Int Arch Occup Environ Health (2012) 85:807–818
DOI 10.1007/s00420-011-0726-4

ORIGINAL ARTICLE

Evaluation of genotoxic effects of lead in pottery-glaze workers using micronucleus assay, alkaline comet assay and DNA diffusion assay

V. Kašuba · R. Rozgaj · M. Milić · D. Želježić · N. Kopjar · A. Pizent · Z. Kljaković-Gašpić · A. Jazbec

Received: 14 September 2011 / Accepted: 27 November 2011 / Published online: 9 December 2011

Abstract
Purpose We investigated genotoxic effects of occupational exposure to lead acetate in pottery-glaze ceramic workers.
Methods The study was carried out in 30 exposed workers and 30 matched controls, to whom several biochemical parameters—the blood lead (B-Pb; range: exposed, 41.68–404.77; controls, 12–52) and cadmium (B-Cd) level, the activity of delta-aminolevulinic acid dehydratase (ALAD), erythrocyte protoporphyrin (EP), the level of vitamin B_{12} and folate in serum—were measured. The genotoxic effects were evaluated by the alkaline comet assay, the DNA diffusion assay and micronucleus test in peripheral blood lymphocytes.
Results Subjects exposed to lead had significantly higher B-Pb level and, consequently, increased values of tail intensity (TI), frequency of apoptotic and necrotic cells, and frequency of micronuclei (MN). In contrast, their activity of ALAD, the level of vitamin B_{12} and folate in serum were significantly lower compared to controls. Poisson regression analysis showed a significant correlation of profession, duration of exposure, smoking, level of cadmium in blood, ALAD and EP with primary DNA damage. A majority of primary damage repairs in a short period after exposure to a genotoxic agent. In addition, the influence of gender and level of vitamin B_{12} and folate in serum MN frequency in exposed group was observed.
Conclusions In this study, DNA diffusion and micronucleus test showed higher influence of tested parameters to DNA damage. The results indicate a need for concomitant use of at least two different biomarkers of exposure when estimating a genetic risk of lead exposure.

Keywords Ceramic workers · Lead exposure · Biological markers · Alkaline comet assay · Micronucleus assay

V. Kašuba (✉) · R. Rozgaj · M. Milić · D. Želježić · N. Kopjar
Mutagenesis Unit, Institute for Medical Research and Occupational Health, Ksaverska c. 2, HR 10000 Zagreb, Croatia
e-mail: vkasuba@imi.hr

A. Pizent · Z. Kljaković-Gašpić
Unit for Analytical Toxicology and Mineral Metabolism, Institute for Medical Research and Occupational Health, Zagreb, Croatia

A. Jazbec
Faculty of Forestry, University of Zagreb, Svetošimunska 25, Zagreb, Croatia

Introduction

Lead is an important environmental chemical contaminant used since ancient times. It enters the environment from natural and anthropogenic sources. It is used in a variety of industrial processes. Increased risk for occupational lead (Pb) exposure also occurs among pottery/ceramic industry workers (ATSDR 1999). The major exposure pathways for workers are inhalation and ingestion of Pb-bearing dust and fumes. Occupational exposure can be evaluated by means of biological monitoring.

Lead and its conjugated compounds are known genotoxic agents affecting the integrity of chromosomes. It is listed as a human carcinogen on the basis of rodent tests (ARC 1994). The most commonly used biomarker of body Pb burden is the concentration of Pb in blood (B-Pb). It mainly reflects current or recent Pb exposure level (Alessio et al. 1981; Telišman et al. 1982, 1990). Additionally, measuring the activity of delta-aminolevulinic acid dehydratase (ALAD) in blood and the erythrocyte protoporphyrin (EP) concentration,

biomarkers of Pb exposure and/or effect, provides even more accurate evaluation of long-term cumulative exposure to Pb (Alessio et al. 1981). ALAD can be inhibited by Pb and is one of the most sensitive indicators of B-Pb accumulation (Schwartz et al. 1990; Wetmur 1994; Kelada et al. 2001). The EP test is especially useful in the detection of mild increases in B-Pb concentration under conditions of occupational exposure. The permissible limit for B-Pb in male Pb workers set by the Occupational Safety and Health Administration (OSHA) is 400 μg/L (1.93 μmol L^{-1}) (OSHA 2002). However, adverse health effects below this standard limit have been increasingly evident (Murata et al. 2009; Mantere et al. 1984; Hogstedt et al. 1983; Seppäläinen et al. 1983). Pb can inhibit the activity of many enzymes and contributes to oxidative stress, increases rate of DNA single- and double-strand breaks, DNA–protein cross-links, induces micronuclei formation, chromosomal aberrations and causes DNA damage (Chen et al. 2006; Woźniak and Blasiak 2003; Silbergeld et al. 2000; Hartwig 1994; Johnson 1998). Moreover, Pb can enhance the genotoxicity of other DNA-damaging agents (such as UV light, X-rays and certain chemicals) and thus act as co-mutagen, predominantly by interfering with DNA replication, fidelity and repair processes (Johansson and Pelliccari 1988; Hartwig et al. 1990; Vaglenov et al. 2001).

This study has been conducted to investigate the relationship between lead acetate occupational exposure and genotoxic effects in glaze pottery ceramic workers. EP concentration and activity of ALAD in blood, biomarkers of Pb exposure and/or effect, were used in addition to B-Pb for better evaluation of long-term cumulative Pb exposure. The influence of serum folate (S-folate) and vitamin B_{12} (S-Vit B_{12}), as known factors of genome stability, was also included. Folate, also known as vitamin B_9, plays an important role in DNA and RNA synthesis (Wagner 1995), production of red blood cells (McKevith 2004) and maintenance of the nervous system (MRC Vitamin Study Research Group 1991). Folate functions as a co-enzyme in many of the reactions during the metabolism of amino acids (Zing and Jones 1997). Beetstra and co-workers found that folate deficiency increases chromosome instability (Beetstra et al. 2005). Vitamin B_{12} or cyanocobalamine is an important co-factor in folate metabolism (Swanson et al. 2001). It is essential for the normal DNA synthesis, especially in erythrocyte development.

To determine the genotoxic risk, the alkaline comet assay (ACA), DNA diffusion assay and the cytokinesis-block micronucleus assay have been used. The alkaline comet assay (ACA) enables an estimation of primary damage of DNA. This highly sensitive method measures single-strand breaks (SSBs) and alkali-labile sites (ALSs) (Singh et al. 1988). The resulting image that is obtained resembles a "comet" with a distinct head and a prominent tail. The head is composed of intact DNA, while the tail consists of damaged (single-strand or double-strand breaks) or broken pieces of DNA. The extent of DNA liberated from the head of the comet is directly proportional to the amount of DNA damage. DNA diffusion assay is designed to quantify cell death by apoptosis (Singh 2005). It detects small DNA fragments from apoptotic cells. The diffused fragments are detected by a fluorescent dye, ethidium bromide.

Primary DNA lesions repair immediately after exposure to genotoxic agent. The cytokinesis-block micronucleus assay, as a comprehensive system for measuring DNA damage and cytotoxicity, was performed to estimate a stable DNA damage. It was evaluated in binucleated lymphocytes and included detection of micronuclei, nucleoplasmic bridges and nuclear buds (Fenech 2007).

