each donor at least 100 metaphases were counted and scored blindly by the same reader (Barch et al., 1997).

2.5. Comet assay

Approximately 8 ml of whole blood was collected by venipuncture and lymphocytes were separated using Vacutainer CPT tubes (Becton Dickinson and Company, Franklin Lakes, NJ, USA). Basal level of lymphocyte DNA damage was evaluated by comet assay under alkaline conditions, performed according to Singh et al. (1988) and described in detail elsewhere (Fracasso et al., 2006). Briefly, the cells were embedded in agarose and layered on a microscope slide, then immersed 1 h at 4 °C in a freshly prepared lysing solution (2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris, pH 10) and supplemented immediately prior to use with 1% N-lauroylsarcosine, 10% DMSO and 1% Triton X-100. Following steps of alkaline unwinding ($pH > 13$) for 40 min, electrophoresis, and neutralization were performed as standard protocol. All steps were conducted under dimmed light to prevent additional DNA damage. Following the electrophoresis run, the slides were neutralized and dipped into cold 100% ethanol. Then the slides were dried at room temperature and kept in a dry atmosphere for a period of up to 3 months until analysis. A total of 50 cells from each of the duplicate slides were examined randomly under fluorescent microscope, and the extent of DNA damage was measured using a software-based analysis of electronic images. DNA damage was quantified as % of DNA in the tail (TI), tail length (TL), tail moment (TM) and the number of cells with comet (Perceptive Instruments, Suffolk, UK). The median of each parameter was used as representative value for each subject, and the mean of medians was used for statistical analysis (Bauer et al., 1998; Rajaee-Behbahani et al., 2001; Mohankumar et al., 2002; Fracasso et al., 2002; Baumgartner et al., 2004).

3. Statistical analysis

Data analysis was performed with the statistical package SPSS (Version 14.0, Chicago, IL, USA). Any normal distribution of the different variables was verified with the Kolmogorov–Smirnov test. Parametric and non-parametric tests were used for the analysis of normally and non-normally distributed variables, respectively. The results were expressed as median (range) or mean ± SD, as indicated. Differences between samples with normal distribution were examined by the Student's *t*-test, whereas the Mann–Whitney *U*-test was used to determine statistical differences between values with non-parametric distribution. Correlation coefficients were calculated using the Spearman's test. A *p*-value lower than 0.05 was considered as statistically significant for all tests.

Table 1
Descriptive parameters of control and exposed subjects (values are mean ± SD).

Group of workers	Subgroup	*n*	Age	Years of employment	Cigarettes/day
Controls	Total	51	37.69 ± 10.19	–	
	Non-smokers	26	35.45 ± 7.61	–	
	Smokers	25	40.67 ± 12.52	–	10.88 ± 4.90
Service-station attendants (SSA)	Total	28	43.79 ± 12.35	14.20 ± 14.13	
	Non-smokers	15	43.87 ± 12.74	14.76 ± 13.40	
	Smokers	13	43.67 ± 12.42	13.28 ± 16.08	15.94 ± 5.20
Gasoline pump maintenance workers (GPMW)	Total	21	38.65 ± 9.77	10.68 ± 10.39	
	Non-smokers	12	41.56 ± 11.52	9.50 ± 11.79	
	Smokers	9	35.38 ± 6.59	12.00 ± 9.17	8.44 ± 2.65
Petrochemical industry operators (PIO)	Total	33	51.33 ± 5.05	25.36 ± 6.12	
	Non-smokers	18	51.60 ± 5.04	26.33 ± 4.64	
	Smokers	15	51.11 ± 5.19	24.49 ± 7.21	17.46 ± 8.95

4. Results

Personal exposure to benzene in air and the levels of metabolites in urine in different groups of exposed workers and in matched controls, including smokers and non-smokers, are reported in Table 2.

In all groups of workers (SSA, GPMW and PIO) the level of personal exposure to benzene was significantly higher as compared with the control group ($p < 0.0001$). In controls we found that personal exposure to benzene was influenced by cigarette smoking, benzene exposure being significantly higher in smokers than in non-smokers. Interestingly, in the exposed groups an opposite trend is observed. The urinary excretion of the two metabolites showed a significant increase in SSA ($p = 0.0258$ and $p = 0.0001$, for *S*-PMA and *t*,*t*-MA, respectively) and in PIO ($p = 0.0013$ and $p = 0.0001$, for *t*,*t*-MA and *S*-PMA, respectively) as compared with the control group, while no increase was observed for GPMW, for whom occupational exposure is not continuous and only occurs in specific working days. The smoking habits did not substantially modify the urine levels of the metabolites in controls or in exposed groups, the difference being statistically significant only for *S*-PMA in SSA ($p = 0.0025$).

