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Vol. 7(29), pp. 2033-2042, 8 August, 2013
DOI 10.5897/AJPP12.805
ISSN 1996-0816 © 2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

A change in metabolic status of glutathione (γ-L-glutamyl-2-cysteinyl-glycine) in lymphocytes by lead compounds

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Accepted 24 June, 2013

A spectrophotometric investigation of the interaction and coordination of lead compounds with glutathione (GSH) in lymphocytes of healthy male volunteer venous blood has been described. The present study was designed to provide insight into the proposed mechanism of action of lead acetate {$Pb(CH_3COO)_2$} and lead acetylacetonate ($C_{10}H_{14}O_4Pb$) with GSH at molecular level. Lymphocytes were separated from blood of healthy human volunteers. Different concentrations of lead acetate and lead acetylacetonate were used and their concentration, time, pH and temperature dependent effects on GSH level was studied. Ellman's method was used for the determination of GSH. With lead compounds, a decreased concentration of GSH was found, which further decreased with increasing concentration of lead compounds. A more decrease in GSH concentration was found with time-dependent (0-90 minutes) incubation of lymphocytes with lead compounds. pH, 8.5 and temperature, 45°C have been found to be most favorable for reactions of lead compound with GSH. It may be suggested that changes in GSH status produced by lead compounds could be due to the formation of conjugates (Pb-SG) between GSH and lead compounds or conversion of GSH to oxidized GSH (GSSG) in lymphocytes. The interaction and coordination of lead compounds with sulphur suggest the sulfhydryl (-SH) group to be the coordinating site of GSH.

Key words: Glutathione (GSH), 5-5- dithiobis 2-nitrobenzoic acid (DTNB, Ellman's reagent), metapfosphoric acid (MPA), lead acetate, lead acetylacetonate (LAA), lymphocytes.

INTRODUCTION

Heavy metal(s) are widespread pollutants of great concern as they are non-degradable and thus persistent (Zouboulis et al., 2004). Lead is a naturally occurring heavy metal found in the earth's crust in trace quantities of approximately 8 to 13 ppm (Rudnick and Fountain, 1995; Taylor and McLennan, 1995). Anthropogenic activities leading to increased air Pb levels include primary and secondary lead smelting, the burning of gasoline containing lead, anti-knocking agents, coal combustion, storage battery manufacture and pigment production (NRCC, 1973). The utility of lead may be derived from its low melting point (327°C), high density (11.4 g/cm^3), malleability, chemical stability and resistance to acid corrosion (Howe, 1981).

Lead occurs in four valence states: elemental (Pbo), monovalent (Pb^+), divalent (Pb^{2+}), and tetravalent ($Pb4^+$); all forms are environmentally important, except possibly Pb^+. In nature, lead occurs mainly as $Pb2^+$; it is oxidized

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to $Pb4^{+}$ only under strong oxidizing conditions (Demayo et al., 1982; Harrison and Laxen, 1982). Lead is a ubiquitous environmental contaminant; nearly 5% of American children are affected by lead poisoning (a blood lead level (BLL) of 10 mgdL_1 or higher) (Lanphear, 1998). Measurements of blood lead reflect exposure to all lead compounds (inorganic and organic) and all routes of exposure (inhalation, oral, and dermal) (Rabinowitz et al., 1976).

Thiol groups are found in all body cells and are indispensable for life (Atmaca, 2004; Moriart and Jones, 2004). Thiols have been of continuing interest for many years because of their important role in several biological processes (Martindale and Reynolds, 1989). It is of clinical, biological, and pharmaceutical importance to determine these compounds in biological fluids and tissues. Thiols are those compounds which contain the sulfhydryl group (- SH) attached to a carbon atom (Chung et al., 2005; Pogocki and Schöneich, 2001). The tripeptide glutathione is naturally occurring low molecular weight non-protein thiol, which is endogenously synthesized in the body and every cell is responsible for its own supply of glutathione. It possesses very important biochemical and antioxidant properties against the oxidative stress due to the presence of sulfhydryl (SH) group (Pastore et al., 2003). Reduced glutathione (GSH) is the most abundant intracellular low-molecular-weight thiol and plays an essential role in protecting cells from toxic species, such as reactive oxygen intermediates and reactive electrophiles (Russell and Rabenstein, 1996). It has been found that decreased concentration of GSH has been associated with aging (Samiec et al., 1998), and the pathogenesis of many diseases like acquired immuno-deficiency syndrome (AIDS) (Pirmohamed et al., 1996), Alzheimer disease (Cecchi et al., 1999), alcoholic liver diseases (Altomare et al., 1988) and pulmonary diseases (Andersson et al., 2001). The ratio of oxidized to reduced GSH has been shown to be an effective measure of the redox state (Schafer and Buettner, 2001). The redox state of a cell is kept within a narrow range under normal conditions, similar to the manner in which a biological system regulates its pH (Droge, 2002). The glutathione (2GSH/GSSG couple) represents the major cellular redox buffer and therefore is a representative indicator for the redox environment of the cell (Schafer and Buettner, 2003). Glutathione couple GSSG)/GSH dominates when describing the global state of reduced and oxidized species in the cell. A 30 mV change in the redox state means a 10-fold change in the ratio between reductant and oxidant species. The intracellular “redox homeo-stasis” or “redox buffering” capacity is substantiated primarily by GSH (Schafer and Buettner, 2003).

