ORIGINAL ARTICLE

Lead-Induced Bioenergetics Disruption and Oxidative Stress in the Cultured Human Retinal Pigment Epithelial Cells

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ABSTRACT

Aim: To study bioenergetics disruption and oxidative stress as underlying mechanisms for lead induced cytotoxicity in human retinal pigmented epithelial (RPE) cells.
Methods: The cytotoxic effect of lead nitrate (Pb) on the human REP cells was evaluated by MTT assay using concentrations range 0.1-100µM in time points from 3-48 hours. The effect of Pb on ATP production, complexes I and III activities, Oxygen consumption rate and lactate production by the cells were evaluated using MTT estimated IC50. For oxidative stress studies, the effect of PB was on the treated cells' reactive oxygen species (ROS)production, catalase (CAT), superoxide dismutase (SOD), reduced glutathione, and lipid peroxidation was evaluated.
Results: Lead was found to be cytotoxic to the cultured HRPE cells with decreased ATP production in a concentration and exposure duration dependent patterns. Lead was found to significantly inhibits the activities of complexes I and III to about 65% and 80% of the control samples activities, respectively (P=0.0002 and 0.0024, respectively). Also, Lead was found to induce significant decrease in the treated cells OCR to about 72 % of the control OCR levels (P=0.0049) with a significant increase lactate production (p=0,004).Pb was found to significantly increase ROS production (p=0.006) with significant decrease in both CAT and SOD activities and intracellular reduced glutathione stores (p=0.0005, 0.0024, 0.003 respectively) and increased lipid peroxidation in (P=0.0052).
Conclusion: lead is cytotoxic to the retinal pigmented epithelial cells. Bioenergetics disruption and oxidative stress may play a major role in Pb induced retinal complications.
Key words: Lead nitrate, Heavy metals, retinal toxicity, cytotoxicity.

INTRODUCTION

Lead and its compounds are widely used in industrial products including gasoline, paint, craft supplies, and plumbing materials resulting in broad exposures and distribution of its hazardous effects[1]. Lead can be absorbed via ingestion, inhalation or even dermal exposure[2]. Researchers have identified a number of adverse health effects of lead including neurotoxic, hematotoxic and nephrotoxic effects[3]. Lead induced neurotoxicity can be represented by cognitive dysfunction with reduced intelligence quotient (IQ) in children aged 3 and 5 years with long-term exposure to low concentrations of lead (even less than 10 µg/dL)[4]. Also, antisocial behavior was reported among children with history of prenatal exposure to lead[5].

Some studies have shown that lead can be accumulated in the human eye with the highest concentration reported in the retinal pigment epithelial cells and choroid[6]. This may cause hazardous effect on vision pathway. In addition to deterious effect on the Fundus coexisting diseases visual fields defects [7].Exposure to Pb was found to induce electrophysiological and biochemical changes in both in-vivo and in-vitro models with effect on both rods and cones dependent electro-retinal waves in a concentration and exposure duration dependent patterns[8].

The retina is the innermost, light-sensitive layer, or "coat", of shell tissue of the eye . The optics of the eye create a focused two-dimensional image of the visual world on the retina, which translates that image into electrical neural impulses to the brain to create visual perception, the retina serving much the same function as film or a CCD in a camera[9]. Very low concentrations of lead were reported to cause serious effects on the retina due to its higher susceptibility to oxidative stress [10-12] and accumulation of higher levels of lead in retinal pigment epithelium (RPE) and choroid [13]. However, relationship between lead and age related maculopathy is controversial. U.S. National Health and Nutrition Examination Survey (NHANES), 2005–2008, reported no association between blood lead levels and age related macular degeneration (AMD) [14]. However, the Korean NHANES, 2008–2011, showed a significant association between lead levels and AMD [15].
Lead induced toxicities were explained via different mechanisms. However, the underling mechanisms of lead retinal toxicity is still poorly understood. This study will evaluate the cytotoxic effect of lead on cultured human retinal cells. Also the effect of lead on mitochondrial functions and redox status of the retinal pigmented epithelial cells will be evaluated.

MATERIALS AND METHODS

Cell culture condition: Human retinal pigmented epithelial HRPE cells were obtained in passage 3 and maintained in 1:1 mixture of Dulbecco's modified Eagles medium and Ham's F12 medium with HEPES buffer containing 20% fetal bovine serum, 56 mM final concentration sodium bicarbonate and 2 mM L-glutamine.

Cytotoxicity assessment using MTT assays: HRPE cells were seeded in 96 well plates (2x104 cells/well). At 90% confluence, cells were treated with Pb nitrates at concentrations of (0.1, 1, 10, 100, and 1000 µM) for 4, 24, 48, or 72 hours. The effect of lead on then cell viability was evaluated by its effect on the reduction of MTT reagent. The assay was done following Elmorsy et al. (2014)[16]. The absorbance for reduced MTT substrate was quantified spectrophotometrically at 590 nm. The experiment was repeated trice with 3 wells for each treatment in each experiment. Viability was expressed as a percent of control assuming that the viability of the control is 100%.

