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Biomonitoring findings for occupational lead exposure in battery and ceramic tile workers using biochemical markers, alkaline comet assay, and micronucleus test coupled with fluorescence *in situ* hybridisation

Vilena Kašuba[1], Mirta Milić[1], Davor Želježić[1], Marin Mladinić[2], Alica Pizent[3], Zorana Kljaković-Gašpić[3], Melita Balija[4], and Irena Jukić[4]

[1] *Institute for Medical Research and Occupational Health, Mutagenesis Unit, Zagreb, Croatia*
[2] *LEO Pharma, Ballerup, Denmark*
[3] *Institute for Medical Research and Occupational Health, Analytical Toxicology and Mineral Metabolism Unit, Zagreb, Croatia*
[4] *Croatian Institute for Transfusion Medicine, Zagreb, Croatia*

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Manufacture of lead-containing products has long been associated with various health risks. To get an insight into the related genotoxic risks, we conducted a biomonitoring study in 50 exposed workers and 48 matched controls using a battery of endpoints that sensitively detect the extent of genome instability in peripheral blood lymphocytes. The levels of primary DNA damage were estimated with the alkaline comet assay, while cytogenetic abnormalities were determined with the cytokinesis-block micronucleus (CBMN) cytome assay. Additionally, CBMN slides of 20 exposed and 16 control participants were subjected to fluorescence *in situ* hybridisation (FISH), coupled with pancentromeric probes to establish the incidence of centromere-positive micronuclei, nuclear buds, and nucleoplasmic bridges. Blood lead levels (B-Pb) were measured with atomic absorption spectrometry. To further characterise cumulative effects of occupational exposure, we measured erythrocyte protoporphyrin (EP) concentrations and delta-aminolevulinic acid dehydratase (ALAD) activity in blood. We also assessed the influence of serum folate (S-folate) and vitamin B_{12} (S-B_{12}) on genome stability. Compared to controls, occupationally exposed workers demonstrated significantly higher B-Pb (298.36±162.07 vs 41.58±23.02), MN frequency (18.71±11.06 vs 8.98±7.50), centromere positive MN (C+ MN) (8.15±1.8 vs 3.69±0.47), and centromere negative MN (C- MN) (14.55±1.80 vs 4.56±0.89). Exposed women had significantly higher comet tail intensity (TI) and length (TL) than control women. Furthermore, workers showed a positive correlation between age and nuclear buds and MN, between MN and years of exposure, and between S-B_{12} levels and TI and ALAD activity, while a negative correlation was found between TI and B-Pb. These findings suggest that occupational settings in the manufacture of lead-containing products pose significant genotoxic risks, which calls for developing more effective work safety programmes, including periodical monitoring of B-Pb and genetic endpoints.

KEY WORDS: blood lead; genetic endpoints; genome damage; human lymphocytes; MN-FISH

Lead (Pb) can damage the liver, kidney, brain, and the nervous, reproductive, cardiovascular, and immune systems (1, 2) by inhibiting the activity of many enzymes and contributing to oxidative stress (3, 4). It damages DNA by increasing the rate of DNA single- and double-strand breaks (5, 6) DNA-protein cross-links (5) and by inducing micronuclei (6, 7) and chromosomal aberrations (8). It can also enhance the genotoxicity of other DNA damaging agents (such as UV light, X-rays, and certain chemicals) by interfering with DNA replication fidelity and repair (9–11). Literature data about mutagenic, clastogenic, and carcinogenic properties of Pb compounds are inconsistent, and the International Agency for Research on Cancer (IARC) has classified inorganic Pb compounds as possible human carcinogens (Group 2B) (12).

Blood lead (B-Pb) level is the most common biological marker of recent lead exposure (13) and is largely used in industry to monitor Pb exposure of workers and protect their health (14). The EU Scientific Committee on Occupational Exposure Limits (SCOEL) (15) has recommended an occupational exposure limit (OEL) of 100 µg/m^3 (fumes and dust) and a biological limit value (BLV) for B-Pb of 300 µg/L (for both men and women). Current EU OEL for B-Pb in lead battery plant workers is 70 µg/mL of blood (16). SCOEL has proposed to lower this limit to 30 µg/mL (17).

Corresponding author: Vilena Kašuba, Institute for Medical Research and Occupational Health, Mutagenesis Unit, Ksaverska cesta 2, 10000 Zagreb, Croatia, E-mail: *vkasuba@imi.hr*

However, even concentrations below this limit have adverse health effects, including the inhibition of haeme synthesis (18), high blood pressure (19, 20), heart disease (21), musculoskeletal diseases (22), and gastrointestinal symptoms (23). Of the three enzymes lead inhibits in haeme biosynthesis, delta-aminolevulinic acid dehydratase (ALAD) is affected the most (24). ALAD activity is a sensitive indicator of early Pb effects and a biomarker of oxidative stress in a lead-exposed haematological system (24–26). Another useful marker is erythrocyte protoporphyrin (EP), as it can detect mild increases in B-Pb concentration under conditions of occupational exposure (27, 28).

Methods for measuring different cytogenetic and molecular endpoints (such as cytokinesis-block micronucleus assay, analysis of structural chromosome aberrations, fluorescence *in situ* hybridisation, or comet assay) have proved themselves useful in the assessment of adverse Pb effects on genetic material (29–35).

The aim of this study was to combine biochemical and cytogenetic markers of Pb exposure, not only to establish exposure to Pb through B-Pb quantification and the above mentioned parameters but to evaluate its genotoxic effects in occupationally exposed battery and ceramic tile workers. To get a more accurate picture of these effects we combined the cytokinesis-block micronucleus (CBMN) and the alkaline comet assay. In a smaller subgroup we also combined CBMN with fluorescence *in situ* hybridisation (FISH) using pancentromeric DNA probes to get a better insight into the content of micronuclei (MN), nuclear buds (NB), and nucleoplasmic bridges (NPB). Our secondary aim was to see how erythrocyte protoporphyrin (EP) concentrations, delta-aminolevulinic acid dehydratase (ALAD) activity, serum folate and vitamin B_{12} levels were related to genotoxic effects.