Oxidative stress has recently been reported as one of the important mechanism of toxic effect of lead (Saxena and Flora, 2004). Lead-induced oxidative stress contri-butes to the pathogenesis of lead poisoning for disrupting the delicate prooxidant/antioxidant balance that exists within mammalian cells. Production of reactive oxygen species (ROS) is increased after lead treatment in *in vitro* studies, moreover other studies *in vivo* suggest that lead exposure cause generation of ROS and alteration of antioxidants defense system in animals (Hsu and Guo, 2002). One of the effects of lead exposure is on glutathione metabolism. Lead-induced depletion of intracellular GSH and increased levels of malondial-dehyde in brain and liver have been demonstrated in animal models (Gong and Evans, 1997). Most experimental research has indicated that after exposure of animals to lead there is an increased amount of oxidized glutathione (GSSG) with a parallel drop of GSH in different organs (Gurer et al., 1999; Sivaprasad et al., 2003). Lead (Pb) may alter the structure of the cell membranes by stimulating the lipid peroxidation process with consequent complex sequences of biochemical reactions (Viarengo, 1989). Droge et al. (1994) have found that GSH deficiency of T-cells is associated with a suppression of NF-kB function. Such GSH deficiency-dependent NF-kB response is observed in certain NF-kB activation systems. For example, suppression of hydrogen peroxide-dependent NF-kB activation has been observed consistently in GSH deficient cells (Droge et al., 1994). Due to the therapeutic applications of glutathione, and its emerging role as bio-markers of pathological condition, there is need to fully investigate the dynamic association between glutathione and toxic metal like lead in the body. Thus, defining the chemistry of glutathione with Pb(II) is important for understanding the toxicity of this element. The present study was undertaken with the objective to examine the effect of two lead compounds (Figure 1) on the metabolic status of GSH in lymphocytes of blood.

MATERIALS AND METHODS

L. Glutathione (GSH) (Fluka), Ellman’s reagent that is, 5-5, dithiobis 2-nitrobenzoic acid (DTNB), (Sigma), sodium hydroxide (Fluka), sodium chloride (Merck), potassium dihydrogen phosphate (Merck), HCl 35% (Kolchlight), lead acetate (Across, Belgium) lead acetyl acetonate (LAA) (Sigma), RPMI-1640 (Sigma), fetal calf serum (Sigma), Ficol paque plus (Sigma), metapfosphoric acid (MPA) (Sigma-Aldrich), distilled water (Double Refined), chloroform (Merck), ethanol (Merck), Spectrophotometer: UV. Visible, 1601 (Schimadzu, Japan), PH Meter: Model NOV-210 (Nova Scientific Company Ltd. Korea), analytical balance AX 200 (Schimadzu, Japan), Centrifuge H-200 (Kokusan Ensink Company Japan), Vortex mixer (Shalimar scientific), Eppendolf’s tubes (Plastic, 101) Oven: Memmert Model U-30,854 (Schwa Bach, Germany), Magnetic Stirrer, Nylon wool, BD Syringe, Gilson and Pastuer pipettes were all used in the current study.