Basal levels of lymphocyte DNA damage evaluated with the alkaline version of the comet assay, chromosomal aberrations and intracellular GSH levels are reported in Tables 3–5, respectively. Table 3 summarizes the results of the baseline DNA damage parameters (tail moment, tail intensity or % of DNA in the tail, and tail length) and the number of cells with comet in peripheral blood lymphocytes of control subjects and benzene-exposed workers. Significant increases were found for TM, TI and TL in SSA ($p = 0.002$, $p = 0.023$, and $p = 0.002$, for TM, TI, and TL, respectively) and for TM and TL in PIO workers ($p < 0.0001$ and $p < 0.0001$, for TM and TL, respectively) when compared with the control group. The PIO group also displayed a significant increase in the number of cells with comet ($p < 0.0001$). The baseline DNA parameters in GPMW were quite similar to controls. The smoking habits did not interfere with the results in all groups (Table 3).

Table 2
Personal benzene exposure and urinary metabolite excretion in control and exposed subjects, including smokers and non-smokers. The values are reported as median (range).

Group of workers (*n*)	Benzene (μg/m^3)	*t*,*t*-MA (μg/g creatinine)	*S*-PMA (μg/g creatinine)
Controls (51)	5.40 (1.97–16.3)	84.00 (3.00–460.50)	1.90 (0.30–10.08)
Non-smokers (26)	4.73 (1.97–10.52)	79.20 (3.00–460.50)	1.88 (0.30–9.62)
Smokers (25)	7.80 (4.00–16.30)	88.60 (13.30–445.00)	2.30 (0.50–10.08)
	$p = 0.0005$[d]	*n.s.*[d]	*n.s.*[d]
SSA (28)	40.00 (8.00–260.00)	117.00 (30.00–418.00)	5.55 (1.55–15.00)
	$p = 0.0001$[a]	$p = 0.0258$[a]	$p = 0.0001$[a]
Non-smokers (15)	62.50 (12.00–260.00)	103.50 (30.00–418.00)	5.10 (1.55–7.20)
	$p = 0.0001$[b]	*n.s.*[b]	$p = 0.0024$[b]
Smokers (13)	29.00 (8.00–68.00)	127.00 (42.00–256.00)	7.20 (3.88–15.00)
	$p = 0.0001$[c]	$p = 0.0204$[c]	$p = 0.0001$[c]
	$p = 0.0026$[d]	*n.s.*[d]	$p = 0.0025$[d]
GPMW (21)	24.20 (4.60–514.90)	92.00 (13.40–242.50)	1.77 (0.21–10.53)
	$p = 0.0001$[a]	*n.s.*[a]	*n.s.*[a]
Non-smokers (12)	80.10 (8.40–165.20)	109.60 (13.40–242.50)	2.01 (0.21–10.53)
	$p = 0.0001$[b]	*n.s.*[b]	*n.s.*[b]
Smokers (9)	9.50 (2.20–514.90)	65.90 (20.20–225.00)	1.05 (0.62–6.76)
	n.s.[c]	*n.s.*[c]	*n.s.*[c]
	$p = 0.0474$[d]	*n.s.*[d]	*n.s.*[d]
PIO (33)	27.80 (1.70–593.50)	128.00 (49.00–422.00)	8.60 (0.40–35.60)
	$p = 0.0001$[a]	$p = 0.0013$[a]	$p = 0.0001$[a]
Non-smokers (15)	33.30 (1.70–593.50)	108.50 (49.00–380.00)	8.65 (0.50–13.20)
	$p = 0.0001$[b]	$p = 0.0384$[b]	$p = 0.0001$[b]
Smokers (18)	22.80 (5.80–482.00)	139.00 (56.00–422.00)	8.60 (0.40–35.60)
	$p = 0.0001$[c]	$p = 0.0046$[c]	$p = 0.0001$[c]
	n.s.[d]	*n.s.*[d]	*n.s.*[d]

n.s. not significant. A Mann–Whitney *U*-test *p*-value < 0.05 was considered as statistically significant.

