Lead acetate genotoxicity in suckling rats

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The mutagenic and carcinogenic potentials of lead are still being investigated. The aim of this study was to evaluate the genotoxic effect of lead acetate in the early period of life, when the organism is extremely sensitive to toxic effects of lead. Six-day-old suckling Wistar rats were exposed to lead (as acetate) either orally for 9 days (daily dose 2 mg Pb/kg b. wt., 18 mg/kg b.wt. total dose) or by a single intraperitoneal injection (5 mg Pb/kg b. wt.). DNA damage was investigated using the comet assay and *in vivo* micronucleus test. The results of the comet assay showed statistically significant differences between the control (unexposed) animals and the two groups of exposed animals by ANOVA weighted for unequal variance (heterogeneity of variances was found by Levene's test), followed by Tukey's *post hoc* test at the level of significance of $p < 0.05$. The two groups of lead-exposed animals were also significantly different from each other. Orally lead-exposed animals showed a significant increase of micronuclei frequencies in reticulocytes and erythrocytes compared to unexposed animals (ANOVA, $p < 0.05$).

Key words: lead acetate, suckling rats, genotoxicity, alkaline comet assay, *in vivo* micronucleus test.

Introduction

Heavy metals rank among the most spread environmental contaminants. The lead consumption in the world was steadily increasing over the period 1965–1990 to about 5.6×10^6 metric tons in 1990 (OECD, 1993). In contemporary world, lead concentrations in the biosphere are 1,000 to 100,000 times above the natural levels (WHO, 1995).

Lifestyle factors (e.g. cigarette smoking), certain occupations, proximity to industrial areas, lead mines or smelters, lead-based paints, and leaded gasoline significantly contribute to lead pollution of the air, food, water, and soil. Lead is absorbed mainly by ingestion and inhalation. The absorption of lead through the gastrointestinal tract is the major exposure pathway in children (WHO, 1995). Gastrointestinal absorption of this toxic metal is significantly higher in infants and young children. In contrast to adults, whose lead

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absorption through the gastrointestinal tract is approximately 14% (RABINOWITZ et al., 1976), children aged 2 to 3 years absorb 42% of ingested lead (ZEIGLER et al., 1978). This data have been confirmed by animal studies (AUNGST et al., 1981; KOSTIAL et al., 1991).

The incidence of childhood cancer has increased, especially for acute lymphocytic leukemia, brain tumor and Wilm's tumour (LANDRIGAN et al., 1998). The last one is suspected to be in relation with paternal exposure to lead (KANTOR et al., 1979; IARC, 1999).

The results described so far demonstrate that lead is genotoxic itself or enhances the effect of other DNA-damaging agents (HARTWIG, 1994; MIADOKOVÁ et al., 1999). The genotoxic effect of heavy metals in bacteria was proved in conditions that enhanced their bioavailability (PAGANO & ZEIGER, 1992; MIADOKOVÁ et al., 2000). However, several *in vivo* and *in vitro* studies confirm the clastogenic action of lead acetate in mammalian models (MURO et al., 1969; DEKNUDT & DEMINATTI, 1978; DI PAOLO et al., 1978; SHARMA et al., 1985; TACHI et al., 1985). There are indications for rather indirect mechanisms of genotoxicity, which may be due to an interaction of lead and DNA repair processes (HARTWIG, 1994; ARIZA et al., 1996). The mutagenic potential of lead in humans has recently been described in lead-exposed battery workers who showed an increase in DNA breakage with an alternate cellular redox state and a significant down-regulation of protein kinase C, suggesting that lead may act as a tumour promotor (FRACASSO et al., 2002).

Although it has been recognized that lead-exposure, even at very low levels, poses an important health risk in young children, especially concerning the central nervous system (BELLINGER et al., 1992; FACTOR-LITVAK et al., 1999; SCHMIDT, 1999), far less is known about the mutagenic effects of lead during early postnatal development.

The aim of this study was to evaluate lead-induced genotoxic damage in suckling rats' peripheral blood lymphocytes using the single cell gel electrophoresis (comet assay) and *in vivo* micronucleus test. There are no similar data for this age group in the literature.