Preparation of stock solutions

To make 0.9% NaCl solution, 90 mg of pharmaceutical grade NaCl was added to 100 ml of distilled water. Lead acetate, 150 µM solution contained 2.84 mg of lead acetate in 50 ml of deionized water, which was again gradually diluted to get 5 different concentrations (30, 60, 90, and 120 µM) of lead acetate. LAA, 150 µM solution contained 3.04 mg of LAA in 50 ml of deionized water,

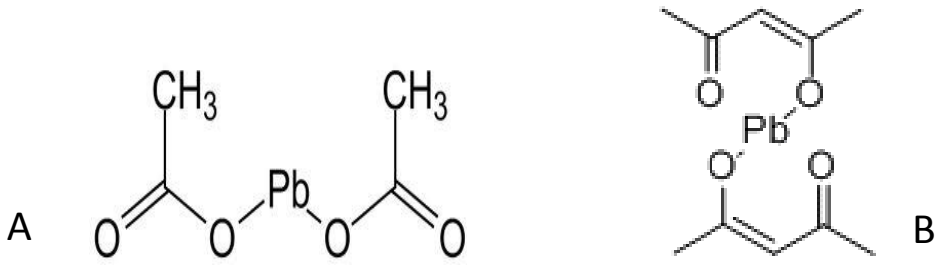

Figure 1. A, Lead acetate; B, lead(II) acetylacetonate (LAA).

which was again gradually diluted to get 5 different concentrations (30, 60, 90, and 120 µM) of LAA. GSH (150 µM) solution was obtained by dissolving 2.304 mg of GSH in 50 ml of phosphate buffer (pH 7.8). 2.97 mg of DTNB, Ellman's reagent was added to phosphate buffer (pH 7.8) to get 50 ml of 150 µM solution of Ellman's reagent. 10% MPA reagent was prepared by dissolving 5 g of metapfosphoric acid in 50 ml deionized water). Balanced salt solution was prepared by mixing 4-parts of fetal calf serum with 45 parts of RPMI-1640. Ficol paque plus was used without further purification.

Isolation of lymphocytes

Fresh venous blood were collected from male human donors of age between 25-30 years. Volunteers were selected with written consent, no disease and no drug use in recent history. Anticoagulant-treated blood (10 ml) was diluted with an equal volume of balanced salt solution (RPMI-1640 plus 10% fetal calf serum) and layered on the Ficoll-Paque PLUS solution. This two-phase system was centrifuged at 400×g for 30-40 min. Differential migration during centrifugation resulted in the formation of layers containing plasma and different cell types. The bottom layer contained erythrocytes which sedimented completely through the Ficoll-Paque PLUS. Due to its lower density, the lymphocytes accumulated at the interface between the plasma and the Ficoll-Paque PLUS. The lymphocytes were then recovered from the interface and subjected to short washing steps with balanced salt solution to remove any Ficoll-Paque PLUS and plasma. Platelet contamination was effectively removed with the 20% sucrose. The lymphocytes sedimented in the form of clump through the sucrose gradient. The clump of lymphocytes was dissolved in 0.5-1 ml or phosphate buffer saline (PBS), pH 7.6) and the red blood cells were lysed, if any, in 0.5 ml of ice-cold high performance liquid chromatography (HPLC)-grade water. Centrifugation at 700 rpm for 5 min with PBS results in sedimentation of lymphocytes. For deproteinization, equal volume of MPA reagent was added to the sample and mix by vortexing in Vortex mixer (10 counts). The mixture was allowed to stand at room temperature for five min and centrifuged at 2000 rpm for 5 min or equivalent. The supernatant was carefully collected into clean sample tubes without disturbing the precipitate. The lymphocytes were then diluted with PBS and transferred from centrifuge tube for application on the same day.

Determination of GSH concentration

In the course of this study, each time, five different concentrations (30, 60, 90, 120 and 150µM) of lead acetate and LAA were used to investigate their reactions with lymphocytes-GSH concentration. Lead compounds were incubated with the lymphocytes solutions in equal volumes, and the un-bound GSH were determined by using Ellman's reagent. The sample mixtures were then processed according to the parameter under study. For maximum confidence on the results, all the experiments were run in triplicate. All the experiments were performed in a nitrogen atmosphere to avoid oxidation of GSH unless indicated otherwise.

Study of lymphocyte fractions incubated with different lead acetate and LAA concentrations was carried out for biochemical estimation of effective toxicity marker compound, GSH in lymphocytes with respect to most influential parameters that is, different concentrations of lead acetate and LAA (10, 20, 30, 40 and 50µM), time intervals (00, 30, 60 and 90 min), pH range (7.0, 7.8 and 8.5) and temperatures (30, 37 and 45°C). The reduced glutathione concentration in lymphocytes was determined by the method of Ellman (1959).

