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Mutation Research 582 (2005) 61–70
Genetic Toxicology and Environmental Mutagenesis
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DNA damage in lymphocytes of benzene exposed workers correlates with *trans,trans*-muconic acids and breath benzene levels

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Received 7 July 2004; received in revised form 25 December 2004; accepted 27 December 2004

Abstract

Benzene causes many kinds of blood disorders in workers employed in many different environments. These diseases include myelodisplastic syndrome and acute and chronic myelocytic leukemia. In the present study, five occupational work places, including six industrial process types, namely, printing, shoe-making, methylene di-aniline (MDA), nitrobenzene, carbomer, and benzene production were selected, and the levels of breath benzene, and *trans,trans*-muconic acids (*t,t*-MA) and phenol in urine were evaluated, as well as hematological changes and lymphocyte DNA damage. The concentration of benzene in breath was less than 3 ppm in the workplaces, and benzene exposure was found to be higher in work places where benzene is used, than in those where benzene is produced.

At low levels of benzene exposure, urinary *t,t*-MA correlated strongly with benzene in air. Highest Olive tail moments were found in workers producing carbomer. Levels of breathzone benzene were found to be strongly correlated with Olive tail

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1383-5718/$ – see front matter
doi:10.1016/j.mrgentox.2004.12.011

moment values in the lymphocytes of workers, but not with hematological data in the six workplaces types. In conclusion, the highest benzene exposures found occurred in workers at a company, which utilized benzene in the production of carbomer. In terms of low levels of exposure to benzene, urinary *t,t*-MA and DNA damage exhibited a strong correlation with breath benzene, but not with hematological data. We conclude that breath benzene, *t,t*-MA and lymphocytic DNA damage are satisfactory biomonitoring markers with respect to benzene exposure in the workplace.

Keywords: Benzene; Comet assay; DNA damage; *t,t*-MA; Lymphocytes; Phenol

1. Introduction

Benzene is an aromatic hydrocarbon substance that has cytotoxic, haematotoxic, immunotoxic, and genitoxic properties [1–16], and is extensively used in industry as a volatile solvent or an assorting material for the synthesis of other chemicals [17]. The level of benzene exposure has been reduced and in some workplaces, where benzene has been supplanted by other solvents. However, industrialization inevitably leads to more benzene emissions in the environment [18].

Benzene can either be converted to phenol or react with glutathione to form premercapturic acid in biological systems. Phenol is then finally converted into the metabolites catechol, hydroquinone, and benzoquinone. Benzene can also be converted into benzene glycol, and then is metabolized by ring opening into muconic acid, which is later excreted in the urine, and constitutes a classical biological monitoring marker [19–22].

These benzene metabolites bind covalently to proteins and DNA in biological systems such as cells or tissues, thereby inducing intracellular toxic effects, such as the inhibition of cell replication or carcinogenesis [17]. Benzene is also believed to act as a mutagen via an indirect mechanism, resulting in oxidative DNA damage through the formation of hydroxyl radicals via hydrogen peroxide [23]. In recent years, comet assay or single cell gel electrophoresis have been used to investigate the level of DNA damage in many biomonitoring studies. This assay has been widely used in order to detect strand breaks, alkali-labile sites, DNA crosslinking, and incomplete excision repair sites. The technique has proven to be a very sensitive method, and a useful tool for the detection of genetic damage at the individual cell level, and in human biomonitoring [24,25]. Guidelines for the use of the comet assay were developed at the International Workshop on Genotoxicity Test Procedures (IWGTP)[26].

In 2000, 9768 individuals in Korea developed malignant blood disorders. Among these, 926, 643, and 480 persons developed acute myelocytic leukemia, myelodisplastic syndrome, and aplastic anemia, respectively. It was also reported that 10–20% of blood disorders were caused by occupational or environmental factors [27].

