WORKERS EXPOSED TO LOW LEVELS OF BENZENE: BIOMARKERS OF EXPOSURE AND EFFECT.

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ABSTRACT

The mechanism(s) by which benzene causes leukaemia still remain unclear, but is generally accepted that for toxicity to occur, benzene must be bioactivated to metabolites by CYP2E1, and these metabolites are known to induce reactive oxygen species that are capable of inducing DNA strand breaks and other cytogenetic effects. This study has been undertaken to identify useful biomarkers of exposure and effect in workers exposed to low levels of benzene. The study consisted of gasoline service-station attendants, pump maintenance workers and controls. Personal exposure was measured to benzene during workshift. Blood samples were collected for cytogenetic tests and comet assay, and urine for determination of *t,t*-MA and S-PMA. GSH lymphocyte concentration was measured by HPLC.

Benzene exposure were significantly higher in gasoline attendants, followed by pump maintenance workers compared to controls. Excretion of *t,t*-MA was similar in benzene-exposed and unexposed subjects, S-PMA showed a significant increase only in gasoline attendants. Comet assay showed a significant increase of all parameters only in gasoline attendants than control group. SCE and CA analysis did not display any difference between workers and controls. Intracellular GSH levels were similar in all groups. Significant correlation was between benzene exposure and comet parameters in gasoline service attendants. Similar trend was in pump maintenance workers. Negative correlations were in all exposed groups between GSH levels and DNA damage.

These results indicate that urinary S-PMA and DNA damage by comet assay are very sensitive and they are significantly affected by low doses of benzene.

INTRODUCTION

Benzene is an ubiquitous environmental contaminant and a component of cigarette smoke, gasoline and automobile emissions. Currently, the progressive reduction of exposure levels in most workplaces has shifted the attention on the health risks to low occupational and environmental exposure levels of benzene. The chronic exposure of humans to low levels of benzene in workplace has been associated with blood disorders, including aplastic anemia and leukaemia (1,2). Microsomal metabolism of benzene results in formation of reactive intermediate *trans,trans*-muconaldheyde, that is further metabolized to *trans,trans*-muconic acid. Benzene oxide also reacts with the thiol groups such as those present in proteins and in glutathione. These products are enzymatically degraded in the formation of S-phenylmercapturic acid, which is excreted in urine, such as the *trans,trans*-muconic acid. Although there are studies showing the toxic effects of benzene, the mechanism(s) by which benzene causes leukaemia still remain unclear. It is however generally accepted that for toxicity to occur, benzene must first be bioactivated to metabolites such as phenol, hydroquinone and catechol by cytochrome P450 2E1 (CYP2E1). In biological systems, inhalated benzene is activated in the liver by CYP2E1 forming benzene oxide, a portion of which is then converted to phenol by non-enzymatic rearrangement. Once in the bone marrow, these metabolites are further bioactivated by myeloperossidases to reactive semiquinones and quinones, which lead to the formation of reactive oxygen species. These toxic metabolites are known to induce sister-chromatid exchanges, micronuclei and chromosomal aberrations in cultured human lymphocytes, even if variable results are reported from cytogenetic survey (3,4). Moreover, active oxygen species are capable of inducing DNA strand breaks and can cause chromosome aberrations (5). Since oxidative DNA damage is subject to a specific repair, the cellular steady-state levels reflect the balance between generation and removal of the lesions. Lesion generation is modulated by cellular antioxidant systems, and among these,

glutathione (GSH) is found an important scavenger of free radicals and it regulates the redox status of many other cellular substances, thus playing an essential role for detossification processes.

This study has been undertaken to investigate benzene exposures to low levels in two different occupations such as in gasoline service attendants and in pump maintenance workers.

Personal air sampling have been used to assess the exposure. Measurement of urinary *trans,trans*-muconic acid (*t,t*-MA) and S-phenylmercapturic acid (S-PMA) provides information on the internal doses. DNA damage as measured by comet assay, and genotoxic effects at chromosomal level, such as chromosomal aberrations and sister chromatid exchanges, provide information on possible early biological effects of benzene exposure and may be indicative of health risks.

MATERIALS AND METHODS

The study consisted of gasoline service-station attendants (n=24: 15 smokers and 9 non-smokers), pump maintenance workers (n=21: 9 smokers and 12 non-smokers) and controls (n=35: 15 smokers and 20 non-smokers). Referent subjects were matched by gender, age and smoking habits. Overall cigarette smoking prevalence was 30%, with a mean daily consumption of 18 cigarettes. Information on health status, smoking habits, diet and chemical exposure was recorded by questionnaires. Personal exposure to benzene was measured by personal diffusive samplers (Radiello ®) worn at the breathing zone for the entire work shift. After desorption with CS_2, samples were analyzed by FID-GC. End of shift *t,t*-muconic acid (MA) and S-phenylmercapturic acid (PMA) were measured in urine by HPLC/diode array and immunochemical technique respectively (6).