Samples of the isolated lymphocytes fractions were separately mixed with each of the 5 different concentrations of lead acetate and LAA in equal volumes. 6 ml from each of the mixtures (lymphocytes incubated with 5 different concentrations of lead acetate and LAA) were added to 3 ml of Ellman's reagent. The final concentrations of lead acetate and LAA (in each 5 samples of lymphocyte fractions) were 10, 20, 30, 40 and 50 µM. The absorbances were determined at 412 nm after incubating for 5 min. The concentration of GSH was obtained from the standard curve of GSH and expressed as µM. Standard curve of GSH was obtained with known GSH concentration, 200, 400, 600, 800 and 1000 µM using Ellman's method. Control for lymphocytes-GSH was prepared by mixing 3 ml of lymphocyte fraction with 3 ml of 0.9%-NaCl solution followed by the addition of Ellman's reagent without treating lead compounds.

RESULTS

Effect of lead acetate on the concentration of GSH in lymphocytes

GSH concentration was determined in isolated lymphocytes, after the addition of different concentrations of lead acetate, having the final concentrations of lead acetate (10, 20, 30, 40, and 50 µM). The spectrophotometric analysis showed significant decrease in lymphocytes-GSH concentration (42.75) with the initial lead acetate concentration that is, 10 µM compared to the lymphocytes-control (49.42). The lymphocytes-GSH concentration continuously decrease with increasing LA concentrations. The maximum used LA concentration (50 µM) brought drop in lymphocytes-GSH concentration up to (35.83) compared to the lymphocytes-control (49.42) as shown in Table 1 and Figure 2.

Solutions of lead acetate having final concentrations of lead acetate (10-50 µM) were incubated for different time

Table 1. Lead acetate in lymphocytes.

S/N	Time (min)		10 µM		20 µM	30 µM	40 µM	50 µM	Control
1	0	Conc.		42.75	40.92	39.17	37.50	35.83	49.42
		PH	7	44.17	41.92	40.33	38.42	37.08	49.42
			7.8	39.75	37.83	36.17	34.50	32.83	49.42
			8.5	37.50	35.67	33.92	32.25	30.58	49.42
		Temp	30°C	42.75	40.92	39.17	37.50	35.83	49.42
			37°C	43.75	41.67	40.33	38.42	37.00	49.42
			45°C	44.58	42.42	41.00	39.58	37.08	49.42
2	30		39.75		37.83	36.17	34.50	32.83	49.42
3	60		37.50		35.67	33.92	32.25	30.58	49.42
4	90		35.42		33.92	31.92	30.42	28.67	49.42

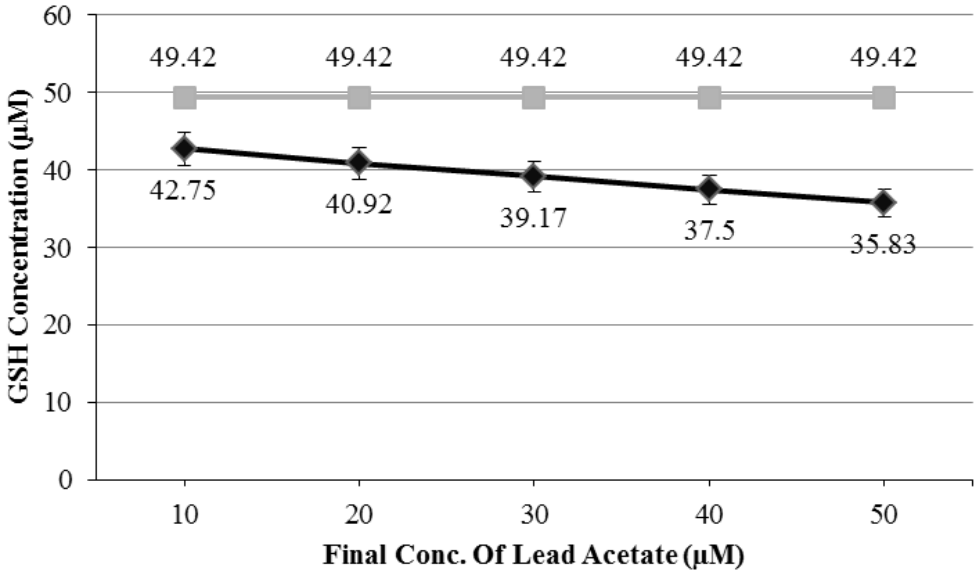

Figure 2. Effect of Lead acetate Concentration on lymphocytes-GSH Content. ■, Lymphocytes-GSH Control (1ml 0.9% NaCl+1ml of lymphocytes); ♦, lead acetate (10-50 µM). Results are the mean ±SE of three experiments.