In the present study, we evaluated benzene exposure levels in several different occupational environments, in order to establish basic data for use in the determination of the incidence of benzene-induced blood disorders. Firstly, we measured breath benzene levels in workers and also the urinary phenol and *t,t*-MA levels, in order to evaluate the extent to which individual workers had been exposed to benzene. We also examined the genotoxic and hematotoxic effects of benzene on the lymphocytes of workers, and explored any correlations between the levels of urinary metabolites, *trans,trans*-muconic acids, and breath benzene concentration, and lymphocyte DNA damage in workers in different occupational environments.

2. Materials and methods

2.1. Subjects

Sixty-one workers from five companies in Ansan, South Korea were subjected to this study. All subjects completed a questionnaire, which included items on, smoking, drinking, age, medication, etc. Workers exposed to benzene were recruited at five companies in July 14 and July 28, 2001. These workers had been exposed to benzene containing organic solvents in six different industrial processes including; printing and shoe-making and the production off nitrobenzene, carbomer, methylene di-aniline (MDA) and benzene.

2.2. Cell preparation

Blood samples, 2–3 ml of heparinized whole blood, were collected by venipuncture from each human subject and delivered immediately to the laboratory. Comet assay was carried out within 3 h. Two milliliters of bloods were mixed with same volume of PBS and loaded onto 4 ml of Histopaque-1077 solution (Sigma Co., USA) in 15 ml of conical tube. The tube containing sample was then centrifuged 1500 rpm for 20 min. The mononuclear cells were collected by pasture pipette and washed with PBS buffer for comet assay.

2.3. Analysis of inhaled benzene in workers

The analysis was carried out according to the NIOSH standard method 1501 with a minor modification [28]. Benzene was collected by using personal sampler from the breathing zones of workers in five companies. Then, charcoal tube section containing benzene was transferred into vials. One-milliliter of carbon disulphide was added and allowed to stand at least 30 min with occasional agitation. The extracts were reduced under nitrogen gas and analyzed by GC. Benzene authentic standard was obtained from Sigma Co. Gas Chromatography-Flame Ionization Detector (GC-FID) analysis was performed with an Agilent 6890 series GC-FID equipped with of Agilent-VOC column (Agilent, USA). A Agilent-VOC column (length 60 m × 0.32 mm i.d.) was employed with helium as carrier gas. A 1 ul of sample was split injected (split ratio 1:30) into the GC-FID. The GC temperature was programmed as follows: first 40 °C for 5 min, then from 100 to 190 °C at 5 °C/min and finally 190 °C for 10 min. The injector and detector temperature were 230 and 250 °C, respectively

2.4. Determination of trans,trans-muconic acid (t,t-MA) in urine

The high performance liquid chromatographic (HPLC) method of Inoue et al. [29] for *t,t*-MA was carried out with a small modification for urine analysis as follows: Urinary *t,t*-MA was measured by HPLC (Gilson) equipped with Spherisorb ODS 5 μm column (4.6 mm in inner diameter and 300 mm in length). 50–100 ml urine samples were collected and centrifuged immediately at 3000 rpm for 5 min. The supernatants were purified by solid phase extraction with disposable C18 column and then injected into a HPLC with UV detector. The mobile phase (one volume of methanol mixed with nine volumes of 1% acetic acid) was allowed to flow at a rate of 1.0 ml/min, and the eluates were monitored at wavelength of 259 nm.

2.5. Determination of phenol and creatinine in urine

The gas chromatographic (GC) method of Inoue et al. [30] for phenol was carried out with a small modification for urine analysis as follows: Phenol authentic standard was obtained from Sigma Co. One milliliter of urine was mixed with 0.5 ml of 15% HCl and heated for 1 h for acid hydrolysis. Then, 3,5-xylenol was added as an internal standard and the mixture was extracted by centrifugation with 2 ml of carbon disulphide. The organic phase was reduced under nitrogen gas and analyzed by GC. GC analysis was performed with an Agilent 6890 series GC equipped with of HP-INNOWAX column (Agilent, USA). A HP-INNOWAX column (length 60 m × 0.32 mm i.d.) was employed with nitrogen as carrier gas. A 1 μl of sample was split injected (split ratio 1:50) into the GC. The oven temperature was 180 °C and analysis was carried out for 15 min for 5 min and phenol was detected at 8.5 min. The injector and detector temperature were 200 and 250 °C, respectively. The urinary creatinine was determined with a Hitachi 747 Computer-Directed Analyzer.

