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Early effects of low benzene exposure on blood cell counts in Bulgarian petrochemical workers

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Key words
Benzene; petrochemicals; hematological effects; genetic polymorphisms

Summary
Objectives: *Only few studies have examined early hematological effects in human populations exposed to low benzene levels and their findings are controversial. We evaluated hematological outcomes (WBC, neutrophils, lymphocytes, monocytes, eosinophils, basophils, RBC, Hb, HCT, MCV, platelets and MPV) in a population of 153 Bulgarian petrochemical workers exposed to benzene (range 0.01-23.9 ppm) and 50 unexposed subjects.* **Methods:** *Written informed consent was obtained and a self-administered questionnaire used to collect information on current smoking habits, lifestyle, and occupational activities. Exposure assessment was based on personal monitoring sampling the day before phlebotomy. Urinary trans-trans-muconic acid (t,t-MA) was determined at the beginning and end of the work shift. Based on individual airborne benzene measurements, study subjects were categorized in three exposure categories (referents, <1 and ≥1 ppm). Mean values of each hematologic outcomes in each exposure category were compared with the referent group using a multiple linear regression model adjusted for age, gender, current smoking habits and environmental toluene level. The influence of the CYP2E1 (RsaI and DraI) and NQO1 609C>T genetic polymorphisms on differential hematological parameters was also investigated.* **Results:** *No dose-response effect was observed for most of the examined hematological outcomes (WBC, lymphocytes, neutrophils, monocytes, RBC, Hb, HCT, MCV, platelets and MPV). The eosinophil count was inversely related to benzene exposure only among smokers. Conversely, basophils increased with increasing exposure. No effect on benzene hematotoxicity was found for any of the investigated polymorphisms.* **Conclusion:** *In our study we did not find a decline in WBC and lymphocytes related to benzene exposure. A myeloproliferative effect of benzene is highly unlikely to explain the observed reduction in eosinophils and increase in basophils as it would lead to a concordant depression in all granulocyte subpopulations. Whether benzene effects at low doses are present in Caucasian populations remains uncertain, thus warranting further investigations.*

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Riassunto

«Effetti ematologici dell'esposizione a basse dosi di benzene in lavoratori petrolchimici bulgari». *Solo pochi studi hanno analizzato le possibili conseguenze ematologiche dell'esposizione a basse dosi di benzene, con risultati contrastanti. Il nostro studio ha indagato parametri ematologici in una popolazione di 153 operai petrolchimici bulgari esposti a benzene e in 50 soggetti non esposti. Dopo raccolta di consenso informato e di dettagliate informazioni individuali attraverso questionario, l'esposizione a benzene è stata valutata mediante l'utilizzo di campionatori personali. L'acido trans-trans-muconico urinario è stato misurato all'inizio e alla fine del turno di lavoro e il giorno seguente ogni soggetto è stato sottoposto a prelievo venoso. In base all'esposizione individuale, i soggetti allo studio sono stati suddivisi in tre categorie (controlli, esposti a meno di 1 ppm, esposti a più di 1 ppm). Modelli di regressione multipla aggiustati per età, sesso, attuale abitudine al fumo e esposizione a toluene sono stati utilizzati per confrontare i valori ematologici in ciascuna delle tre categorie di esposizione. È stata anche valutata l'influenza dei polimorfismi nei geni metabolici CYP2E1 e NQO1 sui diversi parametri ematologici indagati. Per la maggior parte degli indicatori ematologici esaminati (leucociti totali, linfociti, neutrofili e monociti, globuli rossi, Hb, Ht, MCV, piastrine e MPV) non è emersa una relazione dose risposta con l'esposizione a benzene. Il numero di eosinofili è risultato inversamente correlato all'esposizione a benzene solo tra i fumatori, mentre il numero di basofili aumenta all'aumentare dell'esposizione. Tali effetti sono difficilmente attribuibili all'azione mieloproliferativa del benzene che più probabilmente porterebbe a una depressione di tutte le sottopopolazioni granulocitarie. Inoltre, non è emerso nessun effetto dei polimorfismi genetici sulle cellule ematiche. La nostra indagine non sembra evidenziare significativi effetti della esposizione a basse dosi di benzene sui parametri ematologici indagati.*