SUBJECTS AND METHODS

Study population

Our study included 50 Pb-exposed workers (13 women and 33 men) with an average age of 38.4±9.71 years (range: 18–57 years) who voluntarily participated in the study. They spent 10.36±8.94 years (range: 0–34 years) continuously working in battery or ceramic tile production (Zagreb and Hrvatsko Zagorje region, Croatia).

The control group consisted of 48 healthy voluntary blood donors who matched the exposed workers by age, gender, and smoking status [18 women and 30 men with an average age of 38.65±1.53 years (range: 18–57 years)] from the general Croatian population, whose blood samples were collected at the Croatian Institute for Transfusion Medicine, Zagreb, Croatia. They had no history of occupational exposure to Pb or other known genotoxic agents nor did they undergo diagnostic or therapeutic procedures involving ionising or non-ionising radiation.

A smaller, randomly selected subgroup of 20 Pb-exposed workers (8 women and 12 men) and 17 controls (6 women and 11 men) was also assessed with fluorescence *in situ* hybridisation (FISH).

All the participants signed informed consent before entering the study. Information about their health, lifestyle, job conditions, smoking status (smoker or never-smoker; there were no former smokers in either group), alcohol consumption, vitamin supplementation, and X-ray examinations was collected through interviews. Those who had undergone radiotherapy or chemotherapy or had any serious viral infections in the past year were excluded from the study.

Blood sampling

Blood samples of exposed workers were collected in the morning (following the shift of the day before) under sterile conditions by venepuncture in vacutainer tubes (trace element K_2EDTA 10.8 mg vacutainer) for B-Pb (Becton Dickinson and Company, Franklin Lakes, NJ, USA), in clot activator tubes for vitamins (Becton Dickinson, Plymouth, UK), and in LH 170 IU vacutainer tubes for ALAD, MN, and comet assay (Becton Dickinson and Company, Franklin Lakes, NJ, USA) at the Mutagenesis Unit of the Institute for Medical Research and Occupational Health, Zagreb, Croatia. These workers had been working eight-hour shifts a day five days a week and usually came for sampling in the middle of week.

Control blood samples were collected at the Croatian Institute for Transfusion Medicine, Zagreb, Croatia. All blood samples were handled in the same manner. After collection, they were coded, kept refrigerated, and processed as quickly as possible (usually within 2 h of sampling). Further laboratory manipulations with blood samples and all investigations were carried out in accordance with a high standard of ethics. All research procedures complied with the Declaration of Helsinki and the Convention for the Protection of Human Rights and Dignity of the Human Being with regard to the Application of Biology and Medicine. The study was approved by the Ethics Committee of the Institute for Medical Research and Occupational Health, Zagreb, Croatia (28 Feb 2006). One operator scored comet assay slides, one MN assay slides, and one FISH-MN assay slides.

CBMN assay and MN-FISH

The CBMN assay detects DNA damage and abnormalities in human peripheral blood lymphocytes such as chromosomal breakage and loss, nucleoplasmic bridges, nuclear buds, apoptosis, necrosis, and inhibition of cell division (36). Fluorescence *in situ* hybridisation (FISH) has made it possible to reveal the origin of observed micronuclei. An increase in the ratio of centromere-positive micronuclei indicates more pronounced aneugenic activity of the agent of concern (37).

On the same day when samples were collected, standard culturing procedures were employed with small modifications as first described by Fenech and Morley (38) and modified by Fenech (36).

For cytochalasin B blocked micronucleus assay, each 0.8 mL whole blood sample was cultured in 8 mL of RPMI 1640 medium (Sigma-Aldrich, St. Louis, MD, USA) supplemented with 20 % foetal bovine serum (Sigma), 0.2 mL of PHA-M (Biological Industries, Beit-Haemek, Israel), and penicillin and streptomycin (Sigma). Cytochalasin B (Sigma) (in the final concentration of 6 μg/mL) was added at hour 44 to stop cytokinesis, and culturing continued until hour 72. Each culture was centrifuged at 800 rpm for 8 min, and the obtained pellet mixed with cold 110 mmol/L KCl, left at room temperature for 15 min, and fixed with freshly prepared solution of methanol:acetic acid (3:1) with three drops of formaldehyde (formaldehyde was used only in the first fixative). A total of 1000 binucleated cells per sample with a well-preserved cytoplasm was analysed for MN, NB, and NPB frequency according to the criteria proposed by Fenech et al. (39). All samples were coded, so the operator was unaware of the group to which the sample belonged. Cell proliferation was expressed as nuclear division index (NDI) as follows:

$$NDI=(M_1+2M_2+3M_3+4M_4)/N$$

where M_1, M_2, M_3, and M_4 indicate the number of cells with one, two, three, and four nuclei, and N the total number of cells analysed (1000 per sample) (40).

To detect centromeres in MN, FISH was performed using commercial all human centromere satellite, direct labelled probes (Qbiogene, Inc., Irvine, CA, USA)) according to manufacturer's instructions. The FISH assay included a smaller randomly selected group of 20 Pb-exposed workers (8 women and 12 men) and 17 controls (6 women and 11 men). Probes were labelled with a red fluorophore (Texas red spectrum), and slides stained with a solution of 4,6-diamidino-2-phenylindole (DAPI) prepared with antifade solution (Qbiogene). Slides were then analysed with an Olympus AX-70 epifluorescence microscope at 1000x magnification (Olympus Optical, London, UK) to establish the incidence of centromere-positive MNs (C+ MN), centromere positive NBs (C+ NBs), and NPBs (C+ NPBs). At least 1000 binuclear cells per subject were analysed. A C+ MN represents aneugenic events, and a centromere-negative MN (C- MN) represents clastogenic events (41).