Measurements of intracellular ATP levels: Cells were seeded and treated as for MTT assay then the levels of intracellular ATP were then measured by luminescence using kit (Abcam, Cambridge, UK) according to a kit protocol.The experiment was repeated trice with 3 wells for each treatment in each experiment. ATP content was expressed as a percent of control assuming that ATP content of the control is 100%.

Measurements of mitochondrial complex activities: The cells were seeded in 25 T cell flaskand treated with Pb in its estimated IC50s. A mitochondrial enriched fraction was isolated for complex I assay,while cell lysate was prepared for complex III assay according to (Spinazzi et al., 2012)[17]. Then Complex I activity was measured with Dichloroindophenol (DCIP) was used as the terminal electron acceptor following Janssen et al. (2007)[18], while the activities of Complex III were evaluated following (Spinazzi et al., 2012)[17].

Measurements of lactate production: Cells were seeded (5x104 cells per well) in 24-well plates and treated with Pb (55 µM). 24 hours' post-treatment, Cells were trypsinized and counted while the media were collected and its lacatae content was measured using lactate assay kit (Biovision, Mountain View, California, USA) according to the manufacturer's instructions. Finally, Lactate production was normalized to number of cells for each well. Lactate content was expressed as a percentage of control lactate production assuming that control lactate production was 100%.

Measurements of oxygen consumption rate: The effect of Pb nitrate in IC50s on OCR of the HRPE cells was evaluated polarographically using Clark oxygen electrodes (Rank Brothers, Bottisham, UK) followingElmorsy and Smith (2015)[19].Cells were incubated with Pb (55 µM) for 24 hours. Then the cells were harvested, centrifuged and re-suspended in Hank's solution and counted. The OCR was then assessed in the chambers for the treated and control cells for 10 minutes before addition of 2 µl of 6 mM azide to each chamber. The OCR was measured as the change in PO_2 level under the basal condition over a five minutes' period. While the azide effectwas measured after one minute of its addition.

Measurements of reactive oxygen species (ROS): 2,7-dichlorodihydrofluorescein diacetate (DCFDA) assay was used to evaluate the effect of lead (45 µM) on ROS production by HRPE cells following Elmorsy *et al. (*2014)[16]. Antimycin A (10 mM for 30 minutes) was used as positive controls. ROS production was expressed as a percentage of control ROS production assuming that control ROS production was 100%. Experiments were conducted in triplicates with 3 wells for each treatment in each experiment.

Measurements of oxidative stress markers: Cells were treated with PB for 24 hours then Catalase (CAT) and Superoxide dismutase (SOD) were assessed following calorimetricallyas described by Sinha *et al. (*1972)[20]and Beauchamp and Fridovich, 1971[21]. SOD based on its inhibition to reduction of nitroblue tetrazolium. Units of SOD activity were expressed in terms of mg of total protein. While reduced glutathione (GSH) was determined as described by Ullah *et al. (*2011)[22]spectrophotometrically at 412 nm using a plate reader 'TopCount' (Perkin Elmer, Ueberlingen, Germany). The activity of GSH was expressed as nM reduced GSH/g tissue. Finally, Thiobarbituric acid reactive substances (TBARS) were measured following Alam *et al. (*2013)[23] as markers of lipid peroxidation.

Statistical analysis: All statistical analyses were performed using PRISM 5 (GraphPad Software Inc., San Diego, California, USA. Unpaired student t-test was used to compare the raw data sets of the treated and control cells after subtraction of blanks. Wilcoxon test was used to compare OCR of the controls and treated cells samples. p-value <0.05 was considered as statistically significant.

RESULTS

Lead was found to be cytotoxic to the cultured HRPE cells in a concentration and exposure duration dependent patterns. (Figure 1) without significant different between 24 and 48 hours for all tested concentration. Lead found to be significantly cytotoxic to the retinal cells at 10 µM, 3 hours' post-treatment. Even at 0.1 µM, Pb was significantly cytotoxic to osteoblasts 24 hours' post-treatment ($p = 0.021$).