Material and methods

The study included suckling Wistar rats of both sexes (from the Institute's Laboratory Animals Unit, Zagreb, Croatia). The animals were six days old at the beginning of the experiments, and their average body weight was 14 g. During the experiments, pups were kept in litters in individual polycarbonate cages (6–8 per litter; number of pups reduced on second day of life) with their mothers, except when they were receiving cow's milk by artificial feeding from 9.00 a.m. to 4.00 p.m. for 9 consecutive days (VARNAI et al., 2001). The sucklings received lead either through 9-day oral exposure (Experiment I) or by a single intraperitoneal injection on the 6th day of life (Experiment II). In both experiments, artificial feeding with cow's milk was administered to ensure the same nutritional conditions during exposure to lead. Heavy metals (lead, cadmium, and mercury), analysed in the cow's milk by electrothermal atomic absorption spectrometry, were below detection limits.

In the Experiment I, lead (as lead acetate, p.a. grade, "Kemika" Co., Zagreb, Croatia; dissolved in distilled water) was administered to pups by an automatic pipettor (25 μL) in a daily dose of 2 mg lead/kg body weight (in two drops a day, 1 mg in each) (18 mg lead/kg body weight total dose). Twenty-four hours after the last treatment pups were killed by exsanguinations in ether anaesthesia, and their blood was taken from the abdominal aorta, collected into syringes containing heparin, and immediately shaken gently to avoid clotting.

In the Experiment II, lead acetate dissolved in 0.05 mL of distilled water was administered in a single intraperitoneal injection at a dose of 5 mg lead/kg body weight on the 6th day of the pups' life. On the 16th day of their life, the pups were euthanised and blood samples collected as described above. In both experiments lead was administered in doses previously shown not to affect pups' growth and development (VARNAI et al., 2001). Dose levels were determined on the basis of preliminary range finding test.

Untreated pups from two litters from the same breeding and of the same age were used as control.

The procedure used in this study observed the national law on the care and use of laboratory animals and was approved by the Ministry of Agriculture and Forestry of the Republic of Croatia.

Method
Single cell gel electrophoresis (comet assay). We used the method described by SINGH (1988) with some modifications. 10 μL of whole blood was dissolved in 0.5% low melting point agarose (LMP) (Sigma) and spread on fully frosted microscope slides (Surgipath, Richmond, IL, USA) precoated with 0.6% normal melting point agarose (NMP). The slides were immersed in a jar containing cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium lauryl sarcosine, pH 10; 1% Triton X-100 and 10% DMSO were added fresh) at +4 ℃ over night. The cells were exposed to alkali solution (300 mM NaOH and 1 mM Na$_2$EDTA) for 20 minutes to allow DNA to unwind and express the alkali-labile sites. The electrophoresis was applied in an alkaline buffer (300 mM NaOH and 1 mM Na$_2$EDTA) for 20 minutes, using electric current of 25 V and 300 mA. After the electrophoresis, the slides were neutralized with 0.4 M Tris buffer (pH 7.5), stained with 200 μL ethidium bromide (2 μL/mL), and analyzed using

the Leitz Orthoplan fluorescence microscope equipped with a 515–560 nm excitation filter. The images of 100 randomly selected cells were analyzed from each sample. We used image analysis (Perceptive Instruments, Comet Assay II, Release 1.02, Suffolk) to determine the mean tail length of the migrated DNA.

Supravital staining with acridine orange (AO). Acridine orange coated slides were prepared according to Hayashi et al. (1990). Ten μL of aqueous solution (1 mg AO/mL) was spread by glass rod back and forth over a cleaned and warmed glass slide. Five μL of blood was placed without any anticoagulant on the AO-coated glass slide and was covered with a coverslip. Reticulocytes, young RNA containing erythrocytes, supravitally stained by AO were examined using a fluorescence microscope with a blue excitation filter. Reticulocytes were clearly identified by reddish fluorescence of the reticulum structure. Micronuclei were round in shape and emitted a strong yellow-green fluorescence. The recorded frequencies of micronuclei in reticulocytes (MNRETs) and micronuclei in erythrocytes (MNERYs) were based on the observation of 1000 reticulocytes and erythrocytes per animal.

Statistical analysis
Differences between groups were tested by ANOVA followed by post hoc Tukey's HSD test, at the level of significance of $p < 0.05$ (SAS 6.12). Where unequal variances were found (by Levene's test), ANOVA for unequal variances was used.