Alkaline comet assay

The alkaline comet assay is a rapid, sensitive technique for measuring DNA strand breaks, alkali-labile sites, and cross links with DNA in individual cells (42–44). It was performed according to the method described by Singh et al. (45) with minor modifications. Cells were suspended in 0.5 % low melting point (LMP) agarose (Sigma) pipetted onto fully frosted slides (Surghipath, Richmond, IL, USA) precoated with 300 μL of 0.6 % NMA (normal melting agarose). After gelation on ice, 0.5 % LMP was used as a final protective layer. Slides were then placed in cold lysis solution [2.5 mol/L NaCl, 100 mmol/L Na_2EDTA, 10 mmol/l Tris, pH 10, and 1 % sodium lauroyl sarcosinate to which 10 % of dimethylsulphoxide (DMSO) and 1 % Triton X-100 were added immediately before use] overnight. After lysis, slides were placed in electrophoresis solution (300 mmol/L NaOH and 1 mmol/L Na_2EDTA, pH 13) for 20 min to allow DNA to unwind. Electrophoresis took place in a horizontal electrophoresis power tank (Life Technologies, Gaithersburg, MD, USA) with the same fresh buffer by applying an electric current of 300 mA at 25 V for 20 min. Finally, slides were washed with neutralisation buffer (0.4 mol/L Tris, pH 7.5) three times for 5 min and stained with ethidium bromide (20 μg/ mL, Sigma). All steps of the comet assay preceding electrophoresis were performed on ice to prevent repair and effects of metabolic processes. To avoid possible variation 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 a Leitz Orthoplan fluorescence microscope equipped with an excitation filter of 515–560 nm.

A total of 200 randomly selected comets were measured per person (i.e. 100 comets per replicate slide). The two main comet parameters – tail length (TL) (expressed in micrometres) and tail intensity (TI) (i. e. DNA % in tail) – were used as indicators of primary DNA damage.

Biomarkers of Pb exposure

B-Pb levels were determined with electrothermal atomic absorption spectrometry (AAS) with the Zeeman-effect background correction (46). The accuracy of the measurements was controlled daily by analysing three reference blood samples with certified B-Pb values (BCR No. 194–196, Community Bureau of Reference, Commission of the European Communities, Brussels, Belgium) and through laboratory's regular participation in the National External Quality Assessment Scheme (NEQAS, Birmingham, UK).

EP concentration was measured by a spectrofluorometric method described elsewhere (27) and the accuracy controlled through regular participation in an erythrocyte protoporphyrin proficiency-testing program (Pennsylvania Department of Health, Bureau of Laboratories, Lionville, PA, USA).

Erythrocyte ALAD activity was measured using a standardised method described elsewhere (47). The principle of the method is based on the porphobilinogen (PGB) formation in excess of delta-aminolevulinic acid

(ALA). ALAD activity corresponds to the quantity of PGB produced during the incubation of the enzyme at 37 °C in excess of ALA in the phosphate buffer (pH=6.4). PGB was measured with a spectrophotometer (Cary® 50 UV-Vis, Varian, Inc., Palo Alto, CA, USA) at 555 nm after addition of a modified Ehrlich's reagent. The results were expressed as units per litre of erythrocytes (U/L ercs). All samples were measured in duplicate, and the coefficients of variation (CVs) were less than 5 %.

According to Croatian legislation (48), current biological limit values for Pb exposure are as follows: B-Pb<400 µg/L for male and <300 µg/L for female workers; ALAD>15 U/L ercs; and EP<2.67 µmol/L ercs.

Serum folate and vitamin B_{12} concentrations

Folate, also known as vitamin B_9, plays an important role in DNA and RNA synthesis (49), production of red blood cells, maintenance of the nervous system (50), amino acid metabolism (51), and in preserving health by reducing plasma homocysteine levels (52–55). Beetstra et al. (56) found that folate deficiency increases chromosome instability.

Vitamin B_{12} or cyanocobalamin is an important co-factor in folate metabolism (57). It is essential for normal DNA synthesis, especially in erythrocyte development.

Serum (S-) folate and B_{12} were quantified using the electrochemiluminescence immunoassay (ECLIA) in a closed analysis system Elecsys 2010 (Roche Diagnostics Elecsys 2010 Immunoassay System, Mannheim, Germany) following the manufacturer's instructions. All analyses were performed in Clinical Hospital "Sestre milosrdnice", Laboratory for Clinical Chemistry, Zagreb, Croatia. Reference ranges for the Croatian population are 211–911 ng/L and 3.1–17.5 µg/L for S-B_{12} and S-folate, respectively.

Statistical analysis

As the distribution of results was not normal, the differences between the two groups were tested with the non-parametric Mann-Whitney *U* test. Spearman's rank-order correlation test was used to evaluate the influence of age, years of exposure, smoking, B-Pb, S-B_{12}, S-folate, and ALAD and EP activity on the CBMN and alkaline comet assay parameters. Confounding factors were identified with multiple regression analysis. Differences with $p<0.05$ were considered statistically significant. Separate analyses were used for the exposed and control group. All statistics was run on the Statistica 13.2 package (Dell Inc., Round Rock, TX, USA).

RESULTS

Table 1 summarises the general characteristics of the study groups including their age, gender, work experience (years), smoking habit, B-Pb levels, serum vitamin B_{12} and folate concentrations, ALAD activity, and EP concentrations. Table 2 breaks down these findings by gender and smoking status.