[a] Exposed vs. controls.

[b] Exposed vs. controls, in non-smokers.

[c] Exposed vs. controls, in smokers.

[d] Smokers vs. non-smokers, in exposed subjects or in controls.

Table 3
Comet assay parameters in lymphocytes of control and exposed subjects, smokers and non-smokers (mean ± SD).

Group of workers (*n*)	TM	TI	TL	No. of comets
Controls (51)	0.28 ± 0.08	2.26 ± 0.56	17.74 ± 3.75	8.52 ± 4.10
Non-smokers (26)	0.29 ± 0.09	2.23 ± 0.53	18.63 ± 4.48	7.78 ± 4.65
Smokers (25)	0.28 ± 0.08	2.11 ± 0.57	17.93 ± 2.08	6.71 ± 3.85
SSA (28)	0.37 ± 0.13	2.78 ± 0.92	20.30 ± 4.39	9.88 ± 5.84
	$p = 0.0020$[a]	$p = 0.0234$[a]	$p = 0.0017$[a]	*n.s.*[a]
Non-smokers (15)	0.39 ± 0.15	2.97 ± 0.90	19.72 ± 3.51	10.50 ± 5.88
	$p = 0.0172$[b]	$p = 0.0053$[b]	*n.s.*[b]	*n.s.*[b]
Smokers (13)	0.33 ± 0.06	2.47 ± 0.91	21.27 ± 5.66	8.83 ± 5.94
	$p = 0.0490$[c]	*n.s.*[c]	$p = 0.0133$[c]	*n.s.*[c]
GPMW (21)	0.25 ± 0.07	1.99 ± 0.60	17.57 ± 2.64	5.79 ± 3.51
	$p = 0.0146$[a]	$p = 0.0090$[a]	*n.s.*[a]	$p = 0.0117$[a]
Non-smokers (12)	0.27 ± 0.05	2.16 ± 0.47	17.63 ± 2.21	6.06 ± 3.06
	n.s.[b]	*n.s.*[b]	*n.s.*[b]	*n.s.*[b]
Smokers (9)	0.23 ± 0.09	1.80 ± 0.70	17.51 ± 3.21	5.50 ± 4.16
	$p = 0.0350$[c]	$p = 0.0370$[c]	*n.s.*[c]	$p = 0.0330$[c]
PIO (33)	0.54 ± 0.47	2.70 ± 1.21	33.94 ± 13.15	13.27 ± 5.19
	$p < 0.0001$[a]	*n.s.*[a]	$p < 0.0001$[a]	$p < 0.0001$[a]
Non-smokers (15)	0.55 ± 0.35	2.81 ± 1.09	35.23 ± 15.52	13.60 ± 4.85
	$p = 0.0070$[b]	*n.s.*[b]	$p < 0.0001$[b]	$p = 0.0004$[b]
Smokers (18)	0.52 ± 0.55	2.61 ± 1.31	32.86 ± 11.16	13.00 ± 5.58
	$p = 0.0020$[c]	*n.s.*[c]	$p < 0.0001$[c]	*n.s.*[c]

n.s. not significant. A Mann–Whitney *U*-test *p*-value < 0.05 was considered as statistically significant.

[a] Exposed vs. controls.

[b] Exposed vs. controls in non-smokers.

[c] Exposed vs. controls in smokers.

Table 5
GSH levels in lymphocytes of control and exposed subjects, smokers and non-smokers (mean ± SD).

Group of workers (*n*)	GSH (μM/10^6 cells)
Controls (19)	10.76 ± 3.58
Non-smokers (11)	8.98 ± 2.48
Smokers (8)	13.21 ± 3.51
	$p = 0.007$[a]
SSA (22)	11.02 ± 3.42
	n.s.[b]
Non-smokers (15)	10.08 ± 2.05
Smokers (7)	13.04 ± 4.93
	n.s.[a]
GPMW (17)	10.69 ± 3.96
	n.s.[b]
Non-smokers (11)	8.93 ± 3.40
Smokers (6)	13.92 ± 2.87
	$p = 0.008$[a]
PIO (8)	18.83 ± 3.19
	$p = 0.001$[b]
Non-smokers (4)	20.17 ± 1.93
Smokers (4)	17.67 ± 4.59
	n.s.[a]

n.s. not significant. A Student's *t*-test *p*-value < 0.05 was considered as statistically significant.