Blood samples (7 ml), collected by venipuncture in heparinized tube, were used for cytogenetic tests and comet assay. DNA strand breaks were determined by the alkaline comet assay on lymphocytes of each subject performed according to Singh et al. (7) and described in detail elsewhere (8). Briefly, the cells were embedded in agarose and layered on a microscope slide, then immersed one hour at 4°C in a freshly prepared lysis solution (2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris, 1% N-lauroylsarcosine, pH 10 and supplemented immediately prior use with 10% DMSO and 1% Triton X-100). Following steps of alkaline unwinding (pH > 13) for 40 min, electrophoresis (0.66 V/cm, for 20 min), 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 by % of DNA in the tail (TI), tail length (TL), tail moment (TM) and the number of cells with comet (9). Cytogenetic tests were performed on lymphocytes of each subject. Duplicate lymphocyte cultures were set up to analyse chromosomal aberrations (CA) and sister chromatid exchanges (SCE). Cellular GSH concentration was measured by HPLC with a new rapid and sensitive method with orto-phtalaldehyde (OPA) pre-colum derivatization in lymphocytes. An internal standard (glutathione ethyl ester) is added to facilitate quantitation. GSH and GEE peak areas were measured and the GSH/GEE ratio calculated. Values were expressed as μmol GSH/10^6 lymphocytes (10).

All results were expressed as mean ± S.D. Spearman correlation analysis was applied in order to determine correlations between comet parameters and biomarkers of exposure or other variables.

RESULTS AND DISCUSSION

Data of personal benzene exposure were significantly higher in pump maintenance workers, followed by attendants of gasoline service station compared to control group (57.9 μg/m^3, 77.9 μg/m^3 and 7.91 μg/m^3, P=0.0000), although the exposure in working environments was below of occupational limit values adopted for the risk prevention. The group of pump maintenance workers showed higher benzene air measurements (+35%) compared to service station attendants, and this is probably due to the fact that typically this last job exposes the workers to acute high doses of benzene for short time, while the service station attendants are exposed to low levels, but for long time. The urinary excretion of *t,t*-MA was similar in benzene-exposed and unexposed subjects, while S-PMA showed a significant increase only in gasoline service attendants compared to control group (P=0.004), and in both group the smoking habits increased this metabolite (+30%, not significant) (Tab.1). Comet assay carried out to determine lymphocyte DNA damage in benzene exposed and unexposed subjects showed that only gasoline attendants displayed a significant increase of all parameters (tail moment, P=0.008; %DNA in the tail, P=0.03 and tail length, P=0.0006) than control group (Tab.2). The smoking habits did not interfere with the results in all groups, exposed and unexposed: in this case no differences were found in mean values of comet parameters between smokers and non smokers. At these levels of exposure, the results of the analysis of SCE and CA did not display any statistical difference between benzene exposed workers and control subjects, but SCE and CA were increased (15% and 20%, respectively) in smokers compared to non smokers, in agreement with the well-known effect of smoking habits on this cytogenetic end-point.

To evaluate whether the observed DNA strand breaks were secondary to cellular imbalance between antioxidants and pro-oxidants, the lymphocyte GSH concentration was tested by HPLC. Intracellular levels of GSH were similar in all groups (controls: 9.02±2.52, gasoline attendants: 10.32±1.85, pump maintenance workers: 10.02±2.51).

Table 1

Parameters of benzene exposure and urinary excreation of *trans-trans*-muconic acid and S-phenylmercapturic acid.

	Benzene ($\mu g/m^3$)	Ac *t-t*-MA (μg/g creatinine)	Ac S-PMA (μg/g creatinine)
Controls (35)	7.91±3.44	92.88±103.8	2.29±2.37
Non Smokers (20)	7.517±3.25	114.6±136.3	2.53±2.31
Smokers (15)	9.13±3.46	110.0±105.0	3.120±2.65
Service-station attendant (24)	57.87±55.32	135.1±95.4	5.27±1.71
	p<0.0001 [a]		*p<0.0001* [a]
Non Smokers (15)	76.86±64.03	130.6±108.7	4.65±1.51
Smokers (9)	28.33±11.95	142.1±75.74	6.23±1.61
Pump maintenance workers (21)	77.87±115.0	105.4±70.5	2.47±2.66
	p=0.0007 [a]		
Non Smokers (12)	75.07±55.31	196.6±280.8	2.88±2.03
Smokers (9)	79.09±168.3	89.38±68.23	1.93±1.92

[a] Student t-test: exposed vs control group

Table 2

Comet assay parameters in lymphocytes of workers exposed to low levels of benzene and controls.