B-Pb levels in the exposed workers were at least seven times higher than in controls (298.36±162.07 µg/L *vs* control 41.58±23.02 µg/L) (Table 1). They were also significantly higher ($p<0.05$) in exposed men than exposed women ($p<0.05$), in exposed women than control women ($p<0.05$), in exposed smokers than exposed non-smokers ($p<0.05$) and control smokers ($p<0.05$), and in exposed non-smokers than control non-smokers ($p<0.05$) (Table 2).

Mean ALAD activity in peripheral blood lymphocytes in controls was at least twice as high as in the exposed group (Table 1). It was also significantly higher ($p<0.05$) in the exposed women than exposed men, in control women than the exposed ones, in control men than those exposed, in exposed non-smokers than smokers, in control smokers than exposed smokers, and in control non-smokers than the exposed ones (Table 2).

EP concentrations also significantly differed between the groups (Table 1) and respective subgroups (Table 2).

As for S-B_{12} concentrations, the only significant difference between the subgroups was that they were significantly higher in control than exposed women (Table 2).

The same was observed for S-folate concentrations, but we also noticed significantly higher means in exposed non-smokers than smokers. Significantly higher levels were also observed in control vs exposed subgroups (Table 2).

Table 1 General characteristics of lead-exposed workers and controls

Parameters (mean ± SE)	Exposed group (N=50)	Control group (N=48)
Age (years)	38.34±9.71	38.65±1.53
Years of exposure	10.36±8.94	0
B-PB (µg/L)	**298.36±162.07**	41.58±23.02
ALAD (U/L ercs)	**23.32±15.87**	56.32±9.92
EP (µmol/L ercs)	**3.05±3.43**	0.98±0.27
S-B_{12} (ng/L)	446.25±137.98	473.08±166.99
S-folate (ng/L)	**5.83±2.31**	7.69±3.70

Significant differences ($p<0.05$, non-parametric Mann Whitney *U* test) are in bold typeface. ALAD – delta-aminolevulinic acid dehydratase activity; B-Pb – blood lead concentrations; EP – blood erythrocyte protoporphyrin concentrations; ercs – erythrocytes; S-B_{12} – serum vitamin B_{12} concentrations; SE – standard error; S-folate – serum folate concentrations

Table 2 General characteristics of lead-exposed workers and controls by gender and smoking

Parameters (mean±SE)	Exposed group			Control group			
	Women (N=17)	Men (N=33)	Smokers (N=33)	Non-smokers (N=17)	Women (N=18)	Men (N=30)	Smokers (N=26)	Non-smokers (N=22)
Age (years)	36±2.57	40±1.57	36.55±1.75	41.82±2.0	35.17±2.49	40.73±1.87	37.15±2.27	40.41±1.98
Years of exposure	5.05±1.42	13.39±1.60	9.98±1.51	11.07±2.34	0	0	0	0
B-PB (μg/L)	**228.13±25.20**[c,d]	**340.33±30.98**[e]	**299.23±27.08**[g]	**296.66±43.42**[h]	**28.2±3.49**[h]	47.16±5.01	40.49±6.11	43.14±4.22
ALAD (U/L ercs)	**28.21±3.48**[d]	**20.92±2.91**[e]	**22.12±2.63**[f, g]	**25.65±4.24**[h]	55.84±2.81	56.61±2.61	57.85±2.62	53.27±2.15
EP (μmol/L ercs)	**2.11±0.44**[e]	**3.59±0.71**[e]	**2.89±0.57**[g]	**3.35±0.92**[h]	0.98±0.08	0.98±0.06	1.03±0.07	0.9±0.06
S-B_{12} (ng/L)	**410.14±35.82**[d]	467.55±25.81	445.93±26.69	446.93±33.05	537.33±46.75	434.53±24.52	447.38±30	503.45±38.38
S-folate (ng/L)	**6.41±0.66**[b, d]	5.61±0.42	**5.26±0.38**[f, g]	**7.06±0.62**[h]	9.11±0.86	6.84±0.64	7.18±0.56	8.30±0.96

Significant differences ($p<0.05$, non-parametric Mann Whitney *U* test) are in bold typeface. **b** – control women vs control men; **c** – exposed women vs exposed men; **d** – control women vs exposed women; **e** – exposed men vs control men; **f** – exposed smokers vs exposed non-smokers; **g** – exposed smokers vs control smokers; **h** – exposed non-smokers vs control non-smokers. ALAD – delta-aminolevulinic acid dehydratase activity; B-Pb – blood lead concentrations; EP – blood erythrocyte protoporphyrin concentrations; ercs – erythrocytes; S-B_{12} – serum vitamin B_{12} concentrations; SE – standard error; S-folate – serum folate concentrations

Table 3 shows the results of the CBMN and comet assay. Mean MN frequency in peripheral blood lymphocytes of the exposed workers was at least twice as high as in controls (18.71±11.06 vs 8.98±7.50; $p<0.05$). Significantly higher MN frequency was also observed in the exposed vs control women and men, exposed smokers vs non-smokers, exposed vs control smokers, and exposed vs control non-smokers.

Controls and exposed workers did not differ significantly in NB count, regardless of gender or smoking status.

Significant difference in NPB count was observed only at the study group level ($p<0.05$).

Considering NDI, a significant ($p<0.05$) difference was observed between exposed and control men, exposed smokers and non-smokers, exposed and control smokers, and between exposed and control non-smokers.

Spearman's correlation test showed a significant contribution of age to MN frequency in both controls ($r=0.49$, $p<0.05$) and exposed workers ($r=0.36$, $p<0.05$), whose years of exposure also contributed to it ($r=0.31$ $p<0.05$).

B-Pb level negatively correlated with NDI in the control group ($r=-0.51$, $p<0.05$), while ALAD activity correlated with NPB count in the exposed group ($r=0.31$, $p<0.05$).