2.6. Comet assay

The comet assay was performed according to Singh with minor modification [31]. Briefly, lymphocytes were mixed with 50 μl of 1% low melting point agarose (Ameresco, LMA) at 37 °C and then on a fully frosted slides which were already covered with thin layer of 1% normal melting point agarose (Ameresco, NMA) to promote even and firm attachment of second layer. The slides were covered and then kept at 4 °C for 5 min to allow solidification of the agarose. After gently removing the coverglass, the slides were covered with a third layer of LMA and placed in the refrigerator for 5 min. The slides were submersed in the lysing solution (2.5 M NaCl, 100 mM EDTA-2Na, 10 mM Tris–HCl, pH 10; 1% Triton X-100 and 10% DMSO, pH 10 were added fresh) for 1 h. The slides were

then placed in unwinding buffer (1 mM EDTA and 300 mM NaOH, pH 13) for 20 min and electrophoresis was carried out using the same solution for 20 min at 25 V and 300 mA (0.8 V/cm). After electrophoresis, the slides were neutralized by washing three times with neutralization buffer (400 mM Tris–HCl, pH 7.4) 5 min each and were stained with 50 μl of 10 μg/ml ethidium bromide. The slides were examined using a Komet 4.0 image analysis system (Kinetic Imaging, Liverpool, UK) fitted with an Olympus BX50 fluorescence microscope equipped with an excitation filter of 515–560 nm and a barrier filter 590 nm. For each treatment group, two slides were prepared and each 50 cells were randomly chosen (total 100 cells) manually. The Olive tail moment was calculated automatically using the Komet 4.0 image analysis system; [Olive tail moment = ((Tail.mean − Head.mean) × Tail% DNA)/100].

2.7. Hematological analysis

Several factors including serum levels hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell volume distribution width (RDW-CV) in red blood cells, and the levels of white blood cells, neutrophils, lymphocytes, monocytes, and platelet levels, mean platelet volume (MPV), platelet volume distribution width (PDW), and platelet large cell ratio (P-LCR) were measured using a hematological autoanalyzer SYSMEX SF-3000 (TOA Medical Electronics Co., Japan). White blood cells, red blood cells and platelets, and hemoglobin were analyzed by semiconductor laser flow cytometry, DC detection, and sodium lauryl sulfate–hemoglobin method, respectively.

2.8. Statistical analysis

Statistical analyses were performed using SPSS, version 10.0. To test for significant differences between groups, we used the analysis of variance (ANOVA) method. Spearman's correlation test was also applied in determination of correlations between tail moments, and the levels of breath benzene, and *t,t*-MA and phenol in urine.

3. Results

3.1. Characteristics of subjects

Table 1 displays relevant subject characteristics, such as gender, age, smoking habits, and duration of employment at the six sites. Age distributions were practically identical in the six working places. The mean of age of workers was 35.8 ± 8.0 years, ranging from 22 to 59 years. The ages of workers at six sites were not found to be significantly different by the *t*-test ($p > 0.4$). The number of smokers exposed to benzene was higher than the number of non-smokers (Table 1). However, the distribution of smoking duration for workers at the six sites was not found to be significantly different by the Chi-square test ($p > 0.1$), and the mean of number of cigarettes smoked per day was 7.5 ± 6.8, ranging from 5 to 20. The mean duration of employment for workers at the six sites was 8.9 ± 6.7 years. Smoking effects on DNA damage, phenol and *t,t*-MA were evaluated, but no significance was determined ($p = 0.850$, 0.556 and 0.716). We evaluated also alcohol consumption effects on DNA damage in mononuclear blood cells. The Olive tail moments in drinker and non-drinker were 1.85 ± 0.45 and