Introduction

High exposures to benzene in the workplace have been associated in the past to leukemia and different forms of blood changes such as progressive leukocytopenia, anemia, thrombocytopenia and pancytopenia (7). In the last decades, preventive actions have substantially reduced occupational exposures in western countries. Nowadays, in European countries the highest occupational levels of exposure to benzene are likely to occur in gasoline and fuel production, distribution of petroleum products, distillation of coal tar in the coke oven industry (13). The main source of exposure for the general population has been identified in active smoking, followed by passive smoking, auto exhaust and driving or riding in automobiles (21). Current concern mainly regards the possible effects of long term, continuous, low concentrations of exposure to benzene that often show overlapping levels both in the work setting and the environment. Recently, significantly decreased white blood cell (WBC) counts have been detected in Chinese workers exposed to less than 1 ppm benzene (10, 12). This could have important implications for the regulation of benzene exposure (occupational exposure limits are presently in the range of 0.5-1 ppm.) and prevention of benzene associated illness including leukemia. However, other investigations failed to report evidence of hematotoxicity below 1 ppm (3, 4, 19, 20).

Benzene toxicity is mediated by different metabolites produced through steps involving several metabolic key enzymes (17), including cytochrome P4502E1 (CYP2E1), myeloperoxidase (MPO), NADPH quinone reductase (NQO1), and glutathione-S-transferase (GST). Most of these enzymes are polymorphic in the general population (5, 15). In exposed workers genetic variations in activating (CYP2E1) and detoxifying enzymes (NQO1) have been shown to influence susceptibility to benzene toxic effects (12, 16, 22).

We examined hematologic changes in a population of Bulgarian petrochemical workers, in which a significant increase in benzene biomarkers of exposure (t,t-MA and S-PMA) among exposed

workers was previously reported (6). The average benzene exposure, determined by personal sampling was 1.7 ppm (range 0.01-23.9 ppm). We also investigated the role of genetic polymorphisms in the metabolic enzymes CYP2E1 and NQO1 in susceptibility to benzene induced hematotoxicity.

Material and methods

Study population

The study population included 158 workers with at least one year of employment at the Lukoil-Neftochim petrochemical plant in Burgas, Bulgaria and 50 unexposed white collars from the same plant. All subjects who had worked in the same position for at least one year were enrolled between October 1999 and September 2000. The study obtained the approval of the local Bulgarian and the National Cancer Research Institute Ethics Committees. Written informed consent was obtained and a self-administered questionnaire was filled-in collecting information on smoking habits, lifestyle, medical history, recent infections (in the month before phlebotomy) and occupational activities.

Exposure monitoring

Airborne benzene and toluene

Personal exposure to benzene and toluene in air was monitored by active sampling using charcoal sampling tubes and for a sub sample of workers by passive sampling using a stainless steel tube 9 mm internal diameter X 90 mm length, containing Chromosorb 106 equipped with a diffusion chamber. Personal full-shift air monitoring was done during one working day, with both active and passive samplers worn near the breathing zone during a typical work-shift (midweek, from 8:00 a.m. to 2:00 p.m.) (1). Average shift sampling was performed on two-layer-sampling tubes (100/50 mg) filled with charcoal coconut base - type CT-CH-2 (Higitest Ltd - Bulgaria). The absorption of the air sample was performed with low-flow personal sampling pumps type Compur 4903 (Germany) and Gillian 113PS I (USA) with flow of 20 - 30 cm^3/min. The sorbent layers were eluted with carbon disulfide (free from benzene) and aliquots of the solution were injected into a Perkin Elmer Model 8300 gas chromatograph with flame-ionization detector and column DB-1-30 m. Detection limits for benzene and toluene (LOD) were 0.023 and 0.05 ppm respectively at an air sample volume of 30 dm^3. Passive samplers were collected at the end of the day shift, closed with a brass cap and nut, equipped with a polyperfluoroethylene (PTFE) ferule, and kept at −20°C until analysis. Benzene and toluene on these samplers was measured by thermal desorption followed by gas chromatography/flame ionization detector (GC/FID) analysis. The limit of detection was 0.00053 ppm (2 $\mu g/m^3$) and 0.0018 ppm (6 $\mu g/m^3$) for airborne benzene and toluene, respectively. The correlation between benzene air levels (ln ppm) detected by active and passive sampling methods was very good ($r^2 = 0.97$, $p<0.001$).