The control group had a positive correlation between S-B_{12} and the NPB/MN ratio ($r=0.30$ $p<0.05$) and negative correlation between S-B_{12} and MN and TI ($r=-0.29$, $p<0.05$; $r=-0.47$, $p<0.05$, respectively). S-folate negatively correlated only with NBs in the exposed group ($r=-0.31$, $p<0.05$).

The results of the FISH assay are presented in Tables 4 and 5. The MN_{total}, C+ MN, C- MN, total NPB, and C- NPB frequencies were significantly higher in the exposed group than control. The two groups, however, showed no significant differences in the frequency of total NB, C+ NB, C- NB, and C+ NPB.

As expected, MN_{total} frequencies were significantly higher in control smokers than non-smokers. MN_{total} and C- MN frequencies were significantly higher in the exposed vs control smokers. The exposed non-smokers had all three FISH parameters (MN_{total}, C+ MN, C- MN) significantly higher than controls.

Exposed men had significantly higher MN_{total} and C- MN frequencies than control men, whereas exposed women had significantly higher all three parameters (MN_{total}, C+ MN, and C- MN) than control women.

There were no correlations in the FISH-tested control subgroup, but in exposed men age negatively correlated with total NB ($r=-0.66$, $p<0.05$).

As for the comet assay results, the only significant difference was in the tail length between the exposed and control women. Male gender positively correlated with tail length in the control group ($r=0.42$; $p=0.00382$). B-Pb level negatively correlated with TI in the exposed group ($r=-0.30$, $p<0.05$). ALAD activity significantly affected TL and TI in

Table 3 Micronucleus assay and alkaline comet assay endpoints in lead-exposed workers and controls by gender and smoking

Parameters (mean±SE)	Total MNi	NDI	TL	TI	Total NBs	Total NPBs	NPB/MN ratio
Exposed (N=50)	**18.71±11.06[a]**	**2.17±0.23[a]**	16.15±5.33	2.64±3.22	3.65±5.91	**2.02±2.47[a]**	0.12±0.02
Controls (N=48)	8.98±7.50	2.13±0.25	14.27±1.23	1.61±0.74	2.29±2.34	1.14±1.53	0.26±0.08
Control W (N=18)	8.11±1.72	2.22±0.06	**13.67±0.21[b]**	**1.34±0.16[b]**			
Control M (N=30)	9.5±1.41	2.07±0.05	14.67±0.25	1.79±0.14			
Exposed W (N=17)	**20.06±2.09[d]**	2.16±0.07	**16.19±1.28[d]**	**3.04±0.92[d]**			
Exposed M (N=33)	**18.4±2.23[e]**	**2.16±0.05[e]**	16.20±0.99	2.46±0.53			
Control S N=(26)	9.27±1.59	2.13±0.05					
Control NS (N=22)	8.64±1.47	2.14±0.05					
Exposed S (N=33)	**17.87±2.07[f,g]**	**2.21±0.04[f,g]**					
Exposed NS (N=17)	**20.24±2.49[h]**	**2.09±0.08[h]**					

Significant differences (p<0.05, non-parametric Mann Whitney *U* test) are in bold typeface. 1000 binuclear cells per subject were analysed. **a** – controls vs exposed workers; **b** – control women vs control men; **d** – control women vs exposed women; **e** – exposed men vs control men; **f** – exposed smokers vs exposed non-smokers; **g** – exposed smokers vs control smokers; **h** – exposed non-smokers vs control non-smokers. M – men; MNi – micronuclei; NBs – nuclear buds; NDI – nuclear division index; NPBs – nucleoplasmic bridges; NS – non-smokers; S – smokers; SE – standard error; TI – tail intensity; TL – tail length; W – women

the exposed group (r=0.31, p<0.05 and r=0.41, p<0.05, respectively).

EP concentration negatively correlated with TL and TI in the exposed group (r=-0.28, p<0.05 and r=-0.39, p<0.05, respectively).

S-B_{12} levels positively correlated with TL and TI in the exposed group (r=0.48, p<0.05 and r=0.32 p<0.05, respectively).

Multiple regression analysis showed statistically significant interdependence between MN frequency and years of exposure (R^2=0.587, p=0.035) and NDI (R^2=0.587, p=0.0006) as well as between NDI and B-Pb level, and EP and S-folate, all in exposed subjects (R^2=0.841, p=0.0121; R^2=0.711, p=0.0083; R^2=0.543, p=0.000001, respectively).

In the control group age significantly contributed to MN frequency (R^2=0.269, p=0.0076), while ALAD activity significantly contributed to the occurrence of NB (R^2=0.713, p=0.0069) and NPB (R^2=0.713, p=0.0371) counts and NDI (R^2=0.713, p=0.0434).

DISCUSSION

In this study we evaluated the influence of age, years of exposure, smoking status, B-Pb, S-B_{12}, S-folate, and ALAD and EP activity on the occurrence of aberrant chromatin structures (MN, NPB, NB) and the level of primary DNA damage in Pb-exposed workers vs controls.

Over the last two decades, many authors have used B-Pb as the biomarker of lead exposure when reporting about its genotoxic effects. Vaglenov et al. (11) thus reported higher frequency of binucleated cells with MNi in peripheral blood of 103 lead-exposed workers and 78 matched controls and concluded that B-Pb levels higher than 1.2 μmol/L may increase the risk of genetic damage. Relying on the results of the MN assay combined with FISH with pancentromeric probes, sister chromatid exchanges (SCEs), and the comet assay Palus et al. (41) suggested that exposure to Pb and Cd in battery plant workers may have both clastogenic and aneugenic effects. Minozzo et al. (58) also reported significantly higher B-Pb levels and MN frequency in the lymphocytes of workers recycling car batteries and significantly higher NDI values in controls. These results suggest a possible effect of Pb on nuclear proliferation. However, they found no significant differences in MN frequency between smoking and non-smoking workers. Similarly, Danadevi et al. (59) reported significantly higher Pb levels and DNA damage in workers on secondary lead recovery compared to controls and little difference in DNA damage between smoking and non-smoking workers.

