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Genotoxicity in lead treated human lymphocytes evaluated by micronucleus and comet assays

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Lead (Pb) which plays a significant role in modern industry is related to a broad range of physiological, biochemical, behavioural and genetical dysfunctions. Its exposure leads to an increased frequency of genetic aberrations in humans. Hence, this study was designed to assess the genotoxic effect of lead acetate at three dosage levels (10, 25 and 50 µg/mL) by employing: the Cytokinesis Block Micronucleus (CBMN) assay and the Comet assay in Peripheral Blood Lymphocyte Cultures. The results of this study revealed an increased level of DNA damage among treated groups. A significant increase in the tail length of comets and other indices was observed at 25 and 50 µg/mL concentrations comparatively. Thus, lead acetate induced single-strand breaks (SSB) and double strand breaks (DSB) in DNA, alkali-labile sites (ALS), oxidative DNA damage as well as DNA-DNA/DNA-protein/DNA-metal cross linking as evidenced by the Comet assay. The chromosome breakage, DNA misrepair, chromosome loss and telomere end fusion were determined by the Micronucleus assay. Micronucleus frequency in treated lymphocytes was significantly higher as compared to controls. Nucleoplasmic bridges increased significantly and Nuclear buds increased at higher two doses only in exposed cultures. Thus, these assays are better indices for lead induced genotoxicity and metal-nucleus interactions.

Keywords: Chromosome loss, Cytokinesis Block Micronucleus assay, Lymphocyte cultures, DNA damage, Lead acetate, Metal-nucleus interaction, Telomere end fusion

Lead is a ubiquitous common metal found naturally on Earth and occurs in a variety of organic and inorganic compounds with a multitude of additional uses *viz*., manufacturing protective paints for iron and steel, explosives, rodenticides, batteries, solder, fishing weights, pottery, glaze and ammunition, etc.[1-3]. Usage of lead to such an extent has led to increased concentration of lead in air, dust and soil as a consequence of extensive industrialization and environmental pollution[3]. Lead levels in air, water and soil have been increasing and it is considered to be one of the most important environmental pollutants for both urban and semi urban areas. Because of its persistence in the environment, exposure to lead has become a major public health concern[4].

Lead is a well known environmental toxicant that induces mental impairment in children and adolescents[5,6] such as low perception and intelligence quotient (IQ), disturbed personality (aggression)[7] and learning and memory impairments[8]. It damages cellular material and alters cellular genetics and produces oxidative damage. It causes increased production of free radicals and decreased availability of antioxidant reserves to respond to resultant damage. It also interrupts enzyme activation and competitively inhibits trace mineral absorption. Lead binds to sulfhydryl proteins (interrupting structural protein synthesis), alters calcium homeostasis and lowers the levels of available sulfhydryl antioxidant reserves in the body[9]. Once this metal enters the body, it is readily transported in blood and through the blood-brain-barrier, it reaches central nervous system[10]. The freely diffusible plasma fraction is distributed extensively throughout tissues, reaching highest concentrations in bones, teeth, liver, lungs, kidneys, brain and spleen.

Organic lead compounds are actively metabolized in the liver by oxidative dealkylation by cytochrome P-450 enzymes. Metabolism of organic lead consists of formation of complexes with a variety of protein

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and non-protein ligands. It is excreted mainly by renal and gastrointestinal pathways. It is excreted slowly from the body, and hence gets accumulated in the body easily[11-13]. Lead induces Micronuclei[14], DNA damage[15,16] and significant increase both in Chromosomal Aberrations[14] and Sister Chromatid Exchanges[14,17]. *In vitro* studies demonstrated that lead interacts with protein and nucleic acids, particularly at sulfhydryl groups and the phosphate backbone[18,19]. Lead decreases the fidelity of DNA polymerases and disturbs DNA and RNA synthases. Gastaldo *et al.*[20] *in vitro* study in human cells has shown that lead exposure interferes with non-homologous end-joining repair processes by inhibiting DNA-PK kinase activity. Hence, in this study, we investigated the effects of lead in blood cultures using two biomarkers *viz.*, Micronucleus assay for chromosomal anomalies and the Alkaline Comet assay for DNA damage to correlate with three doses of lead. MMC (Mitomycin C) was used as negative control.