Results

Comet assay
Table 1 shows the mean group tail lengths and tail lengths for each animal obtained by a single cell gel electrophoresis (comet assay). Analysis of variance weighted for unequal variances, followed by *post hoc* Tukey HSD test for unequal N, was performed. The heterogeneity of variances was established by Levene's test. Mean tail length (μm) per group was: 12.6 ± 1.66, 17.77 ± 1.33, and 14.8 ± 0.21 for control, Pb-acetate i.p., and Pb-acetate p.o. treated group, respectively. Statistically significant differences between groups were found, at $p < 0.05$ level.

In vivo micronucleus test
Table 2 shows the results of the *in vivo* micronucleus test (MNRETs and MNERYs), number of micronuclei per 1000 reticulocytes, separate for each animal in particular group, and the mean value $\pm$ standard deviation (S.D.) for each group.

Table 1. Tail length (μm) measured by the alkaline single cell gel electrophoresis (comet assay).

Group	Pb-acetate dose (mg/kg b.w.)	No. of animals	Tail length $\pm$ S.D. (μm)
Control	0		13.86
			14.83
			11.32
			11.20
			11.67
Mean		5	12.6 ± 1.66
Pb-acetate i.p.	5 (single dose)		19.20
			16.37
			16.93
			18.56
Mean		4	17.77 ± 1.33[a,b]
Pb-acetate p.o.	2 (9 daily doses)		14.67
			15.01
			14.72
			14.56
			15.02
Mean		5	14.8 ± 0.21[a]

Letters in superscript designate significant differences among the groups (established by ANOVA weighted for unequal variances followed by Tukey's HSD test – post hoc comparisons of means, at the level of significance of $p < 0.05$).
[a] Statistically significant in comparison with control.
[b] Statistically significant in comparison with the group exposed to Pb-acetate p.o.

Table 2. Numbers of micronucleated reticulocytes (MNRETs) and erythrocytes taken from the abdominal aorta of lead-exposed suckling rats.

Group	Pb-acetate dose (mg/kg b.w.)	No. of animals	Retyculoytes MN/1000 MNRETs	Erythrocytes MN/1000 Erythrocytes
Control	0		4	2
			2	3
			1	2
			1	4
			3	6
Mean ± S.D.		5	11/5000	17/5000
			2.2 ± 1.3	3.4 ± 1.7
Pb-acetate i.p.	5 (single dose)		2	3
			3	4
			5	6
			8	10
			3	5
Mean ± S.D.		5	21/5000	28/5000
			4.2 ± 2.4	5.6 ± 2.7
Pb-acetate p.o.	2 (9 daily doses)		5	7
			7	7
			6	8
			5	6
			8	8
Mean ± S.D.		5	31/5000	36/5000
			6.2 ± 1.3 [a]	7.2 ± 0.8 [a]

[a] Statistically significant in comparison with control (established by ANOVA followed by Tukey's HSD test – post hoc comparisons of means, at the level of significance of $p < 0.05$). There was no significant difference between two exposed groups (Pb-acetate p.o. and Pb-acetate i.p.)

Analysis of variance weighted for unequal variances, followed by *post hoc* Tukey HSD test, was performed (for MNRETs: $p = 0.012$, $F = 6.59$; for MNERYs: $p = 0.026$, $F = 5.056$). The heterogeneity of variances was established by Levene's test (for MNRETs: $p = 0.266$, $F = 1.48$; for MNERYs: $p = 0.225$, $F = 1.691$). Statistically significant differences in MNRETs and MNERYs between orally exposed group and controls were found, at $p < 0.05$ level (for MNRETs: Tukey HSD test: control vs. Pb-acetate p.o.: 0.009; and for MNERYs: Tukey HSD test: control vs. p.o. exposed groups: $p = 0.021$).

Discussion

Lead is a weak mutagen in mammalian cell systems, but it is a strong mitogen. It is classified as anticipated human carcinogen (IARC, 1987; ARC, 1994; ROBBIANO et al., 1999), but studies are based on the results of biomonitoring in adults and on rodent data in adult animals (RUSOV et al., 1995; VALVERDE et al., 2001).

Metal-induced genome damage includes DNA single-strand and double-strand breaks, DNA-DNA crosslinks, induction of reactive oxygen intermediates (ARIZA et al., 1998), DNA-protein links, and base modifications. Indirectly, metals may inhibit DNA repair enzymes or DNA replication (ZELIKOFF et al., 1988), and consequently act as co-clastogens or co-mutagens (MIADOKOVÁ et al., 2000).