Urinary t,t Muconic Acid (tt-MA)

Urine spot samples were collected for each study subject at the beginning and end of the work shift. Pre- and post-work shift urine samples were partitioned in plastic tubes for t,t-MA assessment. Determination of urinary t,t-MA was carried out by pre-purification of urine with solid phase extraction using a strong anion exchange column (SAX, 300 mg, Supelco) followed by high performance liquid chromatography and UV detection according to the method described by Buratti and colleagues (2). The detection limit of the procedure was 50 $\mu g/l$.

Blood cell count

Whole blood (2 ml) was collected before the start of the work shift from fasting subjects by venous phlebotomy in a 2 ml EDTA vial on the morning following exposure assessment. Differential white blood cell (WBC) counts, red blood cells (RBC), hemoglobin (Hb), mean corpuscular volume (MCV), hematocrit (HCT), platelets and

mean platelets volume (MPV) were determined by a standardized automatic method (Coulter Counter, Serono 190, Serono Diagnostics, Switzerland) within 3 hours from the time of blood drawing.

Genetic polymorphisms

We examined two single nucleotide CYP2E1 polymorphisms *(RsaI* in the promoter flanking region, and *DraI* in intron 6) and one NQO1 polymorphism (609 C>T intron 6, rs1800566). Cells with a T/T genotype are known to have no NQO1 activity (15).

For polymorphism analysis, DNA was isolated from peripheral blood cells. PCR products were digested with excess Hinf I for 3 h, and then electrophoresed through 1.8% agarose and visualized by ethidium bromide staining.

Statistical analyses

Based on individual airborne benzene, study subjects were categorized in three classes of exposure (referents, exposed to <1 ppm and ≥1 ppm). Individual benzene air measurements below analytical detection were assigned a value corresponding to half of LOD (i.e., 0.0116 ppm) for active samplers (8). Mean values of hematological outcomes (WBC, neutrophils, lymphocytes, monocytes, eosinophils, basophils, RBC, Hb, HCT, MCV, platelets and MPV) in each category of benzene exposure were compared with the referent group using a multiple linear regression model adjusted for age, gender, smoking (current *vs* never and former smokers), and airborne toluene levels (as a log transformed continuous covariate). Test for trend across exposure categories was performed.

Multiple linear regression models, adjusted for the same confounders, were applied to evaluate the effect on blood cells count of individual quantitative benzene exposure data (after log transformation) and of urinary biomarkers of exposure (t,t-MA).

To evaluate the influence of each polymorphism (CYP2E1 *RsaI*, CYP2E1 *DraI* and NQO1) on WBC and lymphocytes, we fitted the regression model (using air benzene as continuous variable) stratifying by genotypes. For each gene, subjects with one or more variant allele were grouped in one category. Formal statistical assessment was performed by adding to the models appropriate interaction terms. All the analyses were performed using the Stata statistical software (version 10.0) (18).

Results

Blood cell count was available for 203 study subjects (98% of total population). Main characteristics of the study population across benzene exposure categories are shown in table 1. Exposure groups were similar for smoking habits, age, and length of employment. The proportion of females was higher in the referent group, although differences across categories did not reach statistical significance. All but one of the referents had benzene exposure below the LOD and only 2 workers were below the LOD.

At the beginning of the work shift workers exposed to benzene levels <1 ppm showed a urinary excretion of *t,t*-MA (277.7 μg/l, SD=345.5) 2.8 times higher than referent workers (97.2, SD=90.4) while workers exposed to ≥1 ppm showed a 3.7 higher level of *t,t*-MA (355.3, SD=426.3). Urinary *t,t*-MA at the end of the working shift was 7.4 and 27 times higher among those exposed to benzene levels <1 ppm (801.3, SD=924.2) and ≥1 ppm (2,917.6, SD=2,993.8), showing a positive exposure related gradient (P_{trend} across exposure categories <0.001, Table 1).