As expected, Pb levels and years of exposure significantly correlated with DNA damage. In a study of battery plant workers, Fracasso et al. (60) pointed to a

Table 4 MN-FISH assay endpoints in lead-exposed and control subgroups

Parameters (mean±SE)	Exposed group N=20	Control group N=16
Age (years)	39.6±2.05	40.31±1.59
Gender	8 women 12 men	5 women 11 men
Smoking	12 smokers 8 non-smokers	10 smokers 6 non-smokers
Time of exposure (years)	11.66±1.91	0
MN_{total}	**22.70±2.27**	8.25±0.84
C+ MN_{total}	**8.15±1.80**	3.69±0.47
C- MN_{total}	**14.55±1.80**	4.56±0.89
NB_{total}	2.75±0.53	4.18±1.01
C+ NB_{total}	0.35±0.21	0.75±0.28
C- NB_{total}	2.40±0.41	3.43±0.74
NPB_{total}	**1.80±0.53**	0.38±0.15
C+ NPB_{total}	0.05±0.05	0
C- NPB_{total}	**1.75±0.54**	0.38±0.15

Significant differences ($p<0.05$, non-parametric Mann Whitney *U* test) are in bold typeface. 1000 binuclear cells per subject were analysed. C + MN – MN with centromeric signal; C- MN – MN without centromeric signal; C+ NB – NB with centromeric signal; C- NB – NB without centromeric signal; C+ NPB– NPB with centromeric signal; C- NPB – NPB without centromeric signal; MN – micronucleus; NBs – nuclear buds; NPBs – nucleoplasmic bridges; SE – standard error

correlation between increased DNA breaks and oxidative stress (increased reactive oxygen species production and low glutathione levels).

In a three-year biomonitoring study Valverde et al. (30) found that recycling battery workers exceeded allowed B-Pb of 10 µg/dL nearly five times, had significant inhibition of ALAD, and about 47 % higher basal DNA damage than controls. Their DNA repair rate was about 28% slower than that of controls. Similarly, Olewinska et al. (61) reported significantly higher tail DNA % and TL in lead-exposed metal workers than controls and a positive correlation between years of exposure and tail DNA %, TL, and tail moment. Balasubramanian et al. (62), in turn, reported that years of exposure to Pb (in organic lead workers) was the only significant feature that resulted in increased genotoxic potential.

As for gender differences, we found significantly higher B-Pb in men than women of either groups (control women had ~40 % lower levels than control men, and exposed women had ~33 % lower levels than exposed men). Similarly, Theppeang et al. (63) observed 41 % lower median B-Pb in women than men aged over 60 ($p<0.01$). Milman et al. (64) reported similar findings and explained them with the higher content of haemoglobin in male blood.

Literature data show that smoking is significantly related to B-Pb, regardless of gender (65–67), which was confirmed by our findings.

ALAD activity and EP concentration reflect cumulative long-term Pb-exposure, which is in agreement with the results of our and several other studies (14, 68). In addition to the negative correlation between B-Pb and ALAD activity, Stoleski et al. (69) also reported a positive correlation of B-Pb levels with age and years of exposure. Huang et al. (70), however, observed no significant effect of age and smoking on ALAD activity in lead workers. In 1973, Piomelli (71) found that EP concentration increases exponentially with linear increase in B-Pb levels. A few years later, Tomokuni and Ogata (72) confirmed these findings in Pb-exposed workers.

As for the protective effects of serum folate and vitamin B_{12}, Fenech and Rinaldi (73) reported no significant correlation between S-folate and MN frequency in healthy men but also reported a significant negative correlation between MN frequency and B_{12} levels. Büyükşekerci et al. (74), in turn, showed that B-Pb negatively correlated with S-folate levels ($r=-0.105$, $p=0.001$) but did not significantly correlate with S-B_{12} ($r=-0.061$, $p=0.062$). Lee et al. (75) showed on a US general population sample that Pb can biologically interact with folate and vitamin B_{12}. In our study the only significant differences we found for S-B_{12} were those between control and exposed women (who had lower S-B_{12}). We did not observe any significant correlation between B_{12} or S-folate and MN frequency in any of the groups (Table 1).

The CBMN assay is a well-established method for biomonitoring chromosome damage in human populations occupationally exposed to different chemical agents. Several recent studies (11, 58, 69, 76) observed significantly higher MN frequency in lead-exposed workers than controls. Some evaluated the influence of smoking on MN frequency in population not occupationally exposed to Pb and came up with inconsistent findings, ranging from

increased in smokers (77–80) over no effect (81) to lower MN frequency than in non-smokers (82).

Several studies have shown that women have higher MN frequencies than men (83–88), possibly due to much higher X chromosome micronucleation (89–91) and higher X-chromosome loss in women with age (92, 93). In our study MN frequency was only higher in the exposed women than men (Table 5). Smoking positively affected MN formation only in the exposed group (Table 3).

We found no significant difference in NB formation between the exposed workers and controls (Table 3). This could be expected, considering how NBs as indicators of genetic instability are formed (94–96). They are the consequence of elimination of amplified genes and unresolved DNA repair complexes (94). Inorganic lead did not activate any detoxification pathway or induce an over-expression of genes and consequently their copy number. Since the primary DNA damage (TL and TI measured by the alkaline comet assay) in exposed workers was not significantly higher than in controls (Table 3), the small increase observed in NB frequency was not a consequence of DNA repair (10, 60). Micronucleus assay detects permanent DNA damage, and elevated NB frequency can sometimes be the result of a later fusion of formed MN within the nucleus. Such conclusion of NB origin is supported by our centromere signal analysis in lead-exposed workers, as most NBs and MNs were centromere-negative (Table 4). This is in line with other studies which demonstrated that DNA fragments in the shape of MNi or NBs may indicate their common origin (36).

