Research Paper

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Evaluation of Dna Damage Induced by Lead Acetate in Human Lymphocytes, Using Comet Assay

Zoology

KEYWORDS : Comet assay; DNA damage; Lead acetate; Lymphocyte cultures

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ABSTRACT

Lead (Pb) is present in the natural and occupational environment and it is one of the most clinically important heavy metals, because it induces a broad range of physiological, biochemical and genetical dysfunctions. Its exposure leads to an increased frequency of genetic aberrations in human beings. Hence, this investigation was conducted to assess the genotoxic effect of lead at three dosage levels. In the present study, the DNA damage was detected by adopting the alkaline single cell gel electrophoresis assay (Comet Assay) in peripheral blood lymphocyte cultures. The result 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 µg/ml 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.

Introduction

Lead is the 5th most abundant, globally well-distributed, dangerous and important environmental metal. It is ubiquitous, versatile, subtle and persistent toxic material encountered in day-to-day life and known to induce toxic effects in several biological systems. It is known to have deleterious effects on nervous 1-3, renal 4, immune 5, 6 and reproductive tissue 7. It has been tested and found capable of eliciting a positive response in tests for enzyme inhibition, fidelity of DNA synthesis, mutation, chromosome aberrations, cancer and birth defects 8. Hence, lead toxicity is a major issue in environmental health in recent years. The results of few *in vitro* and *in vivo* tests detecting the genotoxic effects of lead have been reported 9. The molecular mechanism of lead toxicity are not fully understood, but strong evidences indicate that it can act by competing with endogenous cations on protein binding sites and it can be substituted by calcium and zinc in a variety of proteins 10, 2 , 11. This substitution can promote further changes in these proteins and thus can alter cellular metabolism and induce aberrant gene transcription 12-14.

The genotoxic effects of lead have been studied in a variety of systems 15, 16, 8 and have been found to be contradictory in both *in vivo* and *in vitro* systems. Genotoxic studies, which have examined the genetic effect of occupational lead exposure in humans, are limited and controversial 17-22, 9, 23. Hence, the focal aim of present study was to investigate DNA–damaging potential of lead acetate in normal human peripheral blood lymphocytes using the alkaline single cell gel electrophoresis (SCGE) assay.

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).

Single Cell Gel Electrophoresis (Comet Assay)

The comet assay was performed according to the procedure of Singh et al. 24 with slight modifications. 4.5 ml RPMI-1640 Medium (HiMedia, Mumbai) pre-supplemented with Fetal Bovine Serum (HiMedia, Mumbai) and antibiotics was taken in sterile culture tubes. Whole blood (0.5 ml) and Phytohemagglutinin M (Sigma Aldrich, U.S.A) with final concentration of 10mg/ml was added in each culture tubes. The cells were exposed to different concentrations of lead acetate except control and positive controls. MMC (Mitomycin C) was added as positive control. Lead acetate was added in different concentrations of 10 µg/ml, 25 µg/ml, and 50 µg/ml. Each group had five samples. The cultures were carried out at 37ºC for 72hrs in BOD incubator. Harvesting was done after 72hrs and wash was given of fresh serum free media. Pellet obtained by the centrifugation was used for the assay. Cultured lymphocytes (10 µl) were suspended in 0.5% low melting agarose (HiMedia, Mumbai) and was 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 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, 100mM Na2EDTA, 10mM tris buffer, 1% triton X-100 and 10% DMSO) at 4ºC in dark. After 1 hour, the slides were placed in the electrophoresis buffer (300mM NaOH, 1mM Na2EDTA, 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 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 using 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 quantitized by tail length, tail DNA%, tail moment (TM) and olive tail moment (OTM) in arbitrary units respectively, length of head and length of tail of comet in pixels and % of DNA in comet's head and in the tail.

OTM = [% of DNA in tail] × [Distance between center of gravity of DNA in tail and that of center 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 25. Because our measurement system was not caliberated, tail moment and olive tail moment 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 ac-

cording to Anderson et al. 26.

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 cells25. 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. 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 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. In the present study, four parameters characterizing DNA strand breaks were evaluated: head DNA%, tail length, olive tail moment and tail DNA%. 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 (Figure 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% were significantly ($p<0.001$) increased in treated groups as compared to controls (Figures 2-4). 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 5. A clear significant increase in DNA migration was found in the treated groups comparatively. Among the study groups, significantly more cells of 1, 2, 3 types were observed. The presence of these cells demonstrated greater DNA damage than control groups (Figure 6). 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*.