Results for each hematological outcomes are reported in table 2 by exposure categories: mean value and standard deviation are shown for each exposure category. Mean differences and their 95% confidence interval between exposed and referent groups are also reported. Most of the examined hematological outcomes (WBC, lymphocytes, neutrophils, monocytes, RBC, Hb, HCT, MCV, Platelets, MPV) did not show a dose-response relationship either across categories (see P trend A) or considering benzene as a continuous variable (individual exposure) (see P trend B).

However, statistically significant decrease in eosinophils (P_{trend}=0.003) and increase in basophils

Table 1 - Characteristics of the Bulgarian petrochemical workers by exposure category

	Exposure Category						
	Referents		<1 ppm		≥1 ppm		
Covariates (unit)	No.=50	%	No.=106	%	No.=47	%	P
Gender							
Males	38	76.0	90	84.9	43	91.5	0.09[b]
Females	12	24.0	16	15.1	4	8.5	
Smoking habit							
Non smokers	21	42.0	37	34.9	18	38.3	0.62[b]
Current smokers	29	58.0	69	65.1	29	61.7	
Age at phlebotomy (years)[a]	41.3±10.8		39.6±8.3		40.7±5.6		0.63[c]
Length of employment (years)[a]	13.2±10.9		15.3±8.9		15.7±8.6		0.35[c]
Benzene air level (ppm)[a]	0.024±0.09		0.3±0.2		4.9±5.3		<0.001[d]
Toluene air level (ppm)[a]	0.005±0.01		0.19±0.47		0.41±0.94		0.001[d]
Urinary metabolites							
t.t-MA (start-shift) (μg/l)[a]	97.2±90.4		277.7±345.5		355.3±426.3		0.001[d]
t,t-MA (end-shift) (μg/l)[a]	108.1±135.4		801.3±924.2		2,917.6±2,993.8		<0.001[d]

[a] mean±SD; [b] Chi square test; [c] One way analysis of Variance (ANOVA); [d] Multiple linear regression model adjusted for age (continous), gender, and smoking habits

P values corresponding to the Chi squared test for differences in proportions (gender and smoking habit), one way analysis of variance (age at phlebotomy), and multiple linear regression models (benzene, toluene, t.t-MA start-shift, t.t-MA end-shift) including exposure category (referents, <1 ppm, ≥1 ppm), adjusted for age (continuous), gender, and smoking habits

Table 2 - Hematological variables by exposure category (mean values ± standard deviation), mean differences (Δ) between exposed and referent groups and their 95% confidence intervals (95%CI) detected among Bulgarian petrochemical workers (No.=203)