Materials and Methods

Sample collection

Peripheral blood samples were collected by venipuncture under sterile conditions into heparinised tubes from healthy, non-exposed, voluntary and consented donors (20-35 years).

Cytokinesis Block Micronucleus (CBMN) Assay

Blood samples were used to set up the cultures in sterile conditions using laminar flow cabinet. Lymphocyte cultures were started by adding 0.5 mL whole blood to 4.5 mL RPMI 1640 medium (HiMedia, Mumbai), supplemented with phytohemagglutinin (Sigma, USA), foetal calf serum (HiMedia, Mumbai) and 1% Penicillin and Streptomycin. Lead acetate was added at different doses of 10, 25 and 50 µg/mL. The cultures were incubated at 37°C for 72 h. Cytokinesis was blocked with 20 µL of cytochalasin B (Sigma, USA) added 48 h after PHA stimulation. Cells were harvested by centrifugation at 2000 rpm for 15 min and after a mild hypotonic treatment with 5 mL 0.075 M KCl at 37°C and three successive fixative washes for 15 min at 2000 rpm, the cells were fixed by adding 5 mL fixative solution (glacial acetic acid:methanol, 1:3) to achieve final cell suspension. Later, air-dried preparations were done and stained with 10% Giemsa (HiMedia, India) for 15 min. A minimum of 1000 binucleated cells (from each of two replicated slides) per each group were scored[21].

Single Cell Gel Electrophoresis (Comet Assay)

The alkaline version of Comet assay was performed according to the procedure of Singh *et al.*[22] with slight modifications. About 4.5 mL of RPMI-1640 medium (HiMedia, Mumbai) pre-supplemented with foetal bovine serum (HiMedia, Mumbai) and antibiotics was taken in sterile culture tubes. Whole blood (0.5 mL) and phytohemagglutinin M (Sigma Aldrich, USA) with final concentration of 10 mg/mL was added in each culture tubes. The cells were exposed to different concentrations of lead acetate except control and negative controls. MMC (Mitomycin C) was added as negative control. Lead acetate was added in different concentrations, 10, 25 and 50 µg/mL. Each group had 5 samples. The cultures were carried out at 37°C for 72 h in BOD incubator, harvested at the end and washed with fresh serum free media. Pellet obtained by centrifugation was used for the assay. Cultured lymphocytes (10 µL) were suspended in 0.5% low melting agarose (HiMedia, Mumbai) and were sandwiched between a layer of 1% normal melting agarose (HiMedia, Mumbai) and a top layer of 0.5% low melting agarose on fully frosted slides. The slides were kept at room temperature (37°C) during the polymerization of each gel layer. After the solidification of 0.5% agarose layer, the slides were immersed in lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM tris buffer, 1% triton X-100 and 10% DMSO) at 4°C in dark. After 1 h, the slides were placed in the electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH >13) for 20 min at room temperature to allow the unwinding of DNA and expression of alkali-labile damage sites.

The electrophoresis was performed at 300 mA and 24 V in a horizontal electrophoresis platform for 30 min. The slides were neutralized with 0.4 M Tris-HCl buffer (pH 7.5) and were stained with 10% ethidium-bromide (EtBr) for 10 min. To prevent additional DNA damage, all the steps were conducted under dimmed light or in the dark. Each slide was analyzed by fluorescence microscope (DMLB, Leica, Germany) equipped with appropriate filters and imaging system. For each group, 50 cells (from each of the two replicate slides) were analyzed by a public domain PC-image analysis program (CASP). The slides were examined in 40X magnification. DNA damage was quantified by Tail length, Tail DNA%, Tail moment (TM) and Olive tail moment (OTM) in arbitrary units, length of head and length of tail of

comet in pixels and % of DNA in comet's head and in tail, respectively.