intervals (00, 30, 60 and 90 min) with isolated lymphocytes. Lymphocytes-GSH concentration was further decreased, the maximum decrease shown by the prolonged (90 min) time of incubation is shown in Table 1 and Figure 3. In Figure 3, the results of only the lowest (10 µM) and highest (50 µM) of lead acetate is given. pH related data (pH,7.0, 7.8, 8.5) has also been collected for the effect of lead acetate (10-50 µM) on the lymphocytes-GSH level. The decline in GSH was maximum at pH 8.5, as shown in Table 1 and Figure 4. Results for the effect of temperature (30, 37, 45°C) on the reaction of lead acetate (10-50 µM) with lymphocytes-GSH are shown in Table 1 and Figure 5. In both Figures 4 and 5, the results of only the lowest (10 µM) and highest (50 µM) of lead acetate is given.

Effect of lead acetylacetonate (LAA) on the concentration of GSH in lymphocytes

Isolated lymphocytes were investigated for the change in lymphocytes-GSH concentration after the addition of different concentrations of LAA, having the final concentrations of LAA (10, 20, 30, 40, and 50 µM) in the cuvvette of UV-visible spectrophotometer. The spectrophotometric analysis showed significant decrease in lymphocytes-GSH concentration (37.58) with the initial LAA concentration that is, 10 µM compared to the lymphocytes-control (41.33). The lymphocytes-GSH concentration was continuously decreasing with increasing LAA concentrations. The maximum used LAA concentration (50 µM) brought drop in lymphocytes-GSH

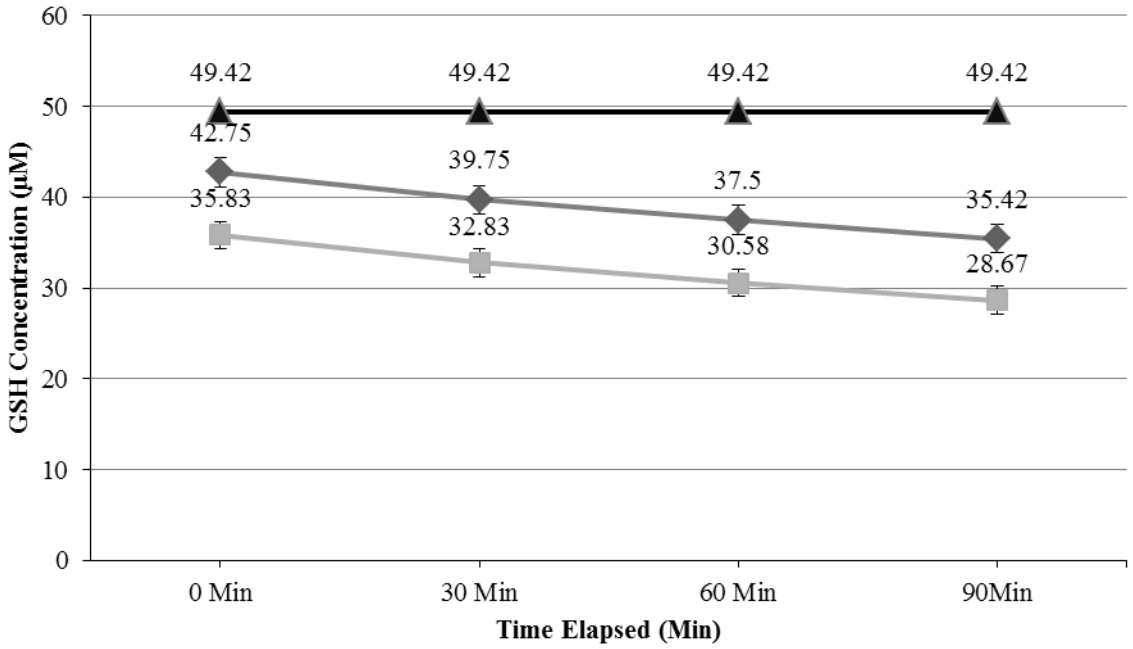

Figure 3. Effect of lead acetate concentration on the lymphocytes–GSH content with time incubation period (0-90 min). ▲, Lymphocytes-GSH control (1ml 0.9% NaCl+1ml of lymphocytes); ♦, lead acetate (10 µM); ■, lead acetate (50 µM). Results are the mean ±SE of three experiments.