Subjects and methods

Studied populations

The exposed group consisted of 30 workers exposed to lead in ceramic industry (average working time, 6.28 ± 1.18 years). Equal number of subjects with no occupational exposure to lead was selected among clerks and newly hired employees as the control group. The exposed and control individuals were matched for age (range 18–57; median: 34.5), smoking habit (18 smokers, 12 non-smokers) and gender (16 women, 14 men). Each blood donor has given a written consent before the investigation for voluntary participation in the study. The examinees were interviewed about work-related exposure to mutagenic agents, use of therapeutic drugs, recent vaccination, smoking and drinking, and therapy-related exposure to ionizing radiation or ultrasound. Subjects who had undergone radiotherapy or chemotherapy, as well as those who had any serious viral infection in the past year, were excluded from the study. Peripheral blood samples were collected by venipuncture under sterile conditions in heparinized vacutainers for comet, DNA diffusion and MN assay, and in vacutainers with EDTA for B-Pb and B-Cd measurements, while vacutainers without an anticoagulant were used for blood samples for hematochemical analysis of folate and vitamin B_{12} plasma concentrations. All blood samples were randomly coded and processed within 2 h following the blood sampling. The research procedures used in the present study were approved by the Ethics Committee of our Institute.

Methods

Biomarkers of Pb and Cd

The B-Pb and B-Cd measurements were performed by electrothermal atomic absorption spectrometry (AAS) method

with the Zeeman-effect background correction (Jurasović and Telišman 1993). The accuracy of the measurements was controlled daily by analyzing three reference blood samples with certified B-Pb and B-Cd values (BCR No. 194–196, Community Bureau of Reference, Commission of the European Communities, Brussels, Belgium) and by the laboratory's regular participation in the National External Quality Assessment Scheme (NEQAS, Birmingham, UK).

EP was measured by spectrofluorimetric method (Chislom and Brown 1975), and the accuracy was controlled by regular participation in the erythrocyte protoporphyrin proficiency-testing program (Pennsylvania Department of Health, Bureau of Laboratories, Lionville, PA, USA).

ALAD activity was measured using the European standardized method (Berlin and Schaller, 1974).

Vitamin B_{12} and folate concentration

Serum folate and vitamin B_{12} were analyzed using the competitive chemiluminescent immunoanalysis electrochemiluminescence immunoassay (ECLIA). Analyses were performed in closed analysis system Elecsys 2010 (Roche Diagnostics Elecsys 2010 Immunoassay System) following the manufacturer's instructions. All analyses were performed in the Laboratory for clinical chemistry, Clinical hospital "Sestre milosrdnice," Zagreb. Referent values for vitamin B_{12} was 211–911 ng L^{-1} and for folate, 3.1–17.5 μg L^{-1}.

The alkaline comet assay

The alkaline version of the comet assay was performed according to the method of Singh et al. (1988) with minor modifications. Fully frosted microscope slides (Surghipath, Richmond, IL, USA) were pre-coated with 1% of normal melting point agarose (NMP) (Sigma). After solidification, this layer was removed and 300 μL of 0.6% NMP agarose was pipetted onto the slides, covered with a cover slip and placed on a metal tray over ice for 10 min to solidify. Aliquots of peripheral blood ($V = 10$ μL) were mixed with 100 μL of 0.5% low melting point (LMP) agarose (Sigma), pipetted over the 0.6 % NMP agarose and placed on ice for 10 min to solidify. As a final protective layer, 0.5% LMP was used. Slides were placed in cold lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10 and 1% sodium *N*-lauroyl sarcosinate, to which 10% of dimethylsulphoxide (DMSO) and 1 % Triton X-100 have been added immediately prior to use) during the night. After lysis, the slides were placed in electrophoresis buffer (300 mmol L^{-1} NaOH and 1 mM Na_2EDTA, pH 13) for 20 min to allow unwinding of the DNA. Electrophoresis was conducted in the same buffer by applying an electric current of 300 mA, at 25 V (1.06 V/cm) for 20 min using a horizontal electrophoresis power tank (Life Technologies). Finally, the slides were washed three times for 5 min with neutralization buffer (0.4 M Tris, pH 7.5) and stained with ethidium bromide (20 mg L^{-1}, Sigma). All steps of the comet assay preceding electrophoresis were performed on ice to prevent repair and effects of metabolic processes. Furthermore, to avoid possible position effects during electrophoresis, two parallel replicate slides per sample were prepared, and each replicate was processed in a different electrophoretic run.

Image analysis was performed using an automatic digital analysis system (Comet Assay II, Perceptive Instruments Ltd., Suffolk, Halstead, UK) fitted with Leitz Orthoplan fluorescence microscope equipped with an excitation filter of 515–560 nm.

Nuclei were excited with green light, and the emitted red spectrum was captured by 25× immersion objective. Images of 200 randomly selected nuclei were analyzed per subject (100 nuclei per slide). Only cells that did not overlap and had a clear margin surrounding them were scored. As a measure of DNA damage, tail length (TL), tail intensity (TI) and tail moment (TM) were selected.

DNA diffusion assay

DNA diffusion assay was performed according to method proposed by Singh (2000, 2005), which detects diffusion of small DNA fragments from apoptotic cells in agarose.

Microgels were made in similar manner as for the comet assay; cells were embedded in low melting agarose and then lysed in a solution of high salt, detergents and alkali. The DNA of the remaining nuclear region was precipitated in microgels by ethanol and spermine. The precipitated DNA and the DNA fragments that diffused in agarose were visualized using ethidium bromide. In healthy cells, the fluorescence is confined to the DNA of the remaining nuclear region, that is, undamaged DNA is supercoiled and does not diffuse very far from the remaining nuclear region. In damaged cells, the alkali lysis treatment unwinds the DNA releasing fragments that diffuse away from the center of the remaining nuclear region, creating a halo surrounding a small compact origin. Therefore, apoptotic cells show a circular gradient or granular DNA with a dense central zone and a lighter and hazy outer zone. Cells with bigger, poorly defined nuclei and clear, defined outer boundary of DNA halo are considered as necrotic cells. For each subject, 2000 cells were analyzed using fluorescence microscope Leitz Orthoplan equipped with an excitation filter of 515–560 nm, under magnification 250×.

The cytochalasin B-blocked micronucleus assay (CBMN)

Standard lymphocyte culture procedure (Fenech and Morley, 1985) with small modifications was performed at the same

day when blood samples were collected. Aliquots of 0.8 mL of whole blood per flask were cultured in 8 mL F-10 medium (Sigma) supplemented with 20% fetal bovine serum (Gibco) and 0.2 mL of PHA-M (Biological Industries, Israel), with penicillin and streptomycin (Sigma). The cytokinesis was stopped by adding cytochalasin B (Sigma) (6 mg L^{-1}) 44th hour after the start of cultivation. Lymphocyte cultures continued to grow until 72 h. Each culture was centrifuged at 800 rpm for 8 min, submitted to cool 110 mM KCl for 15 min at room temperature and fixed with freshly prepared fixative (methanol/acetic acid (3:1). For first fixation, fixative was supplemented with 3 drops of formaldehyde. Fixation step without formaldehyde was repeated until the pellet was white and the resulting cells were re-suspended in a small volume of fixative solution. A total of 1000 binucleated cells per sample with a well-preserved cytoplasm were analyzed for the micronuclei (MN), nuclear buds (NB) and nucleoplasmic bridges (NPB) frequency according to the criteria proposed by Fenech (2000). Cell proliferation is expressed as nuclear division index (NDI); NDI = $(M_1 + 2M_2 + 3M_3 + 4M_4)/N$ (N = 1,000 binuclear cells per sample), where M_1, M_2, M_3 and M_4 indicate the number of cells with one, two, three and four nuclei, and N indicates the total number of cells analyzed (Eastmond and Tucker 1989).