[a] Smokers vs. non-smokers, in exposed subjects or in controls.

[b] Exposed vs. controls.

The analysis of CA was performed in a subgroup of exposed and control subjects only. The data do not display any statistically significant difference between exposed workers and control subjects, whereas this cytogenetic parameter was increased (about 60%) in smokers compared to non-smokers in both groups, although the difference was only statistically significant in controls (Table 4). CA frequency was also significantly correlated with the number of cigarettes smoked ($r = 0.390$, $p = 0.020$, data not shown).

The lymphocyte GSH content was quite similar in controls, SSA and GPMW, and significantly higher in PIO, smokers and non-smokers together, compared to the control group; in this latter group this parameter was only evaluated in a small number of subjects ($n = 8$) because in this group there was not enough cells available. The smoking habits significantly increased GSH level in the control group ($p = 0.007$) and in GPMW ($p = 0.008$) (Table 5).

Correlations between benzene air levels, *S*-PMA or GSH content and genotoxic effect (comet test) are reported in Table 6. Positive, statistically significant correlations were found in all groups between levels of exposure to benzene and comet parameters (TM, $r = 0.509$, $p = 0.007$; TI, $r = 0.524$, $p = 0.005$; and number of comets, $r = 0.476$, $p = 0.012$, in SSA; TM, $r = 0.525$, $p = 0.017$ and $r = 0.420$, $p = 0.046$, in GPMW and PIO, respectively), whereas negative correlations were found between *S*-PMA and TI ($r = -0.465$, $p = 0.017$) or number of comets ($r = -0.469$, $p = 0.016$) in the SSA group. Considering the correlations between GSH cellular content and DNA damage parameters, it is interesting to note that there were negative correlations in SSA and PIO groups, although not statistically significant, and to a significant level in the GPMW group for TM ($r = -0.695$, $p = 0.002$) and TI ($r = -0.678$, $p = 0.003$) and with borderline significance for number of comets ($r = -0.447$, $p = 0.070$). No statistically significant correlation was found between the same parameters in the control group (data not shown).

Table 4
Chromosomal aberrations in lymphocytes of control and exposed subjects, smokers and non-smokers.

Group of workers (*n*)	Percentage of metaphase cells with chromosome damage	
	With gaps (mean ± SD)	Without gaps (mean ± SD)
Controls (16)	5.47 ± 2.53	3.07 ± 1.91
Non-smokers (7)	4.00 ± 1.53	2.57 ± 1.40
Smokers (9)	6.75 ± 2.61	3.50 ± 2.27
	$p = 0.04$[a]	*n.s.*[a]
SSA (19)	4.75 ± 3.32	3.69 ± 2.39
Non-smokers (11)	3.78 ± 2.17	3.00 ± 2.06
	n.s.[b]	*n.s.*[b]
Smokers (8)	6.00 ± 4.24	4.57 ± 2.64
	n.s.[a]	*n.s.*[a]

n.s. not significant. A Mann–Whitney *U*-test *p*-value < 0.05 was considered as statistically significant

[a] Smokers vs. non-smokers, in exposed subjects (SSA) or in controls.

[b] Exposed vs. controls, in non-smokers.

5. Discussion

Personal benzene exposure in exposed workers, although still lower than the current occupational limit value (1600 μg/m^3), is significantly higher in all groups as compared with controls. In this study we found that personal exposure to benzene in the control group is influenced by smoking. Similar results were reported by other authors using passive samplers (Carrer et al., 2000; Aprea et al., 2008). In the exposed groups, however, an opposite trend is observed, and this was probably due to the fact that benzene occupational exposure prevails against exposure to benzene coming from the smoking habits. The correlation found in this study between personal exposure to benzene and the urinary excretion of both metabolites (*S*-PMA and *t*,*t*-MA), but mainly with the former ($r = 0.321$, $p = 0.0001$; $r = 0.250$, $p = 0.0025$, for *S*-PMA and *t*,*t*-MA, respectively, data not shown), indicates *S*-PMA as a more sensitive biomarker of exposure, in agreement with other studies (Choi et al., 2000; Melikian et al., 2002). Moreover, we noted that the more exposed groups (SSA and PIO) are those that showed the highest *S*-PMA urine concentration compared to the control group (+190% and +350%, for SSA and PIO, respectively). The low levels of *S*-PMA found in the GPMW could reflect the non-continuous benzene exposure characterizing their job activity (gasoline pump maintenance). In fact, GPMW may also be exposed to higher concentrations of ben-

Table 6
Correlations between personal exposure to benzene, urinary *S*-PMA, or lymphocyte GSH content and Comet parameters in service-station attendants (SSA), gasoline pump maintenance workers (GPMW) and petrochemical industry operators (PIO).