	Exposure Category					P_{trend}[a]	
	Referents	<1 ppm		≥1 ppm			
Cells type (units)[a]	(No.=50)	(No.=106)	Δ (95%CI)	(No. = 47)	Δ (95%CI)	(A)	(B)
WBC (10^3/mm^3)	8.32±2.37	8.15±1.88	-0.15 (-1.11, 0.82)	8.33±1.92	0.04 (-1.05, 1.13)	0.79	0.77
Neutrophils (10^3/mm^3)	4.62±1.69	4.42±1.43	-0.33 (-1.08, 0.42)	4.52±1.62	-0.23 (-1.08, 0.62)	0.80	0.75
Lymphocytes (10^3/mm^3)	3.37±1.07	3.40±0.93	0.27 (-0.21, 0.75)	3.42±1.07	0.31 (-0.24, 0.85)	0.36	0.29
Monocytes (10^3/mm^3)	0.09±0.15	0.10±0.12	0.05 (-0.02, 0.11)	0.13±0.16	0.07 (-0.001, 0.15)	0.06	0.17
Eosinophils (10^3/mm^3)	0.10±0.12	0.08±0.14	-0.10 (-0.16, -0.03)	0.06±0.13	-0.13 (-0.20, -0.05)	0.003	0.011
Basophils (10^3/mm^3)	0.03±0.07	0.06±0.10	0.04 (-0.01, 0.09)	0.08±0.13	0.06 (0.003, 0.12)	0.047	0.053
RBC (10^6/mm^3)	4.96±0.45	5.06±0.50	0.24 (0.02, 0.45)	5.07±0.51	0.23 (-0.02, 0.47)	0.19	0.32
Hemoglobin (g/dL)	14.20±1.92	14.72±1.60	0.33 (-0.37, 1.03)	14.74±1.24	0.27 (-0.52, 1.06)	0.68	0.92
HCT (%)	42.96±8.84	43.41±4.80	1.43 (-1.66, 4.52)	44.87±7.58	2.71 (-0.79, 6.21)	0.11	0.31
MCV (mm^3)	83.56±9.67	85.70±7.12	1.68 (-2.15, 5.50)	86.91±7.20	2.78 (-1.56, 7.11)	0.21	0.59
Platelets (x10^3)	267.1±53.24	252.8±74.5	1.11 (-31.0, 33.22)	263.4±51.3	14.14 (-22.3, 50.5)	0.31	0.16
MPV (mm^3)	8.48±0.99	8.46±1.56	0.22 (-0.42, 0.85)	8.26±0.82	0.04 (-0.68, 0.77)	0.83	0.63

[a] P values corresponding to the multiple linear regression models including (A) benzene categories and (B) log benzene as a continuous variable adjusted for age (continuous), gender, smoking habits, and environmental toluene levels;

Δ = difference between the mean level for the exposure categories (<1 ppm and ≥1 ppm) and that detected in referent subjects

(P_{trend}= 0.047) across categories of benzene exposure were detected. When benzene was fitted in the model as a continuous variable p for trends were 0.011 for eosinophils and 0.053 for basophils. After stratification for smoking habits (current vs. former and never smokers) the significant associa-

Table 3 - Benzene multiple regression coefficients (β) for white blood cells (WBC) and lymphocytes by workers' metabolic polymorphisms

Genetic polymorphisms	No.[a]	WBC		Lymphocytes	
		β[b]	p	β[b]	p
CYP2E1 RsaI					
CC	188	0.005	0.73	0.006	0.43
C/G or GG	14	0.077	0.73	-0.014	0.88
CYP2E1 DraI					
AA	150	-0.003	0.86	-0.002	0.76
AT or TT	24	0.087	0.21	0.021	0.64
NQO1 609C>T					
C/C	138	0.001	0.96	0.012	0.15
C/T or T/T	63	0.028	0.25	-0.005	0.74

[a] subjects successfully genotyped; [b] regression coefficients are adjusted for age, gender, smoking habits, and toluene air level

tion between benzene exposure and eosinophil count was confined to current smokers only (P_{trend}=0.001; p=0.018 for interaction between smoking and log benzene. Data not shown).

Considering genetics polymorphisms, the observed genotypes frequency of workers carrying the CYP2E1-*RsaI*, CYP2E1-*DraI,* and NQO1 variant genotypes was 7% (No.=14), 14% (No.=24), and 31% (no.=63), respectively (table 3) and respected the Hardy-Weinberg Equilibrium Test. Results for WBC and lymphocytes are reported in table 3. None of the investigated polymorphisms was found to be associated with blood cell counts at any benzene level.

Discussion

Because of its widespread diffusion in the general environment, the possible toxic effects of low benzene levels (<1 ppm) potentially represents a major public health concern. In animal studies, lymphocytes seem to be more sensitive to benzene exposure than other cell types (9), however this selective effect has not been as clearly documented in humans. Studies conducted in human populations gave inconsistent results. Tsai and colleagues (20) found no significant changes in any of the blood indices (WBC, lymphocytes, RBC, Hb, platelet) evaluated in a group of refinery workers repeatedly examined within a medical surveillance program that lasted about 20 years. In this study 84% of personal sample benzene levels were less than 1 ppm with a median of 0.5 ppm. No indication of adverse hematological effects emerged from another surveillance program on exposed petrochemical workers (mean benzene exposure ranged from 0.14 to 1.6 ppm) (19). No differences in hematological parameters were found either in a study comparing unexposed workers with workers exposed to benzene levels in the 0.01-1.4 ppm range at Monsanto Company (St Louis, Missouri) (4). A subsequent evaluation at the same plant did not show lymphopenia or alteration of other hematological endpoints in workers with 0.55 ppm average exposure (3).