OTM = [% of DNA in tail] × [Distance between centre of gravity of DNA in tail and that of the centre of gravity of DNA in head in X-direction]

All these parameters are positively correlated with the level of DNA breakage and/or alkali-labile sites and negatively correlated with the level of DNA cross-links[23]. Because our measurement system was not caliberated, TM and OTM were presented in arbitrary units. DNA damage was further quantified by visual classification of cells into categories of 'Comets' corresponding to the amount of DNA in the tail according to Anderson *et al*.[24].

The most basic way of viewing the data from the comet assay is based on the distribution of cells according to a metric of DNA damage. Such a presentation may provide additional information on different DNA damage in the population of individual cells[23]. Therefore, the results of DNA damage evoked by lead acetate singly assessed by the alkaline version were also presented in the form of histograms of the distribution of tail length.

Statistical analysis

One-way analysis of variance (ANOVA) with Tukey's significant difference post hoc test was used to compare differences among groups. Data were analyzed statistically by Graph Pad Prism 5.0 statistical software. *P* values <0.05 were considered significant.

Results

The genotoxic effects of lead acetate were assessed in normal human lymphocytes using the Micronucleus assay and Comet assay. The distribution of the cells was homogenous indicating that the reaction of individual cells to the metal was typical, without a subpopulation of a distinguishable response. The *in vitro* exposure to lead showed clear evidence of genetic damage in peripheral blood lymphocytes when evaluated by CBMN assay. In this assay, 6 parameters were assessed: Binucleated cells (BN), Multinucleated cells (MN), Binucleated cell with micronuclei (BN with MNi), Multinucleated cell with micronuclei (MN with MNi), Nucleoplasmic Bridges (NPBs) and Nuclear Buds (N-Buds). Fig. 1A shows that Binucleated cells are inversely proportional to the dose of lead acetate. A significant (*P* <0.05) decrease in frequency of occurrence of Binucleated cells was observed with increase in dose concentration. The Multinucleated cells significantly (*P* <0.01) increased in treated groups as compared to control (Fig. 1B). Figure 2 shows the comparison and distribution of Micronucleus in Binucleated and Multinucleated cells. All groups showed significantly higher frequency of Micronucleus as compared to control, but the most significant (*P* <0.001) was observed at the highest dose administered.

A significant (*P* <0.001) higher values of NPBs and N-Buds were observed in the treated groups as compared to control (Fig. 3). Figure 4 indicates percentage of total aberration that includes, BN with MNi, MN with MNi, NPBs and N-Buds. Figure 5

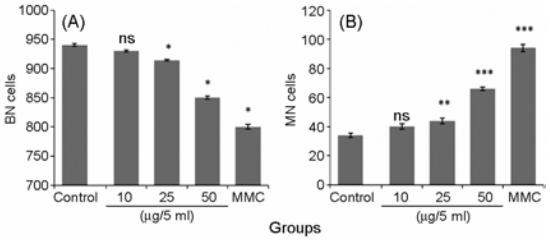

Fig. 1—Frequency of (A) Binucleated cells (BN); and (B) Multinucleated cells (MN) in human lymphocytes incubated for 72 h at 37°C with lead acetate. N=5. [Values are Mean ± SEM, ns = non significant, **P* <0.05, ***P* <0.01, ****P* <0.001]

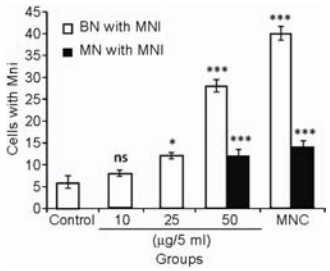

Fig. 2—Frequency of Micronuclei (MNi) in once divided Binucleated and Multinucleated human lymphocyte cells incubated for 72 h at 37°C with lead acetate. N=5. [Values are Mean ± SEM, ns = non significant, **P* <0.05, ****P* <0.001]