	SSA			GPMW			PIO		
Comet assay parameter	Benzene (μg/m^3)	*S*-PMA (μg/g creatinine)	GSH (μM/10^6 cells)	Benzene (μg/m^3)	*S*-PMA (μg/g creatinine)	GSH (μM/10^6 cells)	Benzene (μg/m^3)	*S*-PMA (μg/g creatinine)	GSH (μM/10^6 cells)
TM	$r=$ **0.509** $p=$ **0.007**	$r=-0.377$ $p=0.057$	$r=-0.145$ $p=0.520$	$r=$ **0.525** $p=$ **0.017**	$r=0.024$ $p=0.917$	$r=-$**0.695** $p=$ **0.002**	$r=$ **0.420** $p=$ **0.046**	$r=0.025$ $p=0.895$	$r=-0.323$ $p=0.214$
TI	$r=$ **0.524** $p=$ **0.005**	$r=-$**0.465** $p=$ **0.017**	$r=-0.240$ $p=0.283$	$r=0.408$ $p=0.074$	$r=-0.116$ $p=0.618$	$r=-$**0.678** $p=$ **0.003**	$r=0.428$ $p=0.054$	$r=0.022$ $p=0.910$	$r=-0.571$ $p=0.076$
TL	$r=0.032$ $p=0.874$	$r=0.102$ $p=0.620$	$r=0.400$ $p=0.089$	$r=0.233$ $p=0.321$	$r=0.374$ $p=0.095$	$r=-0.059$ $p=0.821$	$r=0.095$ $p=0.611$	$r=0.071$ $p=0.704$	$r=0.048$ $p=0.467$
No. of comets	$r=$ **0.476** $p=$ **0.012**	$r=-$**0.469** $p=$ **0.016**	$r=-0.243$ $p=0.289$	$r=0.390$ $p=0.089$	$r=0.055$ $p=0.814$	$r=-0.447$ $p=0.070$	$r=0.104$ $p=0.578$	$r=-0.135$ $p=0.469$	$r=-0.140$ $p=0.376$

The bold values shows the data that is highly significant.

zene, but for very short periods during their work shift, compared to the prolonged and continuous occupational exposure of SSA and PIO. The *t*,*t*-MA values measured in non-occupationally exposed smokers were comparable with the reference values of the Italian population reported by Aprea et al. (2008), while, interestingly, in non-smokers they were higher. This may be explained by the fact that *t*,*t*-MA is also a metabolite of sorbic acid present in the diet and therefore its urinary level may be influenced by the diet. The influence of both smoking and diet on *t*,*t*-MA levels may render its application as a reliable biomarker of individual exposure to benzene difficult. On the bases of these observations, we can indicate, therefore, *S*-PMA as a better biomarker of exposure to benzene.

Several studies (Parker, 1996; Whysner et al., 2004) suggest that metabolism of benzene leads to the formation of phenol and other metabolites that may increase ROS production. Moreover, the benzene metabolite hydroquinone may be oxidized to p-benzoquinone, a highly reactive intermediate, which can interact with cellular macromolecules, including DNA, inducing DNA strand breaks, that can be detected by comet assay. The alkaline comet assay, therefore, has been used as a sensitive biomarker that reveals DNA damage caused either directly by reactive oxidant agents, or indirectly by substances that can generate free radicals (Fairbairn et al., 1995; Villani et al., 2000; Fracasso et al., 2002). DNA damage, as shown by the alkaline version of the comet assay, is significantly increased by benzene exposure in the SSA and PIO groups, while the negative data observed in the GPMW group are probably due to the particular pattern of exposure, as above described. As reported in other occupational exposure studies, smoking did not affect comet assay values in the present study, despite it is well-known as a major potential confounding factor when evaluating genotoxic agents (Wojewòdzka et al., 1999; Fracasso et al., 2002; Speit et al., 2003). Using the classical comet parameters to study DNA damage (TM, TI, TL and number of comets) we noted a highly significant increase in TL in exposed worker (SSA and PIO, $p = 0.002$ and $p < 0.0001$, respectively) when compared to the control group. Considering Olive-TM (Olive et al., 1992), i.e. the product of the measure of TL and TI, as an indicator of DNA damage we observed that our higher TM values observed in exposed workers were mainly caused by the prevailing increase of the TL parameter. This specific observation suggests that in our conditions, benzene exposure induces DNA damages, which yield small fragments, that are known to be induced in a prevalent manner as a consequence of an increased ROS production (Olive et al., 1992; Collins et al., 1993; Breen and Murphy, 1995).