Table 1
Characteristics of workers exposed to benzene at the six work sites

Work place	Number of workers	Gender		Age (year)	Smoking status[a]		Working duration (year)
		Male	Female		Smoker	Non-smoker	
Printing	4	4	0	40.0 ± 8.9 (30–48)	1	3	18.8 ± 11.8 (10–35)
Shoe-making	7	6	1	42.6 ± 10.7 (27–59)	1	6	6.3 ± 7.4 (1–20)
Nitrobenzene	9	9	0	30.3 ± 5.6 (24–45)	4	5	5.4 ± 3.2 (1–13)
MDA	18	16	2	35.9 ± 7.9 (27–47)	10	8	7.1 ± 2.1 (4–9)
Carbomer	17	15	2	35.3 ± 5.9 (22–44)	11	6	12.2 ± 5.8 (1–22)
Benzene	6	6	0	39.2 ± 8.0 (30–52)	3	3	7.0 ± 6.4 (2–17)
Total	61	56	5	35.8 ± 8.0 (22–59)	33	28	8.94 ± 6.71 (5–20)

[a] Number of smokers and non-smokers.

1.63 ± 0.39, respectively, but no significant difference was found ($p = 0.11$).

3.2. Analysis of breath benzene

Breath benzene concentration in workers at the six sites was consistently less than 3 ppm. The mean value of breath benzene in workers was 0.268 ± 0.216 ppm, ranging from 0.005 to 2.0321 ppm. Shoe-making and MDA workplaces exhibited the lowest measured levels of breath benzene. The site with the highest breath benzene value was the carbomer production plant, which was seven times higher than that at other sites (Fig. 1A).

3.3. Determination of phenol and t,t-MA in urine

Phenol and *t,t*-MA, the urinary metabolites of benzene were used as biological markers for benzene exposure. Urinary phenol and *t,t*-MA levels were measured in workers at six workplaces, and normalized by creatinine levels (Fig. 1B and C). The mean values of phenol and *t,t*-MA concentration in workers were 10.9 ± 8.66 (2.5–46.6) and 1.02 ± 0.45 (0.24–2.77) mg/g creatinine, respectively. Like breath benzene concentrations, the highest values for phenol and *t,t*-MA were found in workers at the carbomer production sites (Fig. 1B and C).

3.4. Correlations between breath benzene, phenol, t,t-MA and Olive tail moments

Correlations between breath benzene and phenol and *t,t*-MA in urine were evaluated (Table 2). The correlations between breath benzene level and the urinary *t,t*-MA level was found to be significant, as was the correlation between breath benzene levels and phenol