Data analysis

All samples were coded and blindly analyzed. Data were analyzed using the Poisson regression (Frome and Du Frain 1986; Bonassi et al. 1997). Analyses were uni- and multivariate. Poisson regression analysis was done for all subjects as well as for control and exposed subjects separately. The associations between the frequency of micronuclei and comet assay parameters (tail length, tail intensity and tail moment) were considered as dependent variables, while smoking, gender, years of exposure and age were considered as covariates. The level of significance was set at $p < 0.05$.

All analyses were performed using SAS 8.0 (SAS Institute Inc. 1999).

Results

Table 1 shows values of different biomarkers measured in lead-exposed and control groups. The differences between the groups were analyzed by Poisson regression. The blood lead level in exposed group was seven times higher than in control. Consequently, ALAD activity was significantly lower in exposed group. On the other hand, blood Cd level and erythrocyte protoporphyrin concentration showed no significant deviation from control values.

Levels of folate and vitamin B_{12} in serum showed significantly lower values in exposed group.

All three parameters of comet assay showed significantly higher values in exposed group, with the best results for tail intensity. That is why we chose this parameter in further analysis of results.

DNA diffusion assay showed significant increase in apoptotic and necrotic cells in exposed group.

CBMN assay resulted in significantly higher values of MN and NPB frequency in exposed group, while NB in exposed group was insignificant compared to control. NDI did not show difference in cell proliferation between the two groups.

The associations between results of comet assay, DNA diffusion assay and micronucleus assay as dependent variables with gender, age, smoking, vitamin supplementation and years of exposure as covariates, and B-Pb, B-Cd, ALAD, EP, S-folate and S-Vit B_{12} as independent variables analyzed by Poisson regression analysis are presented in Tables 2, 3, 4.

Univariate analysis showed significant association between primary DNA lesions detected by the comet assay and occupational exposure, duration of exposure, smoking and B-Cd (Table 2). In multivariate analysis, association of TI with occupational exposure, age, duration of occupational exposure was observed. The levels of EP and ALAD also influenced DNA damage, that is, ALAD raised the tail intensity, while increase in EP caused significant decrease in tail intensity.

Table 3 shows the results of Poisson regression analysis for the DNA diffusion test. Both uni- and multivariate analysis showed significant association between the incidence of apoptotic cells and occupational exposure (and also duration of exposure). Smoking, vitamin supplementation, as well as B-Pb, B-Cd, S-vit B_{12}, S-folate and ALAD significantly correlated with apoptosis. The significant association between necrosis and occupational exposure was obtained only by univariate analysis. Age, gender and S-folate showed significant association with necrosis in both uni- and multivariate analysis. Multivariate analysis showed a correlation between the incidence of necrotic cells and B-Pb, B-Cd, S-Vit B_{12}, S-folate, ALAD and EP.

Table 4 shows the results of Poisson regression analysis for the frequency of MN, nuclear buds and nucleoplasmic bridges. Occupational exposure to lead, age, smoking, B-Pb and ALAD was statistically significant predictors of the MN, both by univariate and by multivariate analysis. Years of exposure, gender, vitamin supplement, EP and S-folate were significantly associated with MN sole by univariate, while B-Cd was associated with MN sole by multivariate analysis.

Age, smoking, B-Cd, ALAD and EP show significant association with nuclear buds by univariate, and B-Cd and

Table 1 Measured values of different biomarkers in ceramic pottery workers and control group

Variable	Ceramic pottery workers	Controls	Parameter estimate	SE	*p*
B-Pb (μg/L)					
Mean ± SE	220.4 ± 17.75	30.37 ± 2.45	1.9821	0.0424	**<0.0001**
(range)	(41.68–404.77)	(12–52)			
Median	206.40	31.5			
B-Cd					
Mean ± SE	2.13 ± 0.46	1.91 ± 0.75	0.1075	0.2045	0.5993
(range)	(0.06–10.32)	(0.1–14.5)			
Median	1.23	0.43			
ALAD					
Mean ± SE	29.50 ± 2.70	59.38 ± 2.36	−29.884	4.0165	**<0.0001**
(range)	(10.05–69.09)	(46.26–83.23)			
Median	26.17	56.39			
EP					
Mean ± SE	1.74 ± 0.30	0.95 ± 0.06	−0.2170	0.1131	0.0612
(range)	(0.58–7.29)	(0.68–1.57)			
Median	1.01	0.91			
S-Vitamin B_{12}					
Mean ± SE	440.04 ± 27.96	507.73 ± 28.48	−0.1431	0.0125	**<0.0001**
(range)	(237–810)	(325–887)			
Median	390	454			
S-Folate					
Mean ± SE	6.44 ± 0.46	8.34 ± 0.67	−0.2591	0.1011	**0.0103**
(range)	(2.6–12.4)	(4–16.8)			
Median	5.8	7.45			
Tail length[a]					
Mean ± SE	16.66 ± 1.20	14.10 ± 0.20	0.1671	0.0661	**0.0114**
(range)	(11.91–42.85)	(12.45 ± 17.15)			
Median	14.37	13.94			
Tail moment[a]					
Mean ± SE	0.55 ± 0.16	0.21 ± 0.02	0.9473	0.4679	**0.0429**
(range)	(0.09–3.94)	(0.05–0.7)			
Median	0.25	0.19			
Tail intensity[a]					
Mean ± SE	3.21 ± 0.73	1.54 ± 0.14	0.7331	0.1789	**<0.0001**
(range)	(0.78–17.05)	(0.37–3.46)			
Median	1.91	1.36			
Apoptosis[b]					
Mean ± SE	19.56 ± 3.78	5.56 ± 1.09	1.2585	0.1091	**<0.0001**
(range)	(2–90)	(1–18)			
Median	14	4			
Necrosis[b]					
Mean ± SE	3.30 ± 1.04	2.06 ± 0.52	0.4723	0.1956	**0.0158**
(range)	(0–22)	(0–6)			
Median	1	1.5			
Micronucleus[c]					
Mean ± SE	18.17 ± 1.64	7.83 ± 1.12	0.8412	0.0780	**<0.0001**
(range)	(4–39)	(1–24)			
Median	19.5	6			

Table 1 continued

Variable	Ceramic pottery workers	Controls	Parameter estimate	SE	*p*
Nuclear buds[c]					
Mean ± SE	2.87 ± 0.55	2.33 ± 0.44	0.2059	0.1610	0.2010
(range)	(0–11)	(0–11)			
Median	2	2			
Nucleopl. bridges[c]					
Mean ± SE	2.33 ± 0.53	1.07 ± 0.23	0.7828	0.2134	**0.0002**
(range)	(0–10)	(0–4)			
Median	1	1			
NDI					
Mean ± SE	2.16 ± 0.05	2.21 ± 1.12	−0.0213	0.1896	0.9104
(range)	(1.73–2.74)	(1.8–2.63)			
Median	2.17	2.21			