To evaluate whether the observed DNA strand breaks by comet assay were secondary to a cellular unbalance between antioxidants and pro-oxidants, the lymphocyte GSH concentration was also tested. We found that intracellular GSH content was increased in the PIO group, smokers and non-smokers together, and by smoking habits in GPMW and in control subjects. This increase could reflect a compensation mechanism in response to benzene exposure and/or smoking. We and other authors already reported a similar effect in smokers (Fracasso et al., 2002; Muscat et al., 2004).

The consistent negative correlations observed between GSH levels and DNA damage parameters in the GPMW group (Table 6) and, although not statistically significant, also in the SSA and PIO groups, suggest that subjects with lower GSH content may be more susceptible to benzene-dependent DNA damage. Another interesting result is the negative correlation found in this study between the urinary metabolite *S*-PMA and DNA damage parameters (TI and number of cells with comet) in the SSA group and, although to a lesser extent, also in GPMW or PIO. A possible explanation of these data could be that individuals with lower glutathione levels and/or glutathione S-transferase (GSTs) activity and, therefore, presumably with lower *S*-PMA production, may be less protected than subjects with higher activity of these cellular antioxidant systems. Considering both these negative correlations, one could say, that the metabolic pathway via GSH-dependent detoxication (*S*-PMA), a well know defence mechanism, would lead to a decreased risk of DNA damage as evaluated with comet assay. All together, these data suggest that GSH conjugation may be one, if not the main defence mechanism in protecting cells from benzene toxicity.

CA frequency in human peripheral lymphocytes, differently from other biomarkers of genotoxic effect, has been associated with an increased risk of cancer at group level (Bonassi et al., 1995; Hagmar et al., 1998). In our study, CA frequency did not show any significant difference between benzene exposed workers (SSA) and control subjects. Indeed, several studies have reported clastogenic effect in humans following occupational exposure to benzene concentrations higher than 3200 $\mu g/m^3$ (Zhang et al., 2002). Our study, however, did not show any significant difference of CA frequency between benzene exposed workers and control subjects, indicating a lack of clastogenic effect at these levels of occupational exposure. These findings agree with those of other studies performed in workers exposed to low concentrations of benzene (De Jong et al., 1988), particularly those of Carere et al. (1995), who observed no significant increase of CA frequency in SSA workers exposed to mean benzene concentrations of 1500 $\mu g/m^3$ (range 100–13,100 $\mu g/m^3$).

Our results partially agree also with previous studies that investigated the influence of smoking habits on CA frequency. In fact, although cigarette smoking resulted to be a certain genotoxic (De Marini, 2004), studies performed on large human populations showed non-univocal results regarding its influence on CA frequency. Bender et al. (1988) did not observe any influence of cigarette smoking on this cytogenetic endpoint, while another study found that cigarette smoking caused an increase of CA frequency of 10–20% (Nordic Study Group on the Health Risk of Chromosome Damage, 1990).

In conclusion, the present study showed interesting positive and negative correlations between personal benzene exposure, DNA damage, GSH levels in lymphocytes and urinary *S*-PMA levels. Our data confirm that *S*-PMA and DNA damage by the comet assay are both sensitive biomarkers (of exposure and effect, respectively) of occupational exposure to low levels of benzene. Moreover, subjects with an active detoxication system and with a consequent increase in urinary *S*-PMA may be expected to show, for similar levels of exposure to benzene, less DNA damage. Finally, the negative correlations observed here and in previous studies, between lymphocyte GSH content and DNA damage, seem to suggest that GSH may play an important defence role against benzene-dependent DNA damage and, therefore, its use as a potential susceptibility biomarker should be further investigated.

Conflict of interest

There is no conflict of interest for all authors.

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