The cellular bioenergetics assays showed that lead reduced the intracellular production of ATP in the treated HRPE cells in a concentration and exposure duration dependent patterns. Lead found to be significantly reduce the retinal cells ATP content at 1 µM, 12 hours' post-treatment (P=0,002). Since the production of ATP is governed by oxidative phosphorylation through the mitochondrial omplex I and complex III. Hence the effect of lead on both complexes was evaluated. Lead was found to significantly inhibits the activities of complexes I and III to about 65% and 80% of the control samples activities, respectively (P=0.0002 and 0.0024, respectively). Cellular bioenergetics were also assessed using OCR. Lead was found to induce significant decrease in the treated cells OCR to about 72 % of the control OCR levels (P=0.0049). In parallel Pb was found to significantly increase the treated cells lactate production (p=0,004)

Regarding oxidative stress studies, Pb was found to significantly increase ROS production in the treated cells (p=0.006) with significant decrease in both CAT and SOD activities and intracellular reduced glutathione stores (p=0.0005, 0.0024, 0.003 respectively). Also Pb was found to induced significant lipid peroxidation in the treated retinal cells in comparison with the controls (P=0.0052).

Figure 1: The effect of lead nitrate on the human retinal pigment epithelial cells viability (MTT assay) and ATP production in a concentrations range (0.1-100µM) in time points 3, 6, 12, 24 and 48 hours. Data were represented as means ± standard deviations.

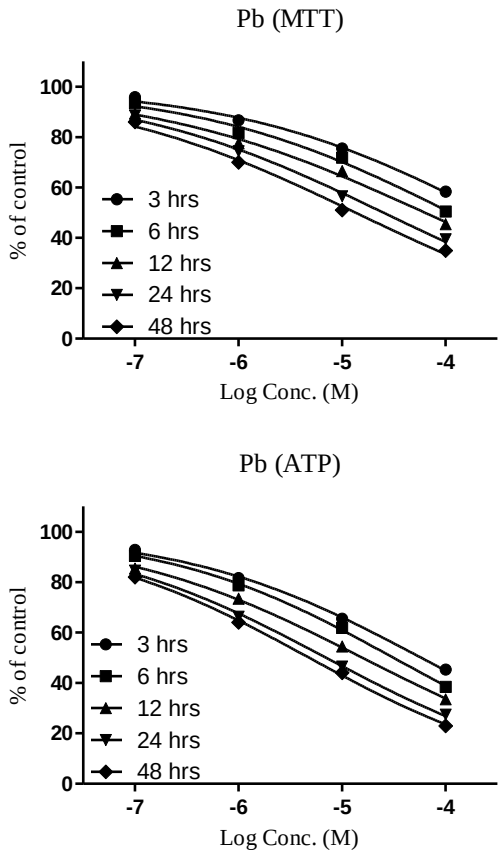


Figure 2: The effect of lead nitrate on the human retinal pigment epithelial bioenergetics via its effect on mitochondrial complexes I and III and its effect on oxygen consumption rate and lactate production. Data were represented as means ± standard deviations. ** means p-value<0.01 and *** means p-value <0.001.

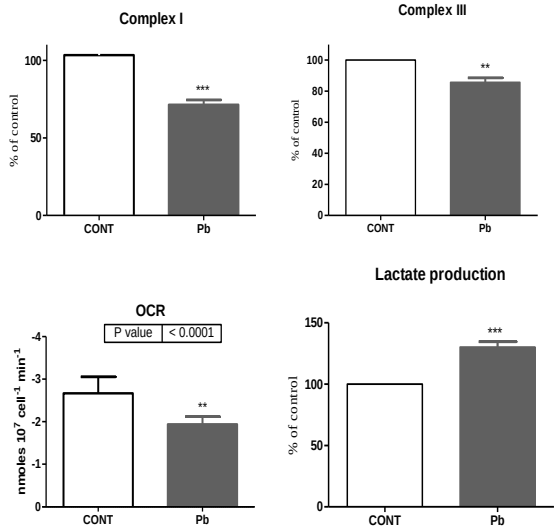
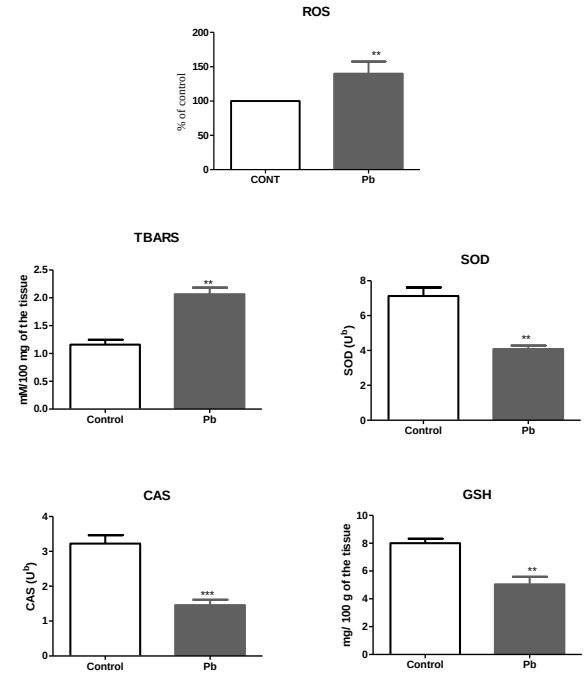


Figure 3: The effect of lead nitrate on the human retinal pigment epithelial reactive oxygen spices production (ROS), Lipid peroxidation (TBARS), super oxide dismutase activity (SOD), Catalase enzyme activity (CAT), and intracellular reduced glutathione content (GSH). Data were represented as means ± standard deviations. ** means p-value<0.01 and *** means p-value <0.001.