The differences between groups are analyzed by Poisson regression

Bold values indicate $p < 0.005$

[a] A total of 200 cells per sample were analyzed (100 per duplicate slide)

[b] Apoptosis and necrosis measured by DNA diffusion assay

[c] A total of 1,000 binucleated cells/sample were analyzed

Table 2 Results of univariate and multivariate Poisson regression for the comet test (tail intensity) in whole examined population

Tail intensity	Univariate analysis			Multivariate analysis		
	Parameter estimate	SE	*p*	Parameter estimate	SE	*p*
Exposure to lead	0.7331	0.1784	**<0.0001**	0.9466	0.4472	**0.0343**
Age	0.0168	0.0091	0.0661	−0.0183	0.0147	**0.02143**
Gender (F)	−0. 2062	0.1676	0.2185	0.3973	0.2969	0.1808
Years of exposure	0.0622	0.0116	**<0.0001**	0.1212	0.0230	**<0.0001**
Smoking (Y/N)	−0.6959	0.1727	**<0.0001**	−0.4414	0.3826	0.2485
B-Pb	0.0007	0.0007	0.3254	0.0010	0.0029	0.7385
B-Cd	−0.0863	0.0418	**0.0393**	0.0417	0.0666	0.5309
S-B_{12}	0.0007	0.0006	0.2301	−0.0011	0.0011	0.2990
S-folate	0.0247	0.02767	0.3547	0.0537	0.0502	0.2847
ALAD	0.0003	0.0047	0.9537	0.0374	0.0161	**0.0200**
EP	−0.1426	0.0893	0.1103	−0.4566	0.1471	**0.0019**

Bold values indicate $p < 0.005$

ALAD by multivariate analysis. Nuclear bridges are significantly associated with exposure (and also duration of exposure), smoking, B-Pb, ALAD and EP by univariate analysis, while only B-Cd was shown to be significant predictor for nuclear bridges by multivariate analysis.

Mitotic activity did not differ between exposed and control groups. NDI values showed almost identical values ($median_{control} = 2{,}21$; $median_{exposed} = 2{,}17$).

Discussion

Human exposure to lead depends on a level of pollution in living and working environment, as well as on life habits. The level of B-Pb in the present study varied in pottery-glaze workers, which is in agreement with other author's results (De Restrepo et al. 2000; Fracasso et al. 2002; Dandevi et al. 2003; Palus et al. 2003; Minozzo et al. 2004). Popović et al. (2005) found a positive correlation with age, bone Pb and alcohol consumption in women. Multiple linear regression for exposed women confirmed B-Pb increase with bone Pb. Age is not a direct predictor of blood Pb in exposed women. On the contrary, Shaik and Jamil (2009) did not found correlation between age and B-Pb. However, they showed that the duration of exposure to lead caused a significant increase in B-Pb. Hamurcu et al. (2001) found that mean concentrations of B-Cd in lead exposed workers were not significantly different compared to controls.

As expected, a sevenfold increase in B-Pb level, observed in the present study, resulted in genetic material damage. The first approach for the assessment of genetic damage in our study was the alkaline comet assay. This assay detects DNA single-strand breaks and alkali-labile sites. The results of our study revealed a statistically significant increase in the level of DNA damage in exposed group

Table 3 Results of univariate and multivariate Poisson regression for DNA diffusion assay parameters (Apoptosis and Necrosis) in whole examined population

	Univariate analysis			Multivariate analysis		
	Parameter estimate	SE	*p*	Parameter estimate	SE	*p*
Apoptosis						
Exposure to lead	1.2585	0.1091	**<0.0001**	1.4944	0.2063	**<0.0001**
Age	0.0285	0.0047	**<0.0001**	0.0415	0.0076	**<0.0001**
Gender (F)	0.2233	0.0801	**0.0053**	0.7227	0.1224	**<0.0001**
Years of exposure	0.0435	0.0056	**<0.0001**	0.0495	0.0118	**<0.0001**
Smoking	−0.3763	0.0800	**<0.0001**	0.7821	0.1874	**<0.0001**
B-Pb	0.0020	0.0003	**<0.0001**	−0.0069	0.0009	**<0.0001**
B-Cd	−0.0433	0.0213	**0.0420**	−0.1296	0.0394	**0.0010**
S-Vit B_{12}	−0.0021	0.0004	**<0.0001**	−0.0031	0.0006	**<0.0001**
S-folate	−0.0752	0.0167	**<0.0001**	0.0641	0.0279	**0.0218**
ALAD	−0.0130	0.0022	**<0.0001**	−0.0301	0.0068	**<0.0001**
EP	0.0261	0.0273	0.3395	−0.1083	0.0571	0.0576
Necrosis						
Exposure to lead	0.4723	0.1956	**0.0158**	0.8718	0.6160	0.1570
Age	0.0379	0.0105	**0.0003**	0.1221	0.0271	**<0.0001**
Gender (F)	−0.7579	0.1927	**<0.0001**	−1.5198	0.3544	**<0.0001**
Years of exposure	−0.0153	0.0162	0.3447	0.0101	0.0516	0.8454
Smoking	0.1625	0.1815	0.3706	1.5388	0.4645	**0.0009**
B-Pb	−0.0006	0.0007	0.3999	−0.0108	0.0039	**0.0055**
B-Cd	−0.0054	0.0432	0.9003	−0.2088	0.0465	**<0.0001**
S-Vit B_{12}	0.0011	0.0007	0.1364	−0.0042	0.0021	**0.0508**
S-folate	0.1253	0.0300	**<0.0001**	0.3404	0.0864	**<0.0001**
ALAD	0.0071	0.0046	0.1246	−0.0383	0.0076	**<0.0001**
EP	0.0970	0.0531	0.0680	−0.02039	0.0628	**0.0012**

Bold values indicate $p < 0.005$

compared with the controls. Results of Poisson regression indicated that all three comet parameters were significant predictors of occupational exposure to lead. The results are consistent with results of de Restrepo et al. (2000), Fracasso et al. (2002), Zhijian et al. (2006) and Manikantan et al. (2010). Basaran et al. (2003) also reported significantly increased DNA damage in peripheral lymphocytes of pottery workers, with stronger effect in smokers. By contrast, Garcia-Leston et al. (2009, 2010) reported that, although occupational exposure is related to higher blood lead level, no increase in the comet assay parameters could be observed in the exposed individuals.

The indicators of lead body burden as well as primary DNA damage and apoptosis/necrosis occurrence may vary due to duration of exposure. On the other hand, frequency of micronuclei in blood lymphocytes is a more stable indicator of chronic exposure to lead. The results of micronucleus assay in the present study indicate a genotoxic activity in pottery-glaze producers exposed to lead. The MN frequency in exposed group was 2.32 times higher than in control. The results support those from our previous study in battery manufacturing workers (Kašuba et al. 2009). Vaglenov et al. (2001) observed a significant correlation between BNMN values and lead levels in workers from a storage battery plant, concluding that exposure to levels of lead higher than 1.20 μM may increase a genetic risk. Significant increase in MN in subjects occupationally exposed to lead was also reported by Minozzo et al. (2004) and Stoleski et al. (2008). On the contrary, Hoffmann et al. (1984) reported insignificant increase in MN in cultured peripheral blood lymphocytes of car radiator repair workers who were exposed to lead.