There are several possible mechanisms how lead (II) might interfere with DNA repair process. Besides a direct interaction with repair enzymes, lead ions may also interfere with calcium-regulated processes involved in the regulation of DNA replication and repair. This might help to explain the conflicting results of epidemiological studies related to the clastogenic and carcinogenic potential of lead compounds (JOHNSON, 1998).

BAUCHINGER & SCHMID (1972) and SCHMID et al. (1972) observed no evidence of increased aberrations yield in workers in lead manufacturing industry. These negative findings have been confirmed in human lymphocytes treated with lead acetate *in vitro* (SCHMID et al., 1972; GASIOREK & BAUCHINGER, 1981) and in mice receiving lead

acetate (LEONARD et al., 1972; JACQUET et al., 1977). HARTWIG and co-workers (1990) found that lead acetate alone did not induce DNA-strand breaks in HeLa cells or mutations at the HPRT locus, nor did it induce sister-chromatid exchange (SCE) in V79 Chinese hamster cells. When combined with UV irradiation, lead ions inhibit the removing of DNA-strand breaks and enhance the number of UV-induced mutations and SCE, indicating an inhibition of DNA repair. VALVERDE et al. (2001) did not detect direct induction of DNA strand breaks by lead acetate at low non-cytotoxic concentrations (0.01, 0.1 and 1.0 μM). They found an induction of lipid peroxidation and an increase in free radical levels in different organs of CD-1 male mice after inhalation of lead acetate for 1 hour. They also suggest the induction of genotoxicity and carcinogenicity by indirect interactions, such as oxidative stress. Recent results show that low-level lead acetate exposure *in vitro* can induce significant cytogenetic damage in human melanoma cells (B-Mel) (POMA et al., 2003).

The comet assay is a very sensitive method that has been used in a number of studies for the evaluation of genome damage caused by chemical and physical agents. VALVERDE et al. (2002) implemented the lead inhalation model in mice in order to detect the induction of genotoxic damage in several mouse organs, assessed by the comet assay. They found the correlation between the length of exposure, DNA damage, and the metal tissue-concentrations in the lung, liver and kidney. YUAN & TANG (2001) also used the comet assay to observe the accumulation effect of lead in three generations of blood cells in mice. The results showed a significant damage in the second and the third generations, suggesting the accumulation effect of lead was very significant starting from the second generation. To the best of our knowledge, a comet assay has not yet been described in suckling animals so that the results of this study may be the first results to show a significant increase in the tail length, Pb-acetate i.p. group vs. control group; Pb-acetate p.o. group vs. control group, and between Pb-acetate exposed groups. These results suggest that lead acetate can produce DNA damage detectable by this method.

An increase in chromosome aberrations has been reported in several studies (SPERLING et al., 1970; SCHWANITZ et al., 1975; ZELIKOFF et al., 1988; CHEN, 1992, ARIZA & WILLIAMS, 1996) in population occupationally exposed to lead, as well as in human lymphocytes treated with lead acetate *in vitro* (VAGLENOV et al., 1998). VA-GLENOV and coworkers (2001) observed a significant increase in the frequency of binucleated cells with micronuclei in occupational exposure to lead and found that the lead exposure at levels higher than 1.20 μM may pose an increase in genetic risk. RUSOV and co-workers (1995) found significantly increased ($p < 0.001$) micronuclei in polychromatic erythrocytes of the bone marrow in BALB/c mice receiving lead acetate in doses of 50, 250, and 500 mg/kg body weights. It is still a matter of speculations whether the differences in the results of genotoxic studies are due to different concentrations of Pb, different sensitivity of cells, or different maturity of cell systems.

In our study, orally lead acetate-exposed animals showed a significant increase in micronucleus frequency in peripheral blood reticulocytes and erythrocytes.

Since little is known about the genotoxic effect of lead on children, it should be one of the most important aims in research of environmental contaminants in this decade.

Our preliminary results suggest that low doses of lead acetate, which do not affect growth and development, cause a detectable genome damage in suckling rats exposed to lead both subchronically (for 9 days) or acutely (in a single intraperitoneal injection). Further studies should include different lead doses and different age groups of animals to gain a better insight in the significance of our findings and to elucidate the mechanisms of lead genotoxicity.

Acknowledgements

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