DISCUSSION

The current study was conducted to evaluate the effect of lead on the retinal pigmented cells bioenergetics and redox state as underlying mechanisms of lead induced retinal effects. Lead was found to be cytotoxic to the cultured HRPE cells in a concentration and exposure duration dependent patterns. The cellular bioenergetics assays showed that lead reduced the intracellular production of ATP in the treated HRPE cells with significant inhibition for mitochondrial complexes I and III and OCR and increased lactate production. PB-induced bioenergetics disruption was shown to be associated with increased ROS production and decrease in both CAT and SOD activities and intracellular reduced glutathione stores. Also Pb was found to induced significant lipid peroxidation in the treated retinal cells in comparison with the controls.

Human RPE cells were used as a model of the study to avoid the effect of species difference. Pb toxicity was evaluated using a wide range of concentrations as there is no definite safe levels of lead exposure with initial clinical symptomology that includes kidney dysfunction at a blood lead level (BLL) of 5-10 µg/dL (0.24-0.48 µM). A spectrum of health impact is observed above 11 µg/dl, with cognitive impairment at 40-79 µg/ dl, neuropathy at >80 µg/ dl (≈4 µM), and encephalopathy at 100-120 µg/dl (≈6 µM) (Sachdeva *et al*., 2018)[24]. Since levels of human intoxication from lead exposure are correlated with BLL, we examined a broad concentration range that included symptomatic BLL concentrations (0.1 µM -1mM), as well as gross intoxication (>10 µM). Although BLL concentrations of >10 µM would be considered overdose levels, exposure of cells *in vitro* at

relatively high concentrations is used to model more chronic and cumulative exposure within a limited time frame of study. Furthermore, HMs including lead are non-degradable, and have a slow rate of elimination (blood lead has a half-life of about 40 days in humans, cadmium an elimination half-life of 20-30 years); they therefore can progressively accumulate in tissues such as bone that sequesters HMs[2].

Lead was found to be cytotoxic to the cultured HRPE cells in a concentration and exposure duration dependent patterns. This is in accordance with the previously published data. Pb cytotoxic to HRPE cells in a concentrations and exposure durations dependent manner, as assessed using a MTT assay. This is in accordance with the previously published dat. Lead acetate was reported to be cytotoxic to primary cultured rat astrocytes in concentration 50 and 100 µg/ml after 96 hrs of exposure, while lead acetate was found to be cytotoxic to schwann cells after 24hrs of exposure to concentrations 1, 5 and 10 µg/ml [25]. Also lead nitrate was reported to be cytotoxic to Human Leukemia (HL-60) Cells at concentration of 10, 20, 30, 40, and 50µg/mL with estimated IC50 as 34.6±5.2 µg/mL upon 24 hrs postexposure[26]. Moreover, Lead was reported to be cytotoxic to rat pheochromocytoma (PC12) cells in a dose dependant manner after 24hrs postexposure[27].

Our study demonstrated that lead when applied to cells at 45 µM damaged osteoblast bioenergetics. This is in line with the published data. Lead induced morphological changes in mitochondrial was revealed by (Watrach, 1964)[28] who reported that mitochondria of swine liver which had given lead acetate contaminated food for 3–6 months were elongated with atrophic cristae arrays of lamellar formations in their matrix. Moreover, Lead effect on mitochondrial was explained as it opens the transition pore, resulting in cytochrome c release, which leads to caspase activation, and apoptosis. Oxidative stress was suggested to open these transition pores in Pb induced apoptosis (Liu et al., 2012)[29].

The ability of toxic agents to induce damage to mitochondria and nullify ATP production is a common mechanism of cellular damage, and contributes to cellular redox stress and induction of apoptosis[19]. Hence oxidative stress can be supposed as amechanism of Pb-induced retinal toxicity. The ability of Pb to induce oxidative stress was reported in previous studies with preventive and curative effect of the antioxidants on lead toxicities [30].

CONCLUSION

Leadis cytotoxic to human RPE cells in a concentration and exposure duration dependent manner. Pb-induced bioenergetics disruption and oxidative stress were shown to have a major role in Pb- induced retinal toxicities, which can explain the role of both mechanisms in Pb-induced retinal pathologies and electrophysiological changes

Conflict of interest: The authors declare no conflict of interest regarding the published results.

Acknowledgement: The authors are grateful to the scientific research deanship of the Northern Border University, Saudi Arabia for funding this project

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