Evaluating a possible association between MN and ALAD and EP as biomarkers of Pb exposure assuming that they better reflect long-term cumulative Pb exposures to humans, as compared to B-Pb, we observed a negative correlation between ALAD and MN frequency, and positive with nuclear bridges, while EP positively correlated with all three parameters—MN, nuclear buds and nuclear bridges. In our earlier study, in workers from battery manufacturing industry (Kašuba et al. 2009) we found negative correlations between MN frequency and B-Cd, and ALAD and EP in lead-exposed non-smokers. Ademuyiwa et al. (2005) observed a highly significant ($p < 0.001$) negative

Table 4 Results of univariate and multivariate Poisson regression for cytochalasin B-blocked micronucleus assay parameters in examined population

	Univariate analysis			Multivariate analysis		
	Parameter estimate	SE	*p*	Parameter estimate	SE	*p*
MN						
Exposure to lead	0.8412	0.0780	**<0.0001**	0.6441	0.1859	**0.0005**
Age	0.0265	0.0039	**<0.0001**	0.0153	0.0066	**0.0202**
Gender (F)	0.1977	0.0726	**0.0065**	0.0545	0.1130	0.6298
Years of exposure	0.0478	0.0052	**<0.0001**	0.0039	0.0111	0.7268
Smoking	−0.2879	0.0717	**<0.0001**	−0.4813	0.1531	**0.0017**
B-Pb	0.0027	0.0003	**<.0001**	0.0021	0.0010	**0.0274**
B-Cd	−0.0194	0.0144	0.1766	0.0800	0.0259	**0.0020**
S-B_{12}						
S-folate	−0.0392	0.0129	**0.0023**	0.0067	0.0212	0.7611
ALAD	−0.0137	0.0127	**<0.0001**	0.0127	0.0063	**0.0423**
EP	0.1649	0.0212	**<0.0001**	−0.0073	0.0525	0.8899
Nuclear buds						
Exposed group	0.2059	0.1610	0.2010	−0.6236	0.4022	0.1211
Age	0.0216	0.0087	**0.0129**	0.0240	0.0156	0.1229
Gender (F)	0.0206	0.1606	0.8978	0.1233	0.2310	0.5937
Years of exposure	0.0081	0.0140	0.5659	−0.0642	0.0298	**0.0314**
Smoking	−0.3806	0.1608	**0.0179**	−0.2135	0.3251	0.5114
B-Pb	−0.0000	0.0007	0.9962	−0.0023	0.0022	0.2968
B-Cd	−0.1758	0.0504	**0.0005**	−0.1753	0.0868	**0.0434**
S-B_{12}	−0.0004	0.0006	0.5087	−0.0000	0.0010	0.9930
S-folate	−0.0434	0.0292	0.1373	−0.0755	0.0515	0.1430
ALAD	0.0041	0.0043	0.3464	−0.00378	0.0148	**0.0108**
EP	0.1480	0.0487	**0.0024**	0.0590	0.1405	0.6745
Nucleoplasmic bridges						
Exposure to lead	0.7828	0.2134	**0.0002**	0.9716	0.5909	0.1001
Age	−0.0004	0.0110	0.9737	0.0067	0.0211	0.7521
Gender (F)	0.1029	0.1994	0.6060	−0.2452	0.3521	0.4863
Years of exposure	0.0336	0.0154	**0.0285**	0.0361	0.0356	0.3109
Smoking	0.4487	0.2086	**0.0315**	0.2794	0.4826	0.5626
B-Pb	0.0025	0.0008	**0.0029**	0.0049	0.0030	0.1059
B-Cd	0.0450	0.0318	0.1577	0.1845	0.065	**0.0045**
S-B_{12}	−0.0007	0.0008	0.3623	−0.0002	0.0015	0.9045
S-folate	−0.0001	0.0341	0.9978	0.0668	0.0681	0.3270
ALAD	−0.0099	0.0057	0.0802	0.0362	0.0186	0.0516
EP	0.1889	0.0574	**0.0010**	−0.1410	0.1904	0.4589

Bold values indicate $p < 0.005$

correlation between ALAD activity and B-Pb levels on the one hand and between ALAD activity and EP on the other hand in Pb-exposed and control non-smokers.

Ergurhan-Ilhan et al. (2008), evaluating the level of oxidative stress in subjects working in auto-repairer workshops, found no correlation between B-Pb and ALAD. Mean B-Pb measured in exposed group was 79 μg L^{-1}. The authors suggest that ALAD may not be a reliable parameter of Pb-induced oxidative stress for chronic low-level exposure.

Evaluating folate and vitamin B_{12} status, we found a negative correlation between S-folate and MN frequency in whole examined population. Fenech et al. (1994a, 1997) found a negative correlation between MN frequency and Vit B_{12} status, and no significant correlation between folate status and MN frequency in normal healthy men.

Duration of exposure in this study was strongly correlated with primary DNA damage both in whole studied population and in exposed group. It also showed a strong influence to a MN frequency. The results are in agreement

with other authors' results (Dandevi et al. 2003; Manikantan et al. 2010). Pinto et al. (2000) reported that occupational exposure time, but not the levels of lead, found in blood was significantly associated with cytogenetic damage.

Age and gender are the other confounding factors, which possibly modulate the level of DNA damage. There are few studies investigating the effect of age of the study subjects on comet assay parameters. Of these, only few studies found a significant correlation between age and the level of DNA damage, whereas Grover et al. (2003) found a significant effect of this confounding factor only in control, but not in exposed subjects. Our results showed age as significant predictor of tail intensity in exposed group, while gender did not show any influence to primary DNA damage. Age appeared as significant predictor of MN in both control and exposed groups in this study, which is in agreement with other authors' results. An increase in incidence of MN in humans with age was reported Fenech and Morley 1986; Huber et al. 1983; Migliore et al. 1991. Many studies show an increase in spontaneous incidence of MN with age (Kažimirova et al. 2006; Zijno et al. 2003, Thierens et al. 1996; Ganguly 1993), as well as an increased incidence concerning exposure to different physical or chemical agents. It was also observed that older women have higher MN levels than men (Fenech et al. 1994b; Barale et al. 1998; Bolognesi et al. 1999). Our results indicate gender as one of the factors influencing MN frequency with higher values in women. The results are in agreement with our previous and other authors' results (Rozgaj et al. 2001, Milić et al. 2008; Fenech and Bonassi 2011). Fenech et al. (1994a, b) suggested higher frequency of MN in women as result of possible loss of X chromosomes.