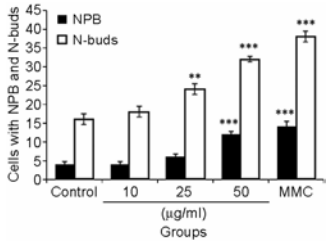

Fig. 3—Frequency of Nucleoplasmic Bridges (NPBs) and Nuclear Buds (N-Buds) in once divided human lymphocyte cells incubated for 72 h at 37°C with lead acetate. [The results were detected by Cytokinesis Block Micronucleus Assay. The number of cells scored in each treatment was 1000. The figure shows mean results from five independent experiments. Values are Mean ± SEM, ns = non significant, ***P* <0.01, ****P* <0.001]

presents the microscopically 100X magnitude of a BN cell, BN cell with MNi, MN cell with MNi, cell with NPB and cell with N-Bud in Giemsa stain. Genetic damage induced due to exposure to lead was higher than that for control, indicating that the *in vitro* supplementation of lead crucially affected the lymphocytes. Thus, the pooled data can be considered as a good reference control. When the overall control data was compared with the obtained exposed data, the differences were significant among control and exposed (P <0.001), indicating a clear genotoxic effect of lead exposure. The genetic effects detected in the treated group are being considered due to effects of lead acetate.

Employing the comet assay, 4 parameters characterizing DNA strand breaks were evaluated: Head DNA%, Tail DNA%, Tail length and Olive tail moment. Lead showed a significantly higher DNA content in peripheral blood lymphocyte cultures because of healthy consented volunteers and there was a significant (P <0.05) head DNA damage observed at high and MMC added groups compared to control cultures (Table 1). It also exerted an increase in Tail length of comets in treated groups. The mean values of Tail length, Olive Tail Moment and Tail DNA% significantly (P <0.001) increased in treated groups as compared to controls (Table 1). It should be taken into account, that computer program, we used, calculated the values of Comet Tail moment for each scored cells and we calculated the mean of it.

The number of cells with damaged DNA are classified as types 0, 1, 2 and 3 (comets) in the cells from treated and control cultures as summarized in figure 6. A clear significant increase in DNA migration was found in the treated groups comparatively. Among the study groups, significantly more cells of 1, 2 and 3 types were observed. The presence of these cells demonstrated greater DNA damage than control groups (Fig. 7). This indicates that double strand breaks contributed to the observed increment in the tail length in the presence of lead. We report here that lead acetate can directly modulate DNA damage in human lymphocytes *in vitro*.

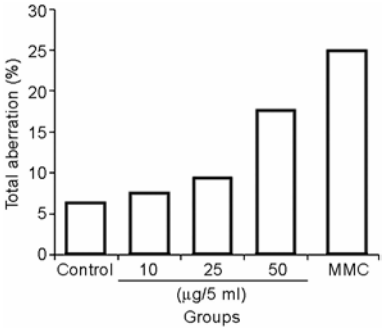

Fig. 4—Characterize the Total Aberration Percentage in reference to dose of lead acetate. [The data indicates dose-dependent aberration as compared to control]

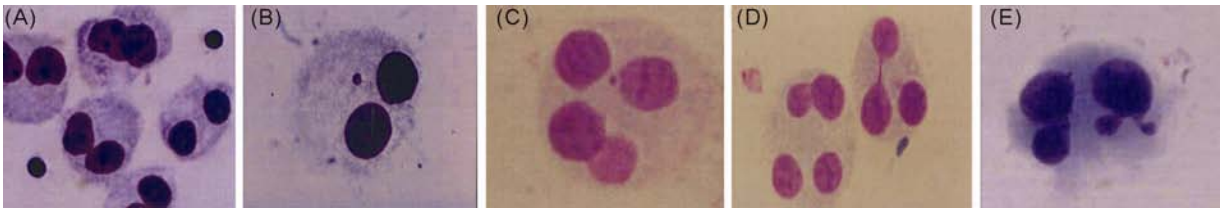

Fig. 5—Image showing (A) Binucleated cells, (B) Binucleated cell with Micronuclei, (C) Multinucleated cell with Micronuclei, (D) Cell with Nucleoplasmic Bridge, (E) Cell with Nuclear Bud.