	TM	TI	TL	N° comets
Controls (35)	0.28±0.081	2.26±0.563	17.74±3.745	8.52±4.104
Non Smokers (20)	0.29±0.085	2.23±0.529	18.63±4.480	7.78±4.650
Smokers (15)	0.27±0.077	2.11±0.570	17.93±2.080	6.71±3.846
Service-station attendant (24)	0.37±0.130	2.78±0.922	20.30±4.387	9.88±5.835
	p= 0.008 [a]	*p= 0.030* [a]	*p= 0.0006* [a]	*n.s* [a]
Non Smokers (15)	0.39±0.146	2.970±0.904	19.72±3.510	10.50±5.889
Smokers (13)	0.33±0.096	2.47±0.913	21.27±5.664	8.83±5.937
Pump maintenance workers (16)	0.25±0.072	1.99±0.596	17.57±2.637	5.794±3.509
Non Smokers (8)	0.27±0.050	2.16±0.466	17.63±2.206	6.06±3.056
Smokers (8)	0.23±0.088	1.80±0.700	17.51±3.213	5.5±4.158

[a] Mann Whitney U-test: exposed vs control group

However, it is to note that the GSH in lymphocytes of gasoline attendants and pump maintenance workers was higher than controls of 15% and 11%, respectively. Even if not significant, this increase could reflect a compensation mechanism in consequence of the benzene exposure, as well as it occurs for other exposures (11).

Significant correlations were found in the gasoline service attendants between exposure levels of benzene and comet parameters (tail moment: r=0.489, P=0.018; %DNA in the tail: r=0.497, P=0.016; number of cells with comet: r=0.452, P=0.03), and negative significant correlations were associated with the same comet parameters and S-PMA. Similar trend was observed in the pump maintenance workers between personal benzene exposure and comet parameters, but only tail moment was significantly correlated (r=0.525, P=0.017). Interestingly negative correlations were found in both exposed groups between lymphocyte GSH levels and DNA damage by comet assay, but no correlation was found between the same parameters in the control group.

On the whole, these findings indicate that, in the research of useful biomarkers for monitoring exposure to benzene, urinary S-PMA levels and the evaluation of DNA damage by comet assay are very sensitive and they are significantly affected by benzene exposure at low doses. The results show clear correlations between DNA damage, personal benzene exposure and urinary S-PMA levels.

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REFERENCES

1. IARC, Benzene. Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans,Suppl. 7. International Agecy for Research on Cancer, Lyon, France, 1986; 120-122.
2. Snyder R, Benzene and leukaemia, Crit Rev Toxicol 2002; 32: 155-210.
3. Whysner J, Reddy MV, Ross, PM, Mohan M, Lax EA. Genotoxicity of benzene and its metabolites. Mutat. Res. 2004; 566: 99-130.
4. Anwar WA. Monitoring of human populations at risk by different cytogenetic end points.Environ Health Perspect. 1994; 102 (4): 131-134.
5. Andreoli C, Leopardi P, Crebelli R. Detection of DNA damage in human lymphocytes by alkaline single cell gel electrophoresis after exposure to benzene or benzene metabolites. Mutat Res 1997; 377: 95-104.
6. PJ Boogaard, NJ van Sittert. Biological monitoring of exposure to benzene: a comparison between S-phenylmercapturic acid, *trans, trans*-muconic acid, and phenol. Occup. Envoron. Med. 1995; 52: 611-620.
7. N.P Singh, L.T. McCoy, R.R Tice, E.L. Schneider. A simple technique for quantitation of low levels of DNA damage in individual cells, Exp Cell Res. 1988; 175: 184-191.
8. Fracasso ME, Perbellini L, Soldà S, Talamini G, Franceschetti P. Lead induced DNAstrand breaks in lymphocytes of exposed workers: role of reactive oxygen species and protein kinase C. Mutat Res. 2002; 515: 159-169.
9. Fracasso ME, Franceschetti P, Doria D, Talamini G, Bonetti F. DNA breaks as measured by the alkaline comet assay in exfoliated cells as compared to voided urine cytology in the diagnosis of bladder cancer: a study of 105 subjects. Mutat Res. 2004; 564: 57-64.

10. Cereser C, Guichard J, Drai J, Bannier E, Garcia I, Boget S, Parvaz P, Revol A. Quantitation of reduced and total glutathione at the femtomole level by high-performance liquid chromatography with fluorescence detection: application to red blood cells and cultured fibroblasts. Journal of chromatography 2001; 752: 123-132.
11. Cohen, GM. Pulmonary metabolism of foreign compounds: its role in metabolic activation. Environ Health Perspect 1990; 85: 31-41.