Surprisingly, smoking and B-Cd showed a negative correlation with DNA damage, by the comet assay, but also by DNA diffusion and micronucleus assay. Generally, data on smoking influence to genetic damage are inconsistent. Some investigators describe increased comets in leukocytes of smokers (Betti et al. 1995; Palus et al. 1999), and others did not find a difference in smokers compared to non-smokers (Collins et al. 1997; Hartmann et al. 1998). Dandevi et al. (2003) found that cigarette smoking significantly increases the percentage of cells with comets among controls. They observed a positive correlation of B-Pb with the DNA damage in smokers and non-smokers. Fracasso et al. (2002) and Manikantan et al. (2010) reported the lack of smoking influence on the comet assay results in subjects occupationally exposed to lead.

Statistical analysis of our results emphasizes a negative correlation of smoking and micronuclei, nuclear buds and nucleoplasmic bridges. Results are in agreement with some other reports. Calderón-Ezquerro et al. (2010) reported significantly lower MN frequency in smokers compared to non-smokers. Pinto et al. (2000) did not find influence of smoking or alcohol intake to cytogenetic damage in outdoor painters exposed to lead-containing pigments. In a recent report of an international collaborative group (Bonassi et al. 2003) on the study of the impact of smoking on micronucleus frequency in human lymphocytes, a small decrease in micronucleus frequencies in current smokers and in former smokers compared to non-smokers was described. A significant increase in micronucleus frequencies among smokers was found only in heavy smokers (smoking 30 cigarettes or more per day) who were not occupationally exposed to genotoxins. Some other studies excerpt smoking as an influential factor on MN frequency (Milić et al. 2008; Huang et al. 2009). Considering the effects of smoking, the impact of passive smoking should not be excluded. Zalata et al. (2007) reported DNA damage in children exposed to environmental tobacco smoke (ETS). The association between ETS and lung cancer has been demonstrated (Zhou et al. 2006). According to the results of Bermúdez et al. (1994), ETS particles, which are smaller than mainstream particles, can reach more distal alveolar spaces of the lung, causing DNA damage. Asomaning et al. (2008) found that individuals first exposed to ETS before 25 years of age have a higher lung cancer risk compared to those exposed in later years.

Apoptotic cells occurred 3.5 times and necrotic cells 1.6 times more often in exposed group compared to control. Regression analysis of DNA diffusion assay parameters excerpted exposure, years of exposure, age, gender and B-Pb as significant predictors of apoptosis. Significantly negative correlations were observed between smoking, vitamin supplementation, B-Cd, ALAD and level of S-folates and S-B_{12}. While in control group only age showed significant influence to apoptosis and necrosis, all studied parameters significantly affected both apoptosis and necrosis (with exception of EP for necrosis).

Exposure to lead in that population examined in this study did not influence mitotic activity. NDI of exposed and control groups were almost identical ($median_{control}$ = 2,21; $median_{exposed}$ = 2,17). Palus et al. (2003) reported similar results. Assessing genotoxic effects of lead in workers in the recycling of automotive batteries, Minozzo et al. (2004) showed significantly higher NDI values in the control group. Some other authors reported (Forni et al. 1976; Sarto et al. 1978; Al-Hakkak et al. 1986) that occupational exposure to lead is associated with increased mitotic activity in peripheral blood lymphocytes, increased rate of abnormal mitosis and increased incidence of chromosomal aberrations at blood lead levels. Al-Hakkak et al. (1986) showed that occupational exposure to lead is associated with increased mitotic activity in peripheral blood lymphocytes, increased rate of abnormal mitosis and increased incidence of chromosomal aberrations at blood lead levels ranging from 220 to 890 μg L^{-1}.

In conclusion, the present study indicates that pottery-glaze producers exposed to lead show evidence of genotoxic activity in their lymphocytes. All three used tests excerpted exposure to lead, duration of exposure, smoking and B-Cd as predictors of DNA damage. Comet assay appeared as a less sensitive test compared to DNA diffusion and MN test, which might be due to a prompt repair of the damage caused by a lead exposure. On the other hand, cytogenetic tests detect stable injury, which indicates cumulative genetic material damage.

Acknowledgments This investigation was supported by the Croatian Ministry of Science, Education and Sports as a part of the Projects No: 022-0222148-2137 and No: 022-0222411-2408. We thank Krešimir Nekić from Unit for analytical toxicology and mineral metabolism for performing ALAD and EP measurement.

Conflict of interest None of authors had any personal or financial conflict of interest.

References

Ademuyiwa O, Ugbaja RN, Ojo DA, Owoigbe AO, Adeokun SE (2005) Reversal of aminolevulinic acid dehydratase (ALAD) inhibition and reduction of erythrocyte protoporphyrin levels by Vitamin C in occupational lead exposure in Abeokuta. Nigeria Environ Tox Pharmacol 20:404–411

Agency for toxic substances and disease registry (ATSDR) (1999) Toxicological profile for Lead. US Department of Health and Human Services, Atlanta

Alessio L, Castoldi MR, Odone P, Franchini I (1981) Behaviour of indicators of exposure and effect after cessation of occupational exposure to lead. Br J Ind Med 38:262–267

Al-Hakkak ZSH, Hamamy HA, Murad AMB, Hussain AF (1986) Chromosome aberrations in workers at a storage battery plant in Iraq. Mutat Res 171:53–60

ARC (Seventh Annual report on carcinogens) (1994) National toxicology program. National Institute of environmental Health Sciences. Research Triangle Park, North Carolina

Asomaning K, Miller DP, Liu G, Wain JC, Lynch TJ, Su L, Christiani DC (2008) Second hand smoke, age of exposure and lung cancer risk. Lung Cancer 61:13–20

Barale R, Marrazzini A, Bacci E, Disibio A, Tessa A, Cocchi L, Scarcelli V, Lubrano V, Vassalle C, Landi S (1998) Sister chromatid exchange and micronucleus frequency in human lymphocytes of 1650 subjects in an Italian population: I. Contribution of methodological factors. Environ Mol Mutagen 31:218–227

Basaran N, Shubair M, Ündeger Ü, Kars A (2003) Monitoring of DNA damage in foundry and pottery workers exposed to silica by the alkaline comet assay. Am J Ind Med 43:602–610

Beetstra S, Thomas P, Salisbury C, Turner J, Fenech M (2005) Folic acid deficiency increases chromosomal instability, chromosome 21 aneuploidy and sensitivity to radiation-induced micronuclei. Mutat Res 578:317–326

Berlin A, Schaller H (1974) European method for determination of deltaamino-levulinic acid dehydratase activity in blood. Z Clin Chem Clin Biochim 2:389–390

Bermúdez E, Stone K, Carter KM, Pryor WA (1994) Environmental tobacco smoke is just as damaging to DNA as mainstream smoke. Environ Health Perspect 102:870–874

Betti C, Davini T, Giannessi L, Loprieno N, Barale R (1995) Comparative studies by comet test and SCE analysis in human lymphocytes from 200 healthy subjects. Mutat Res 343:201–207

Bolognesi C, Lando C, Forni, Landini E, Scarpato R, Migliore L, Bonassi S (1999) Chromosomal damage and aging: effect on micronuclei frequency in peripheral blood lymphocytes. Age Aging 28:393–397

Bonassi S, Ceppi M, Fontana V, Merlo F (1997) Multiple regression analysis of cytogenetic human data. Mutat Res 313:69–80

Bonassi S, Neri M, Lando C, Ceppi M, Lin Y, Chang WP, Holland N, Kirsch-Volders M, Wushou PC, Zeiger E, Fenech M (2003) The HUMN collaborative group, effect of smoking habit on the frequency of micronuclei in human lymphocytes: results from the human MicroNucleus project. Mutat Res 543:155–166