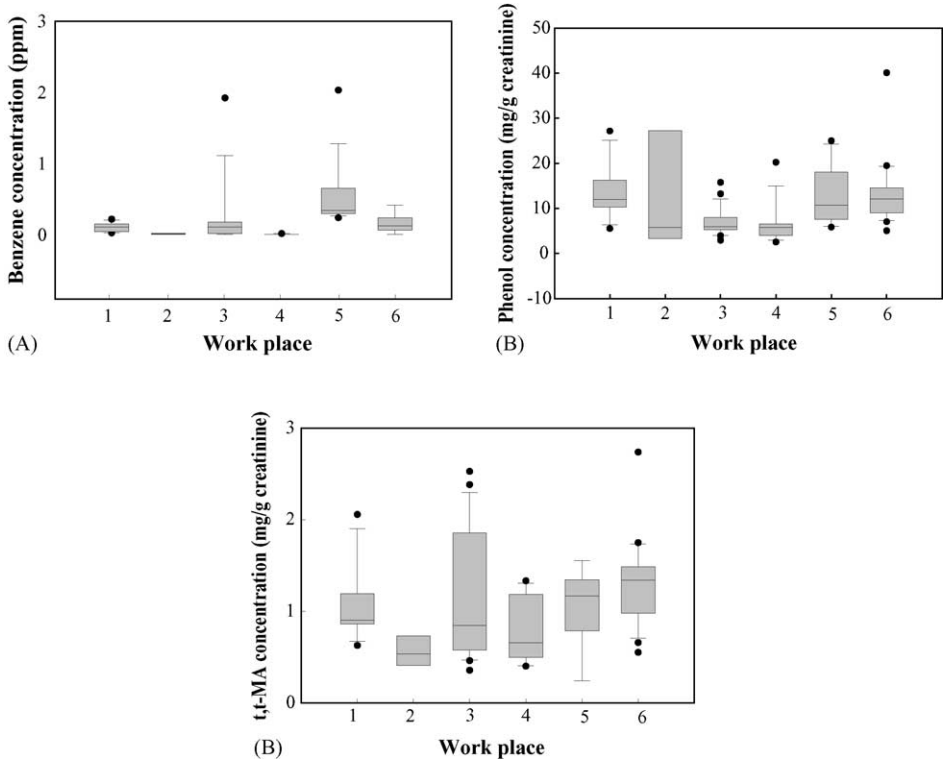

Fig. 1. (A) The levels of breath benzene in workers at the six work sites. Breath benzene was collected using a personal sampler, and analyzed by GC. (B) The levels of phenol in the urine of workers at the six work sites. Urinary phenol was analyzed by GC. (C) The levels of *t,t*-MA in the urine of workers at the six work sites. Urinary *t,t*-MA was analyzed by HPLC. 1: Printing; 2: shoe-making; 3: nitrobenzene; 4: MDA; 5: carbomer; 6: benzene.

Table 2
Spearman correlation between exposure measurement factors in all subjects

Measurement factors	Rho values	*p*-value
Breath Benzene		
Phenol	0.305	0.056
t,t-MA	0.701	0.001**
Olive tail moments	0.574	0.001**
Olive tail moments		
Phenol	0.142	0.388
t,t-MA	0.455	0.002**
Phenol		
t,t-MA	0.455	0.005**

levels. However, the level of *t,t*-MA ($r = 0.701$, $p = 0.01$, $n = 61$) in urine displayed a better correlation than did the level of phenol in urine with of breath benzene levels ($r = 0.305$, $p > 0.05$, $n = 61$) (Table 2).

The spearman correlation rho value was 0.574 with regard to the relationship between lymphocyte Olive tail moment and breath benzene level. This relationship was found to be statistically significant ($p = 0.001$, $n = 61$) (Table 2). The correlation between lymphocyte Olive tail moment and the level of *t,t*-MA in urine was also statistically significant ($p = 0.002$, $n = 61$), with a rho value of 0.455 (Table 2). However, the correlation between lymphocyte Olive tail moment and the urinary phenol level did not exhibit a statistically significant association (rho value = 0.142) ($p = 0.388$, $n = 61$) (Table 2).

3.5. Effects of benzene on hematological components

Hematological analyses were carried out in workers according to breath benzene concentrations. The sites were separated into three groups; low breath benzene (less than 0.1 ppm), moderate breath benzene (0.1–1.0 ppm), and high breath benzene levels (more than 1 ppm). With regard to red cells, the hemoglobin values, the number of red blood cells, hematocrit, MCV, MCH, and RDW-CV exhibited no significant differences, but RBC MCHC was shown to decrease significantly with higher breath benzene values ($p = 0.02$) (Table 3). Hematological analysis of white blood cells and platelets yielded no consistent results with regard to breath benzene concentrations, though platelet MPV underwent significant variation (Table 3).

3.6. DNA damage analysis

Comet assays were carried out in order to evaluate DNA damage occurring in the mononuclear cells

Table 3
The profile of hematological parameters in workers exposed to benzene at the six work sites