Decreased blood cell counts were reported in two independent studies in Chinese workers. In both studies, exposure was measured as the 4-week average benzene exposure levels before blood drawing. Qu and colleagues (14) reported a depression in RBC, WBC, and neutrophils in the lowest exposed group (≤0.25 ppm) when compared to controls. Lan Q and colleagues (12) found that all types of WBCs and platelets were significantly decreased in workers exposed to <1 ppm benzene compared to controls. Two genetic variants in key metabolizing enzymes, (MPO -463GG and NQO1 465CT) were found to influence the observed relation. NQO1 609C>T did not affect white blood cell counts.

We examined early hematological changes in a group of Bulgarian petrochemical workers exposed to a range of benzene levels between 0.01-23.9 ppm (median 0.46 ppm) and in an unexposed reference group. Exposure assessment was based on personal monitoring sampling the day before phlebotomy, an indicator of current exposure. Major confounding factors such as age, gender, smoking and air toluene were taken into account. In our study, eosinophil count was inversely associated with benzene exposure among current smokers. Conversely, basophils showed an increase with increasing exposure.

No differences across exposure categories were observed for all the other hematological outcomes examined.

Considering genetics polymorphisms, polymorphic allele frequencies for CYP2E1 and NQO1 were low as expected in an European population (5). No effect on benzene hematotoxicity was detected for any of these metabolic genes polymorphisms.

We have no clear explanation for the observed eosinophil and basophil trends which might represent chance variation. A myeloproliferative effect of benzene is highly unlikely to explain our results as it would lead to a concordant depression in either all granulocyte sub-populations and monocytes.

Our results are not coherent with those of Lan and colleagues (12) who found a consistent decline in all blood cell types at benzene exposure levels below 1 ppm.

The occupational exposure range of Chinese workers was characterized by higher values (0.2 to 75 ppm) and exposure monitoring was based on repeated measurements in the month before phlebotomy. This difference in individual exposure levels and in exposure assessment could explain the different effect of exposure to benzene on hematological changes detected in Chinese and Bulgarian workers. However, the lack of a clear hematological toxicity on the white blood cells components and platelets among Bulgarian workers it is unlikely to be attributable solely to an exposure misclassification since urinary levels of *t,t*-MA clearly show a difference in benzene exposure between referent and exposed subjects, with markedly increased urinary levels of this benzene metabolite at the end of the working shift in exposed but not in referent workers.

A possible explanation could be that the hematotoxicity detected among Chinese workers reflects a higher exposure to benzene that occurred during the months prior to phlebotomy resulting in a specific toxicity for hematopoietic progenitor cells leading to the dose-dependent decline of the peripheral white blood cell counts among workers exposed between 0.2 and 75 ppm reported by Lan and colleagues (11,12).

Other differences between Chinese workers and our study subjects may explain the different results. Our study has fewer exposed individuals (153 vs. 250) and a smaller group of referent workers (50 vs. 140). Moreover, most of the Bulgarian workers were males (84%) whereas the Chinese population was close to 70% females. Also other unmeasured factors affecting blood cell count and possibly benzene susceptibility as alcohol consumption and nutritional status could be differently distributed among Bulgarian and Chinese workers.

Given the above mentioned limitations, benzene hematological effects were not found in Caucasian workers exposed to low-doses of benzene. The small number of subject included in the study requires further investigations in order to better understand benzene hematotoxicity and to evaluate current public health and regulatory measures in order to protect workers and the public from possible harmful exposures.

NO POTENTIAL CONFLICT OF INTEREST RELEVANT TO THIS ARTICLE WAS REPORTED

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