Calderón-Ezquerro C, Guerrero-Guerra C, Sansores-Martínez R, Calderón-Segura ME, Villalobos-Pietrini R, Amador-Muñoz O, Gómez-Arroyo S (2010) Genotoxicity in lymphocytes of smokers living in México City. Rev Int Contam Ambient 26:47–63

Chen Z, Jianlin L, Shijie C, Wei Z, Wei W, Lifen J, Hongping D, Jiliang H (2006) Evaluating the genotoxic effects of workers exposed to lead using micronucleus assay, comet assay and TCR gene mutation test. Toxicology 223:219–226

Chislom JJ, Brown DH (1975) Micro-scale photofluorimetric determination of "free erythrocyte protoporphyrin" (protoporphyrin IX). Clin Chem 21:1669

Collins AR, Dusinska M, Franklin M, Somorovska M, Petrovska H, Duthie S, Fillion F, Panayiotidis M, Raslova K, Vaughan N (1997) Comet assay in human biomonitoring studies: reliability, validation, and applications. Environ Mol Mutagen 30:139–146

Dandevi K, Rozati R, Banu BS, Rao PH, Grover P (2003) DNA damage in workers exposed to lead using comet assay. Toxicology 187:183–193

De Restrepo HG, Sicard D, Torres MM (2000) DNA damage and repair in cells of lead exposed people. Am J Ind Med 38:330–334

Eastmond DA, Tucker JD (1989) Identification of aneuploidy-inducing agents using cytokinesis-blocked human lymphocyttes and antikinetochore antibody. Environ Mol Mutagen 13:34–43

Ergurhan-Ilhan I, Cadir B, Koyuncu-Arslan M, Arslan C, Gultepe FM, Ozkan G (2008) Level of oxidative stress and damage in erythrocytes in apprentices indirectly exposed to lead. Pediatr Int 50:45–50

Fenech M (2000) The in vitro micronucleus technique. Mutat Res 455:81–95

Fenech M (2007) Cytokinesis-block micronucleus cytome assay. Nat Protoc 2:1084–1104

Fenech M, Bonassi S (2011) The effect of age, gender, diet and life style on DNA damage measured using micronucleus frequency in human peripheral blood lymphocytes. Mutagenesis 26(1):43–49

Fenech M, Morley AA (1985) Measurement of micronuclei in lymphocytes. Mutat Res 147:29–36

Fenech M, Morley AA (1986) Cytokinesis-block micronucleus method in human lymphocytes: effect of in vivo ageing and low dose X-irradiation. Mutat Res 161:193–198

Fenech M, Rinaldi J (1994) The relationship between micronuclei in human lymphocytes and plasma level of vitamin C, vitamin E, vitamin B12 and folic acid. Carcinogenesis 15:1405–1411

Fenech M, Neville S, Rinaldi J (1994) Sex is important variable affecting spontaneous micronucleus frequency in cytokinesis-blocked lymphocytes. Mutat Res 313:203–207

Fenech MF, Dreosti LE, Rinaldi JR (1997) Folate, vitamin B12, homocysteine status and chromosome damage rate in lymphocytes of older men. Carcinogenesis 18:1329–1336

Forni A, Camiaghi G, Sechi GC (1976) Initial occupational exposure to lead: chromosome and biochemical findings. Arch Environ Health 31:73–78

Fracasso ME, Perbellini L, Solda S, Talamini G, Franceschetti P (2002) Lead induced DNA strand breaks in lymphocytes of exposed workers: role of reactive oxygen species and protein kinase C. Mutat Res 515:159–169

Frome EL, Du Frain RJ (1986) Maximum likelihood estimation for cytogenetic dose-response curves. Biometrics 42:73–84

Ganguly BB (1993) Cell division, chromosomal damage and micronucleus formation in peripheral lymphocytes of healthy donors: related to donors age. Mutat Res 295:135–148

Garcia-Leston J, Laffon B, Roma-Torres J, Teixeira JP, Costa C, Coelho P, Monteiro S, Mayan O, Valdiglesias V, Pasaro E, Mendez J (2009) Evaluation of genotoxic effects of occupational exposure to lead bay means of the comet assay. Abstract book of ICEM 2009, August 20–25, Firenze, Italy, pp 287–288

Garcia-Leston J, Mendez J, Pasaro E, Laffon B (2010) Genotoxic effects of lead: an updated review. Environ Int 36:623–636

Grover PK, Danadevi K, Mahboob M, Rozati R, Saleha B, Rahman MF (2003) Evaluation of genetic damage in workers employed in pesticide production utilizing comet assay. Mutagenesis 18:201–205

Hamurcu Z, Donmez H, Saraymen R, Demirtas H (2001) Micronucleus frequencies in workers exposed to Lead, Zinc, and Cadmium. Biol Trace Elem Res 83:97–102

Hartmann A, Fender H, Speit G (1998) A comparative biomonitoring study of workers of a waste disposal site using cytogenetic tests and the comet (single cell gel) assay. Environ Mol Mutagen 32:17–24

Hartwig A (1994) Role of DNA repair inhibition in lead- and cadmium-induced genotoxicity: a review. Environ Health Perspect 102:45–50

Hartwig A, Schlepergrell R, Beyersmann D (1990) Indirect mechanism of lead induced genotoxicity in cultured mammalian cells. Mutat Res 241:75–82

Hoffmann M, Hagberg S, Karlsson A, Nilsson R, Ranstam J, Hogstedt B (1984) Inorganic lead exposure does not effect lymphocyte micronuclei in car radiator repair workers. Hereditas 101:223–226

Hogstedt C, Hane M, Agrell A, Bodin L (1983) Neuropsychological test results and symptoms among workers with well-defined long-term exposure to lead. Br J Ind Med 40:99–105

Huang P, Huang B, Weng H, Nakayama K, Morimoto K (2009) Effects of lifestyle on micronuclei frequency in human lymphocytes in Japanese hard-metal workers. Prev Med 48:383–388

Huber R, Streng S, Bauchinger M (1983) The suitability of the human lymphocyte micronucleus assay system for biological dosimetry. Mutat Res 111:185–193

Johansson L, Pelliccari CE (1988) Lead induced changes in the stabilization of the mouse sperm chromatin. Toxicology 51:11–24

Johnson FM (1998) The genetic effects of environmental lead. Mutat Res 410:123–140

Jurasović J, Telišman S (1993) Determination of lead and cadmium in human seminal fluid by electrothermal atomic absorption spectrometry. J Anal At Spectrom 8:419–425

Kašuba V, Rozgaj R, Milić M, Želježić D, Kopjar N, Pizent A, Kljaković-Gašpić Z (2009) Evaluation of lead exposure in battery-manufacturing workers with focus on different biomarkers. J Appl Tox 30:321–328

Kažimirova A, Barančokova M, Krajčovičova-Kudlačkova M, Volkovova K, Staruchova M, Valachovičova M, Paukova V, Blažiček P, Wsolova L, Dušinska M (2006) The relationship between micronuclei in human lymphocytes and selected micronutrients in vegetarians and non-vegetarians. Mutat Res 611:64–70