Discussion

Lead has been used since pre-historic times and is one of the major hazards for human health due to its wide distribution in the environment. It is considered to be a toxic agent with serious health implications to human 27. 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 24 ,28, 29. In the present work, we studied the ability of lead acetate to induce DNA damage in normal human lymphocytes using single cell gel electrophoresis assay (Comet Assay). Lead acetate at all concentrations tested caused an increase of the fractions of comet with longer tail length, as compared to controls. Under our experimental conditions, we found a significant increase in the mean tail length after exposure to lead acetate. The main metrics of DNA damage showed mean values that were significantly higher than in the unexposed groups. These results suggested the possibility of 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 acetate could arise from DNA degradation connected with the cell death or from inactivation of some proteins, like, repair enzymes 30.

Our data further suggested 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 lead. At higher concentrations of metal, deletions were predominant types of mutation. Probably lead-induced gene mutations may not be the result of direct damage to DNA, but rather, may occur by indirect mechanism such as inhibition of DNA repair15, 31. Similar findings were observed by Palus *et al*. 32 who noted that the incidence of lymphocytes with DNA fragmentation in lead exposed groups were significantly increased as compared to the controls. The results were also supported by few *in vitro* and animal experiments using comet assay 33, 34, 30. These findings suggest that lead exposure reduces the cellular antioxidant reserves, and this shift in GSH-depleted lymphocytes produces a condition known as oxidative stress that may be a cause of DNA damage displayed by alkaline comet assay. This study also supported the findings of others 23, 35, 36.

In conclusion, the present data permit an estimate of genetic risk of lead exposure by employing one of the biomarkers of exposure using comet assay. Further lead induced DNA damage is a consequence of complex biological events that follow one another. By comparing the results with opulence of data available, it is suggestive for molecular analysis to understand exact site of DNA damage.

Acknowledgement

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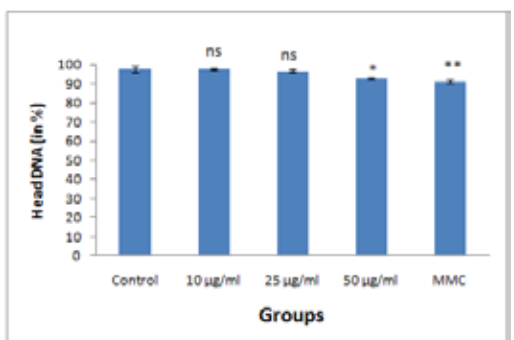

Figure 1. Percentage head DNA of human lymphocytes incubated for 72 hrs at 37°C with lead acetate and analyzed by the comet assay in the alkaline version. The number of cells scored in each treatment was 50. The figure shows mean results from five independent experiments. Values are Mean ± S.E.M. *P<0.05, **P< 0.01, ns = non significant.

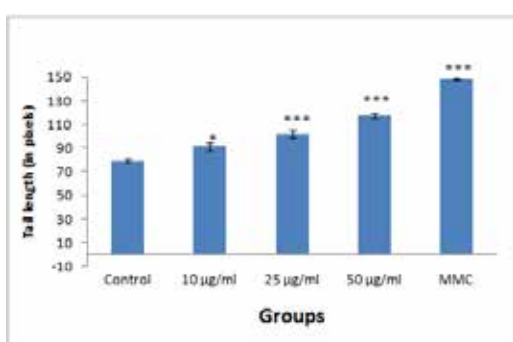

Figure 2. Comet tail length of human lymphocytes incubated for 72 hrs at 37°C with lead acetate and analysed by the comet assay in the alkaline version. The number of cells scored in each treatment was 50. The figure shows mean results from five independent experiments. Values are Mean ± S.E.M. *P<0.05, **P< 0.01, ***P<0.001.

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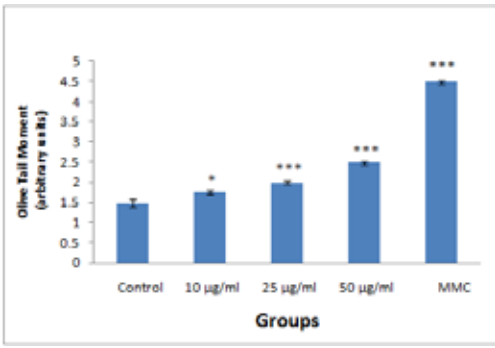


Figure 3. Olive Tail Moment of human lymphocytes incubated for 72 hrs at 37°C with lead acetate and analyzed by the comet assay in the alkaline version. The number of cells scored in each treatment was 50. The figure shows mean results from five independent experiments. Values are Mean ± S.E.M. *P<0.05, **P< 0.01, ***P<0.001.