Parameters	Concentration of breath benzene			*F*-value	*p*-value
	<0.1 ppm (26)	0.1–1.0 ppm (28)	1–3 ppm (7)		
RBC					
Hemoglobin (mg/dL)	14.85 ± 0.78	14.70 ± 1.11	14.80 ± 0.66	0.185	0.832
RBC Number (10^6cells/cc)	4.69 ± 0.29	4.70 ± 0.39	4.86 ± 0.34	0.668	0.516
Hematocrit (%)	43.15 ± 2.19	43.76 ± 3.23	43.9 ± 2.39	0.407	0.667
MCV (fL)	92.10 ± 3.36	93.17 ± 3.28	90.50 ± 2.42	2.118	0.129
MCH (pg)	31.70 ± 1.37	31.31 ± 1.28	30.53 ± 1.21	22.94	0.110
MCHC (g/dL)	34.43 ± 0.99	33.61 ± 1.12	33.76 ± 1.08	4.182	0.020
RDW-CV (%)	13.46 ± 0.34	13.50 ± 0.54	13.37 ± 0.29	0.264	0.192
WBC					
WBC number (cells/ul)	6866 ± 1822	6463 ± 1607	6161 ± 1464	0.650	0.526
Neutrophils (cells/ul)	1982 ± 900	2150 ± 1138	2019 ± 1140	0.183	0.833
Lymphocyte (cells/ul)	2825 ± 848	2464 ± 845	2543 ± 743	1.299	0.281
Monocyte (cells/ul)	1907 ± 1016	1599 ± 819	1341 ± 516	1.467	0.239
Platelet					
Platelet number (10^4 cells/ul)	25.9 ± 3.96	21.5 ± 5.34	24.1 ± 3.66	6.345	0.003
MPV (fL)	10.68 ± 0.88	11.44 ± 1.20	11.04 ± 0.58	3.771	0.029

p-value generated by ANOVA.

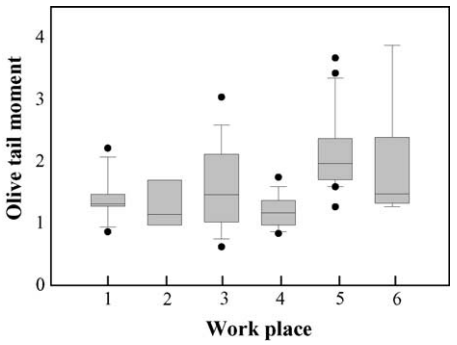

Fig. 2. Distribution of Olive tail moments of mononuclear cells in benzene exposed-workers at the six work sites. 1: Printing; 2: shoe-making; 3: nitrobenzene; 4: MDA; 5: carbomer; 6: benzene.

of workers. Olive tail moments were used to determine the degree of DNA damage. In mononuclear cells, the levels of DNA damage in workers in printing, shoe-making, and nitrobenzene, MDA, carbomer and BTX production were: 1.41 ± 0.41, 1.34 ± 0.53, 1.82 ± 1.10, 1.19 ± 0.29, 2.05 ± 0.54 and 1.98 ± 0.29, respectively (Fig. 2). The mean value of DNA damage was 1.73 ± 0.81. Dose-dependent DNA damage occurred at higher levels of exposure, and DNA damage exhibited a strong correlation with other exposure measurement factors, including breath benzene and *t,t*-MA, but not including phenol (Table 2). DNA damage was highest at the carbomer site, which was also the site of the highest level of breath benzene in workers, but DNA damage had occurred to the least extent in the workers at the printing site. A significant difference in DNA damage was apparent between the carbomer production site and the other sites ($p < 0.05$). DNA damage and haematological parameters were not significantly correlated.

4. Discussion

In recent years, progressive reductions in overall exposure levels in the majority of work places have caused a shift in the focus of biological monitoring, from monitoring only of heavy exposures, to a new evaluation of the health risks associated with low occupational or environmental exposure. Benzene has been established to be a carcinogen of note, and results in panoply of blood disorders, most notably myelodisplastic syndrome, and acute and chronic myelocytic leukemia, in workers in various industries. Therefore, the major concern with regard to benzene centers on the effects of long-term exposure to low concentrations of benzene both occupationally and environmentally [32].

Many benzene metabolites, including S-phenyl-3lmercapturic acid, *t,t*-MA, hydroquinone, catechol, and phenol have been utilized as biomarkers in humans exposed to benzene [33–35]. In addition, benzene exposure can be measured by head-space gas chromatography in environmental air samples, using a personal air sampler, and also can be measured in biological blood samples [36–38]. However, the current limits of detection for these methods are 2 ng/ml in blood and 0.1 ppb in a 5-l sample of air or breath were attained [39]. Many combined measurements, using urine, blood, and breath and air samples have been performed, in order to evaluate benzene exposure more precisely.