For NPBs we observed significantly higher frequencies in the exposed group than controls (Table 2). So far, Pb exposure has not been reported to induce significant formation of dicentric or ring chromosomes that form nucleoplasmic bridges following karyokinesis. We can only speculate that the observed NPB formation was mediated by telomere fusion due to premature telomere shortening as the effect of chronic genome burdening (36). However, this theory still remains to be confirmed.

We also calculated the NPB/MN ratio, which may provide a fingerprint of specific genotoxic exposure (98). It was 0.12±0.02 (median: 0.08) in the exposed group and 0.26±0.08 (median: 0.11) in the control group. To the best of our knowledge, no similar data exist for lead-exposed workers but only for people exposed to ionising radiation and pesticides as in Thomas et al. (97), who associated NPB/MN ratio close to zero with exposure to a strong aneugenic agent such as spindle poison and much higher ratio (0.77) with exposure to a strong clastogenic agent such as ionising radiation. In a study by Želježić et al. (98) that evaluated genotoxic effects of acute carbofurane poisoning in men, the NPB/MN ratio was 0.19, and the authors concluded that carbofuran acted mostly as an aneugen.

Exposure to lead in this study did not affect mitotic activity, as both groups had similar NDI (NDI_{exp}=2.17 vs $NDI_{cont=}$2.13), which is in line with the report by Palus et al. (41). Minozzo et al. (58), in contrast, found significantly higher NDI in battery recycling workers than controls and other studies (99–101) showed increased mitotic activity in lead exposure.

The correlation analysis of all 50 exposed subjects in our study showed that years of exposure and age correlated with MN frequency in the exposed workers. That group also revealed negative correlation between B-Pb and TI, ALAD correlation with NPBs and both TL and TI, S-B_{12} correlation with TL and TI, and negative correlation between S-folate and NBs. In controls, age positively correlated with MN frequency and NDI, and B-Pb correlated with NDI. Fenech and Rinaldi (73) reported a similar correlation between age and MN frequency, as did many other authors (75, 85, 87–88, 102–104).

Popović et al. (67) showed that age positively correlated with B-Pb only in control women. Our earlier study (104) in pottery glaze workers showed significant associations of years of exposure and gender with EP and S-folate. NBs correlated with age, smoking, ALAD, and EP. NPBs significantly correlated with exposure, years of exposure, smoking, B-Pb, ALAD, and EP (104).

Exposed subjects in our study showed a significant interdependence between MN and NDI with years of exposure. The same was observed for associations between NDI and B-Pb level, and EP and S-folate in the exposed group. In the control group, age significantly influenced MN frequency, and ALAD activity significantly affected occurrence of NBs, NPBs, and NDI.

We also observed a significantly higher frequency of C+ MN and C- MN in the exposed workers than controls. These findings point to both aneugenic and clastogenic effects of lead exposure. Studies of hospital and power plant workers exposed to ionising radiation (105–108) showed that age increased the baseline MN frequencies, which can be almost completely attributed to C+ MN. Palus et al. (41) reported both clastogenic and aneugenic effects in Pb and Cd-exposed battery workers.

It is widely accepted that age and gender are important factors influencing MN levels (47, 86, 109–110). The frequency of C- MN frequency in our study correlated with age, yet Norppa and Falck (111) suggest that age mainly increases C+ MN and rarely C- MN frequency. Gorbunova and Seluanov (112), in turn, suggest that age-dependent C- MN increase could be related diminished DNA repair ability with age.

Unlike some studies (7, 59, 60) reporting association between primary DNA damage (TL and TI) established by the comet assay and Pb-exposure (B-Pb levels) in lead workers, we found no such correlation. The only (negative) correlation we found was the one between EP levels and both TL and TI in the lead-exposed group. These findings are in agreement with Garcia-Leston et al. (113, 114) who confirmed that occupational exposure was related to higher B-Pb but observed no increase in comet assay parameters in the exposed individuals.

Table 5 MN-FISH assay endpoints in lead-exposed and control subgroups by gender and smoking status

Parameters (mean±SE)	Exposed group				Control group			
	Women (N=8)	Men (N=12)	Smokers (N=12)	Non-smokers (N=8)	Women (N=5)	Men (N=11)	Smokers (N=10)	Non-smokers (N=6)
MN_{total}	22.38±2.33	22.92±3.53	24.08±3.17	20.14±2.78	**6.6±1.4**[c]	**9.00±1.00**[d]	**9.8±1.03**[c, f]	**5.67±0.56**[g]
C+ MN_{total}	**9.25±2.13**[b]	7.42±1.38	8.46±1.30	7.57±2.45	**2.4±0.4**[c]	4.27±0.59	**4.3±0.62**[f]	**2.67±0.56**[g]
C- MN_{total}	13.13±2.00	15.5±2.72	15.62±2.52	12.57±2.17	**4.2±1.46**[c, d]	4.73±1.15	**5.5±1.36**[f]	**3.00±0.00**[g]
NB_{total}	**2.88±0.61**[b]	2.67±0.81	2.85±0.77	2.57±0.61	2.4±1.12	5.00±1.33	5±1.48	2.83±1.01
C+ NB_{total}	0.13±0.13	0.5±0.34	0.46±0.31	0.14±0.14	0.2±0.2	1±0.38	1±0.42	0.33±0.21
C- NB_{total}	2.75±0.56	2.17±0.58	2.38±0.57	2.43±0.53	2.2±0.97	4±0.96	4±1.06	2.5±0.85
NPB_{total}	1.5±0.42	2±0.85	1.69±0.58	2±1.13	0.6±0.4	0.27±0.14	0.5±0.22	0.17±0.17
C+ NPB_{total}	0.13±0.13	0	0.08±0.08	0	0	0	0	0
C- NPB_{total}	1.38±0.46	2±0.85	1.62±0.59	2±1.13	0.6±0.4	0.27±0.14	0.5±0.22	0.17±0.17