Table 1—Head DNA, Tail DNA, Tail Length and Olive Tail Moment of Comets in the alkaline version obtained from the human lymphocytes incubated with lead acetate for 72 h at 37°C.

DOSE	Head DNA (in %)	Tail DNA (in %)	Tail Length (in pixels)	Olive Tail Moment (arbitrary units)
Control	97.74 ± 1.98	2.35 ± 0.04	79.37 ± 1.03	1.48 ± 0.10
10 µg/mL	97.55 ± 0.88	2.56 ± 0.06**	91.40 ± 3.31*	1.76 ± 0.07*
25 µg/mL	96.98 ± 0.95	3.23 ± 0.05***	101.1 ± 2.03***	1.97 ± 0.03***
50 µg/mL	91.96 ± 1.00*	4.19 ± 0.03***	116.4 ± 1.88***	2.49 ± 0.04***
MMC	90.84 ± 0.96**	5.75 ± 0.02***	148.1 ± 1.19***	4.48 ± 0.04***

[The number of cells scored in each treatment was 50. The table shows mean results from five independent experiments. Values are Mean ±SEM *P <0.05, **P < 0.01, ***P <0.001]

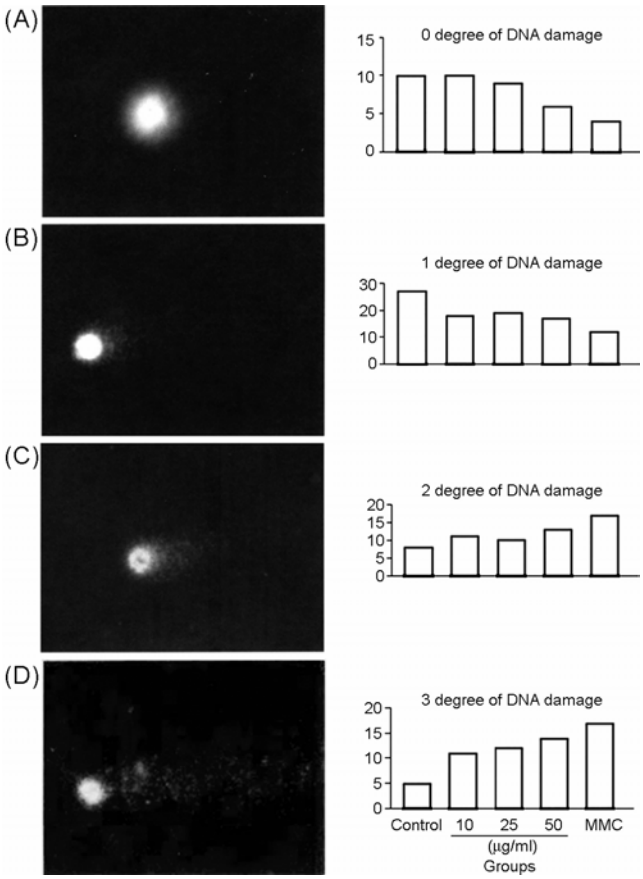

Fig. 6—Histograms with figures (A-D) of the distribution of Comet Tail Length in different classes in Ethidium bromide stained human lymphocytes incubated for 72 h at 37°C with lead acetate at 10, 25 and 50 µg/mL concentrations. [The numbers of cells scored in each treatment was 50. A= Type 0 Comet; B= Type 1 Comet; C= Type 2 Comet; D= Type 3 Comet]

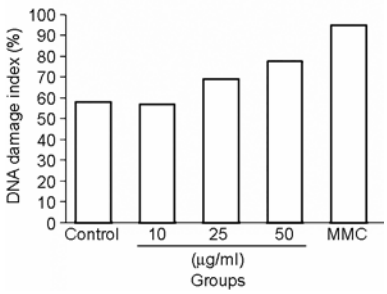

Fig. 7—Characterize the calculated DNA Damage Index as determined by the Alkaline Comet Assay. [The data indicates dose-dependent DNA damage index as compared to control]