Kelada SN, Shelton E, Kaufmann RB, Khoury MJ (2001) Δ-Aminolevulinic acid dehydratase genotype and lead toxicity: a HuGE. Am J Epidemiol 154:1–13

Manikantan P, Balachander V, Sasikala K (2010) DNA damage in workers occupationally exposed to lead, using comet assay. Int J Biol 2:103–110

Mantere P, Hanninen H, Hernberg S, Luukkonen R (1984) A prospective follow-up study on psychological effects in workers exposed to low levels of lead. Scand J Work Environ Health 10:43–50

McKevith B (2004) Folate and folic acid. British Nutrition Foundation, www.nutrition.org.uk

Migliore L, Parrini M, Sbrana I, Biagini C, Battaglia A, Loprieno N (1991) Micronucleated lymphocytes in people occupationally exposed to potential environmental contaminants: the age effect. Mutat Res 256:13–20

Milić M, Kašuba V, Oreščanin V, Želježić D, Kopjar N, Rozgaj R (2008) Chromosome damage in workers in cigarette manufacturing industry. J Appl Tox 28:399–404

Minozzo R, Deimling LJ, Petrucci Gigante L, Santos-Mello R (2004) Micronuclei in peripheral blood lymphocytes of workers exposed to lead. Mutat Res 565:53–60

MRC Vitamin Study Research Group (1991) Prevention of neural tube defects: Results of the Medical Researc Council Vitamin Study. Lancet 338:131–137

Murata K, Iwata T, Dakeishi M, Karita K (2009) Lead toxicity: does the critical level of lead resulting in adverse effects differ between adults and children? J Occup Health 51:1–12

OSHA (2002) Employee Standard Summary—1910.1025 App B.U.S. Department of Labor, Occupational Health and Safety Administration. Available at: http://www.osha.gov/pls/oshaweb/ovaolisp.show_document?_table=STANDARDS&p_id=10032

Palus J, Dziubaltowska E, Rydzynski K (1999) DNA damage detected by the comet assay in the white blood cells of workers in a wooden furniture plant. Mutat Res 444:61–74

Palus J, Rydzynski K, Dziubaltowska E, Wyszynska K, Natarajan AK, Nilsson R (2003) Genotoxic effects of occupational exposure to lead and cadmium. Mutat Res 540:19–28

Pinto D, Ceballos JM, García G, Guzmán P, Del Razo LM, Vera E, Gómez H, García A, Gonsebatt ME (2000) Increased cytogenetic damage in outdoor painters. Mutat Res 467:105–111

Popović M, McNeill FE, Chettle DR, Webber CE, Lee CV, Kaye WE (2005) Impact of occupational exposure on lead levels in women. Environ Med 113:478–484

Rozgaj R, Kašuba V, Jazbec A (2001) Preliminary study of cytogenetic damage in personnel exposed to anesthetic gases. Mutagenesis 16:139–143

Sarto F, Stella M, Acqua A (1978) Cytogenetic studies in twenty workers occupationally exposed to lead (in Italian). Med Lav 69:120–180

SAS Institute Inc (1999) SAS/STAT® user s guide, Version 8. SAS Institute Inc, Cary

Schwartz J, Landrigan PJ, Baker EL Jr, Orenstein WA, von Lindern IH (1990) Lead-induced anaemia: dose-response relationships and evidence for a threshold. Am J Pub Health 80:165–168

Seppäläinen AM, Hernberg S, Vesanto R, Kock B (1983) Early neurotoxic effects of occupational lead exposure: a prospective study. Neurotoxicology 4:181–192

Shaik AP, Jamil K (2009) Individual susceptibility and genotoxicity in workers exposed to hazardous materials like lead. J Hazard Mater 168:918–924

Silbergeld EK, Waalkes M, Rice JM (2000) Lead as carcinogen: experimental evidence and mechanisms of action. Am J Ind Med 38:316–323

Singh NP (2000) A simple method for accurate estimation of apoptotic cells. Exp Cell Res 256:328–337

Singh NP (2005) Apoptosis assessment by the DNA diffusion assay. Chemosensitivity Vol.2: In vivo models. Imaging Mole Regulat 111:55–67

Singh N, McCoy M, Tice R, Schneider E (1988) A simple technique for quantification of low levels of DNA damage in individual cells. Exp Cell Res 175:184–191

Stoleski S, Karadžinska-Bislimovska J, Stikova E, Risteska-Kuc S, Mijakovski D, Minov J (2008) Adverse effects in workers exposed to inorganic lead. Arch Ind Hyg Toxicol 59:19–29

Swanson DA, Liu MJ, Baker PJ, Garrett L, Stitzel M, Wu J, Harris M, Banerjee R, Shane B, Brody LC (2001) Targeted disruption of the methionine synthase gene in mice. Mol Cell Biol 21:1058–1065

Telišman S, Keršanc A, Prpić-Majić D (1982) The relevance of arguments for excluding ALAD from the recommended biological limit values in occupational exposure to inorganic lead WHO 1980. Int Arch Occup Environ Health 50:397–412

Telišman S, Prpić-Majić D, Keršanc A (1990) Relationships between blood lead and indicators of effect in cows environmentally exposed to lead. Toxicol Lett 52:347–356

Thierens H, Vral A, De Ridder L (1996) A cytogenetic study of radiological workers: effect of age, smoking and radiation burden on the micronucleus frequency. Mutat Res 360:75–82

Vaglenov A, Creus A, Laltchev S, Petkova V, Pavlova S, Marcos R (2001) Occupational exposure to lead and induction of genetic damage. Environ Health Perspect 109:295–298

Wagner C (1995) Biochemical role of folate in cellular metabolism. In: Bailey LB (ed) Folate in health and disease. Marcel Dekker, New York, pp 23–42

Wetmur JG (1994) Influence of the common human delta-aminolevulinate dehydratase polymorphism on lead body burden. Environ Health Perspect 102(suppl. 3):215–219

Woźniak K, Blasiak J (2003) In vitro genotoxicity of lead-acetate; induction of single and double DNA strand breaks and DNA-protein cross-links. Mutat Res 535:127–139

Zalata A, Yahia S, l El-Bakary A, Elsheikha HM (2007) Increased DNA damage in children caused by passive smoking as assessed by comet assay and oxidative stress. Mutat Res 629:140–147

Zhijian C, Jianlin L, Shije C, Wei Z, Wei W, Lifen J, Hongping D, Jiliang H (2006) Evaluating the genotoxic effects of workers exposed to lead using micronucleus assay, comet assay and TRC gene mutation test. Toxicology 223:219–226

Zhou W, Heist RS, Liu G, Asomaning K, Miller DP, Neuberg DS, Wain JC, Lynch TJ, Christiani DC (2006) Second hand smoke exposure and survival in early-stage non-small-cell lung cancer patients. Clin Cancer Res 12:7187–7193

Zijno A, Andreoli C, Leopardi P, Marcon F, Rossi S, Caiola S, Verdina A, Galati R, Cafolla A, Crebelli R (2003) Folate status, metabolic genotype, and biomarkers of genotoxicity in healthy subjects. Carcinogenesis 24:1097–1103

Zing JM, Jones PA (1997) Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis. Carcinogenesis 18:869–882