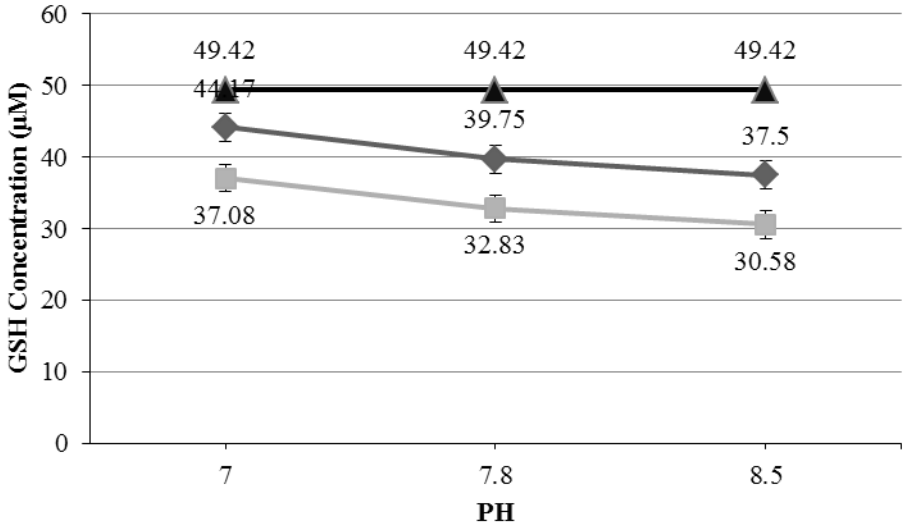

Figure 4. Effect of PH on the lymphocytes–GSH content due to lead acetate. ▲, Lymphocytes-GSH Control (1ml 0.9% NaCl+1ml of lymphocytes); ♦, lead acetate (10 µM); ■, Lead acetate (50 µM). Results are the mean ±SE of three experiments.

concentration up to (32.00) compared to the lymphocytes-control (41.33) as shown in Table 2 and Figure 6.

Different solutions of LAA (10-50 µM) were incubated for different time intervals (00, 30, 60 and 90 min) with isolated lymphocytes. Lymphocytes-GSH concentration was further decreased, the maximum decrease shown by the prolonged (90 min) time of incubation is shown in Table 2 and figure 7. In Figure 7, the results of only the lowest (10 µM) and highest (50 µM) of lead acetate is given. Results for the pH (7.0, 7.8, 8.5) related effect of LAA (10-50 µM) on the lymphocytes-GSH level showed the most decline in lymphocytes-GSH at pH, 8.5 as shown in the Table 2 and Figure 8. Results for the effect of temperature (30, 37, 45°C) on the reaction of LAA (10-50µM) with lymphocytes-GSH are shown in Table 2 and