These analytical studies of benzene exposure are bolstered by other bioassays, which probe the genotoxic effects of benzene on blood cells, micronuclei, sister chromatid exchanges, and comet assays [40–45].

In 1997, Andreoli et al., demonstrated significantly higher DNA damage occurring in the lymphocytes of subjects occupationally exposed to low levels of benzene, versus matched unexposed controls [23]. They also reported that DNA damage was detected in the T- and B-lymphocytes and granulocytes of 41 workers in a printing company, who had all been exposed to benzene. B-lymphocytes, incidentally, may also constitute a useful target for the biomonitoring of human exposure to low levels of benzene [41].

In the present study, five companies, involving six working processes, namely, printing, shoe-making, and MDA, nitrobenzene, carbomer and BTX manufacture were selected, and levels of breath benzene, *trans,trans*-muconic acids (*t,t*-MA) and phenol in urine were determined, as well as DNA damage in lymphocytes in order to evaluate the levels of benzene exposure occurring in workers at these six sites. Working processes were classified into three broad groups; (1) industries using the materials containing benzene (printing and shoe-making) (2) industries using benzene to produce other related compounds (nitrobenzene, MDA and carbomer) (3) industries producing benzene from charcoal. Of the three groups, the lowest values for breath benzene, *t,t*-MA and phenol in urine, and lymphocyte DNA damage were found in workers at sites using materials containing benzene; namely, the print-

ing and shoe-making industries. Breath benzene levels were found to be highest in workers at the site using benzene to produce carbomer, which has not been reported to be a genotoxic compounds. These results appear to accurately reflect environmental conditions at the workplaces. However, this present study has the weakness of the lack of information on dietary status of the subjects. In general, dietary factors including folate, other vitamins and minerals from fruit and vegetables have been associated with decreased risk of colorectal adenoma, carcinoma and leukemia in human [46,47].

Otherwise, it has been reported that *t,t*-MA and S-phenylmercapturic acid levels are more suitable indicators for benzene exposure than are the levels of urinary phenol, hydroquinone, catechol, or of breath benzene. The latter usually measured within the 0.1 to 1.0 ppm range in vehicle mechanics, and other workers exposed to low concentrations of benzene [34]. However, in this study, correlation studies on the levels of breath benzene, *t,t*-MA and phenol in urine, and DNA damage in lymphocytes, demonstrated that levels of breath benzene and *t,t*-MA in workers are, indeed, good indicators for benzene exposure in the 0.05–2.0 ppm range. In other studies, phenol, hydroquinone, and catechol were revealed to exhibit inadequate sensitivity with regard to their use as biomakers for low levels of benzene exposure, when compared with *t,t*-MA and S-phenylmercapturic acid [33,34]. In the present study, urinary phenol exhibited only weak correlations with other biomarkers, including breath benzene, *t,t*-MA and Olive tail moments. Moreover, we found that phenol was an insensitive marker in workers exposed to low levels of benzene.

According to the results of comet assays, DNA damage in the lymphocytes exhibited a strong correlations with the breath benzene and urinary *t,t*-MA levels, but not with phenol levels. It was also reported that DNA damage in individual T and B-lymphocytes is clearly associated with *t,t*-MA levels in workers exposed to low levels of benzene at the low level in a printing company [41].

In conclusion, the highest benzene exposures were found to be occurring in workers at a company using benzene to produce carbomer. With regard to low levels of exposure to benzene, urinary *t,t*-MA exhibited a strong correlation with breath benzene, but not with urinary phenol. We conclude that breath benzene, *t,t*-MA and lymphocytic DNA damage constitute satisfactory biomonitoring markers with regard to the low levels of benzene exposure occurring at contemporary industrial work sites.

Acknowledgment

This work was supported by the Medical Research Center for Environmental Toxico-Genomics & Proteomics of Korea University.

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