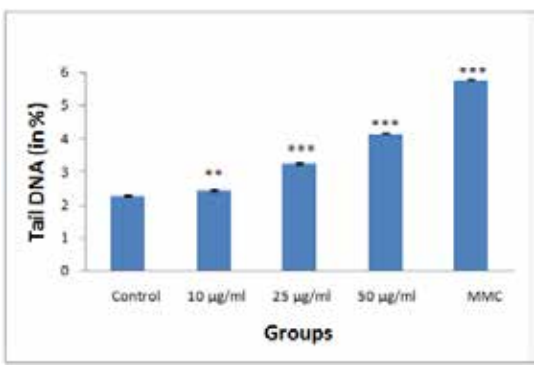
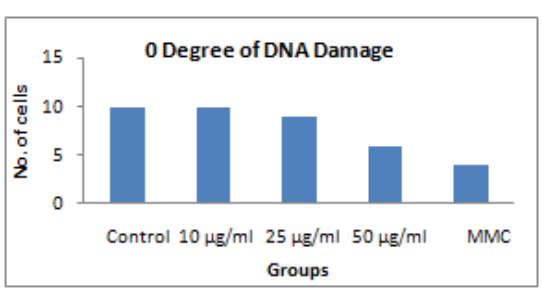
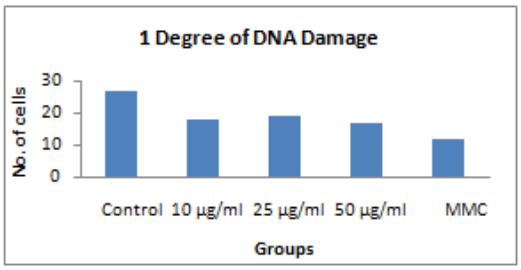


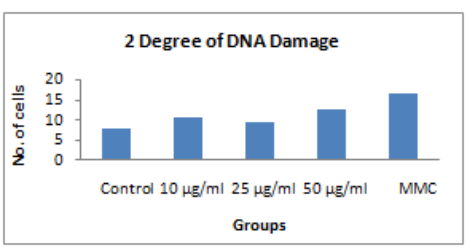
Figure 4. Percentage Tail DNA of human lymphocytes incubated for 72 hrs at 37°C with lead acetate and analyzed by the comet assay in the alkaline version. The number of cells scored in each treatment was 50. The figure shows mean results from five independent experiments. Values are Mean ± S.E.M. *P<0.05, **P< 0.01, ***P<0.001.



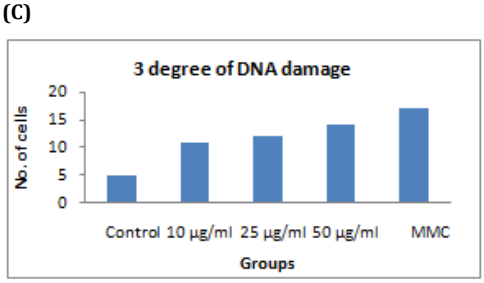
(A)

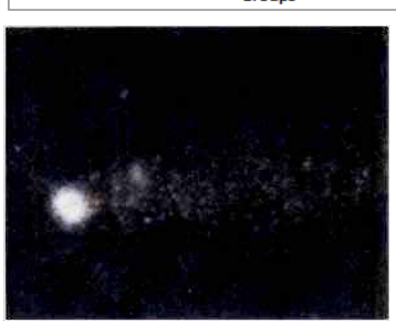


(B)



(C)





(D)

Figure 5. Histograms with figures (A to D) of the distribu-

tion of comet tail length in different classes in ethidium bromide stained human lymphocytes incubated for 72 hrs at 37oC with lead acetate at 10µg/ml, 25µg/ml 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.

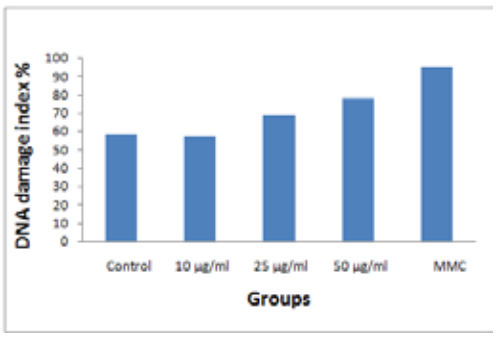


Figure 6. Characterizing the calculated DNA damage index. The data indicates dose-dependent DNA damage index as compared to controls.

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