Discussion

Lead (Pb) is perhaps the longest used and best recognized toxic environmental chemical. Heavy metals potency to cause genome damage is still a thrust area of research. The lymphocytes are highly suitable cell type for analysis of cytogenetic damage induced by several environmental or occupational agents due to their relatively long half-life[22,25]. The ability of lead acetate to induce DNA damage in normal human lymphocytes was studied using Single Cell Gel Electrophoresis assay (Comet Assay) and Cytokinesis Block Micronucleus (CBMN) assay at three different dosage levels.

Genotoxicity of lead has been investigated by CBMN assay in cultured human lymphocytes using the Cytokinesis-Block method[26] as it is a reliable and sensitive technique for quantifying and analyzing DNA damage induced by physical and chemical agents[27,28]. An increased frequency of Multinucleated cells was noted in our study which supports the findings of previous studies by Beek and Obe[29] and Obe *et al.*[30]. Frequency of Micronucleus (MNi) is a stable indicator of chronic exposure to lead and index of both chromosome breakage and chromosome loss. Clearly significant increase in MNi frequency was observed in the exposed groups as compared to control group indicating a possible genotoxic action of lead. Similar findings were noted by Vaglenov *et al.*[4] and Palus *et al.*[31] in support of our data. NPB is a measure of chromosome rearrangement which is probably due to dicentric chromosomes in which the two centromeres are pulled to opposite poles of cell and DNA in resulting bridge covered by nuclear membrane. N-Bud is a biomarker of elimination of amplified DNA and/or DNA repair complexes.

Lead acetate at our concentrations tested caused an increase of the fractions of comet with longer tail length, as compared to controls which is another biomarker of our investigation. Under our experimental conditions, we found a significant increase in the mean Tail length after exposure to lead ions. The main metrics of DNA damage showed mean values that were significantly higher than in the unexposed groups. These results suggested the possibility of an induction of DNA breakage and/or its alkali-labile sites due to the toxicant exposure. The DNA single- and double-strand breaks induced by lead ions could arise from DNA degradation connected with the cell death or from inactivation of some proteins, like repair enzymes[32]. DNA-DNA and DNA-protein cross links formed in the presence of Pb manifest chromatin condensation rendering higher Olive tail moments and thus higher DNA damage[32,33]. Our investigation revealed a good co-relation with dose dependent system. An increased levels of DNA damage observed in the present study group in

comparison to control justifies the genotoxic effects of Pb. Similar findings were observed by Palus *et al*.[31] who noted higher incidence of DNA fragmentation and increased incidence of Micronuclei in lead exposed groups compared to controls. The results were also supported by few *in vitro* and animal experiments using Comet assay[17,34].

DNA damage observed on exposure to Pb may be due to a direct effect of Pb on DNA structure and oxidative mechanisms. Indirect mechanism involved activation of caspases in process of cell death[15,35]. Evidences indicated that Pb ions can apparently take part in Fenton reaction to generate damaging oxygen radicals and can cause DNA damage[36]. Moreover, these ions might decrease the fidelity of DNA synthesis. Some indirect mechanisms also caused inhibition of DNA polymerase B by Pb induced reactive oxygen species (ROS), possibly indicating the failure of DNA repair mechanisms[37]. Snow[38] attributed the genotoxic effects after Pb exposure to several mechanisms, such as the induction of cellular immunity and oxidative stress, inhibition of DNA metabolism and repair and the formation of DNA and/or protein cross-links.

In conclusion, the present data with two markers employed, indicated a significantly higher genetic damage of lead in treated peripheral blood lymphocytes than controls. By comparing the results with opulence of data available, it is suggestive for molecular analysis to understand exact site of DNA damage by the toxicant and the need for preventive action that would improve condition of occupationally exposed cases at work place.

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