Significant differences ($p<0.05$, non-parametric Mann Whitney *U* test) are in bold typeface. 1000 binuclear cells per subject were analysed. **b** – exposed men vs exposed women; **c** – control women vs exposed women; **d** – control men vs exposed men; **e** – control non-smokers vs control smokers; **f** – exposed smokers vs control smokers; **g** – exposed non-smokers vs control non-smokers. C+ MN – MN with centromeric signal; C- MN – MN without centromeric signal; C+ NB – NB with centromeric signal; C- NB – NB without centromeric signal; C+ NPB– NPB with centromeric signal; C- NPB – NPB without centromeric signal; MN – micronucleus; NBs – nuclear buds; NPBs – nucleoplasmic bridges; SE – standard error

However, in our earlier studies (104, 115) we found a significant association between primary DNA damage (TI) detected by the comet assay and years of exposure.

Age and gender generally represent major confounding factors that may affect DNA damage. Previous studies (116–118) reported conflicting results regarding the influence of gender between healthy and exposed populations. Some comet assay studies found a positive association between age and DNA damage (119, 120). In our study we found higher TL values in control women than control men. Control men, in turn, had higher TI values. In contrast, Bajpayee et al. (117) (in a healthy Indian population) and Hofer et al. (121) (in young healthy individuals) reported lower DNA damage in healthy women than men. However, it is difficult to compare their findings with ours, because our controls were older and had different living and eating habits. Even so, these differences could partly be explained by oestrogen binding to its receptors and promoting higher expression of genes that encode for antioxidant enzymes. As a result, mitochondria in female cells produce fewer reactive oxygen species (122 – 124). In their biomonitoring studies, however, Dušinska et al. (125, 126) did not find any differences in primary DNA damage between men and women in control groups. Moller (120) also found no correlation between primary DNA damage and gender or smoking, but did find a positive correlation between DNA damage and age.

To conclude, our study has confirmed earlier reports that occupational Pb exposure increases DNA damage. As expected, MN frequency as an index of genetic stability was affected by age and years of exposure in the exposed group and only by age in the control group. MN-FISH revealed that the adverse effects in Pb-exposed workers are both aneugenic and clastogenic.

These findings once again call for improving safety programmes for Pb-exposed workers and for periodical monitoring of genetic endpoints in addition to B-Pb level monitoring.

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Conflicts of interest

None to declare.

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Rezultati biomonitoringa radnika profesionalno izloženih olovu u industriji izrade baterija i keramičkih pločica dobiveni primjenom biokemijskih markera, alkalnoga komet-testa i mikronukleus-testa u kombinaciji s fluorescencijskom *in situ* hibridizacijom

Profesionalna izloženost u industriji olova štetno utječe na zdravlje radnika. Ovo je istraživanje provedeno na 50 radnika izloženih olovu te 48 ispitanika kontrolne skupine primjenom testova za procjenu genomske nestabilnosti u limfocitima periferne krvi. Razine primarnoga oštećenja DNA procijenjene su alkalnim komet-testom, a citogenetičke abnormalnosti utvrđene su citohalazin blokiranim mikronukleus-testom (CBMN). Na podskupini od 20 izloženih i 16 kontrolnih ispitanika dodatno je proveden mikronukleus-test u kombinaciji s fluorescencijskom *in situ* hibridizacijom (MN-FISH). Primjenom pancentromernih sonda istražili smo učestalost mikronukleusa pozitivnih na centromere, nuklearnih pupova i nukleoplazmatskih mostova. Razine olova u krvi (B-Pb) izmjerene su atomskom apsorpcijskom spektrometrijom. Kako bismo utvrdili kumulativne učinke profesionalne izloženosti, izmjerili smo koncentracije eritrocitnoga protoporfirina (EP) i aktivnost dehidrataze delta-aminolevulinske kiseline (ALAD) u krvi. Također smo procijenili utjecaj serumskoga folata (S-folata) i vitamina B_{12} (S-B_{12}) na stabilnost genoma. U usporedbi s podudarnim kontrolama, profesionalno izloženi radnici imali su značajno višu razinu B-Pb (298,36±162,07 vs. 41,58±23,02), učestalost MN-a (18,71±11,06 vs. 8,98±7,50), mikronukleusa pozitivnih na centromere (C+ MN) (8,15±1,8 vs. 3,69±0,47) i mikronukleusa negativnih na centromere (C- MN) (14,55±1,80 vs. 4,56±0,89). Izložene radnice imale su značajno veći intenzitet (TI) i duljinu (TL) repa u komet-testu od ženskih kontrola. Nadalje, u radnika je utvrđena pozitivna korelacija između učestalosti jezgrinih pupova i MN-a s dobi, između MN-a i godina izloženosti te između TI-ja i aktivnosti ALAD-a i razina S-B_{12}. Negativna korelacija utvrđena je između TI-ja i B-Pb. Dobiveni rezultati upućuju na to da profesionalna izloženost olovu predstavlja značajan genotoksični rizik, što zahtijeva razvoj učinkovitijih programa zaštite na radu, uključujući povremeno praćenje B-Pb i genetičkih markera.

KLJUČNE RIJEČI: genetički markeri; ljudski limfociti; MN-FISH; olovo u krvi; oštećenja genoma