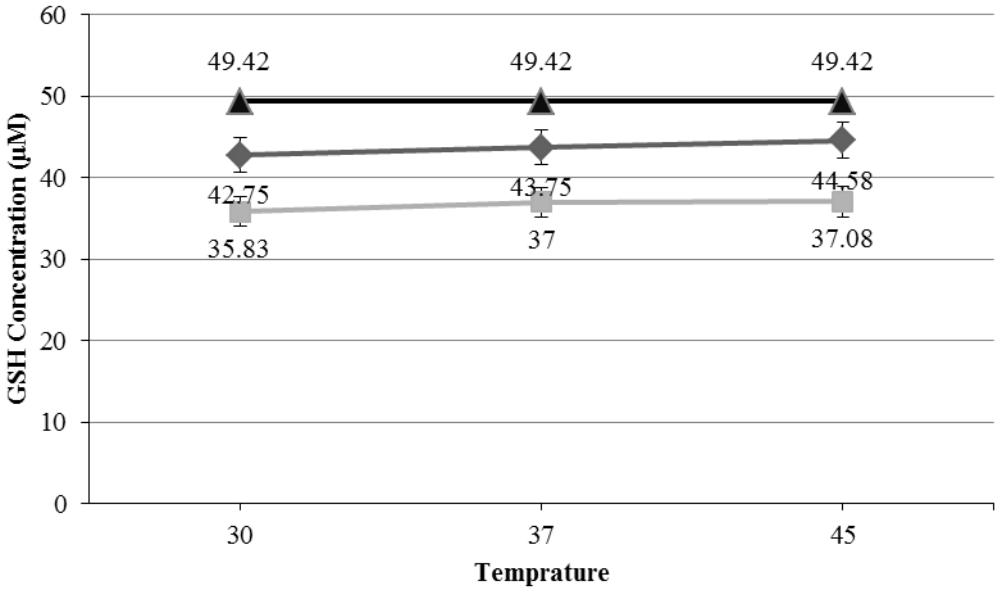

Figure 5. Effect of temperature on the lymphocytes–GSH content due to lead acetate. ▲, Lymphocytes-GSH control (1ml 0.9% NaCl+1ml of lymphocytes); ♦, lead acetate (10 µM); ■, Lead acetate (50 µM). Results are the mean ±SE of three experiments.

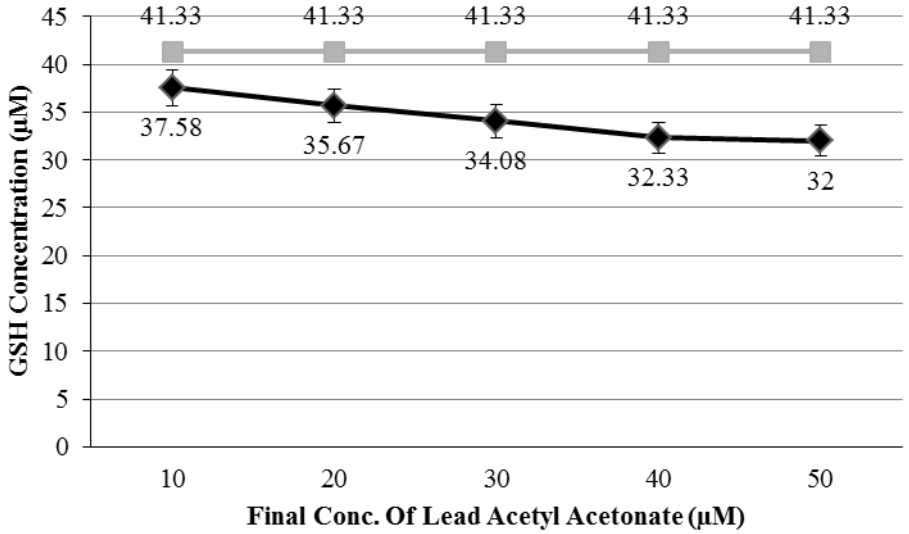

Figure 6. Effect of LAA concentration on lymphocytes-GSH content. ■, Lymphocytes-GSH control (1ml 0.9% NaCl+1ml of lymphocytes); ♦, LAA (10-50 µM). Results are the mean±SE of three experiments.

Figure 9. In both Figures 8 and 9, the results of only the lowest (10 µM) and highest (50 µM) of LAA is given.

DISCUSSION

Glutathione is found in various cells of the whole blood including lymphocytes. Sulphydryl group (SH) in glutathione is highly essential for its activity. GSH, the major non protein thiol present in animal cells is an extremely important biological reducing agent, involved in the detoxification processes of exogenous material (Rabenstien et al., 1989). The binding of lead to glutathione has been investigated, however little information is available regarding the detailed molecular nature of the binding in lymphocytes. Concentrations of glutathione in the blood have been reported to be significantly lower than control levels both in animal studies of lead exposure and in lead-exposed children and adults (Hsu, 1981; Hunaiti et al., 1995). It is believed

Table 2. Lead acetylacetonate (LAA) in lymphocytes.

S/N	Time (min)		10 µM		20 µM	30 µM	40 µM	50 µM	Control
1	0	Conc		37.58	35.67	34.08	32.33	32.00	41.33
		PH	7.0	39.00	36.67	35.25	33.25	33.75	41.33
			7.8	34.50	32.67	31.00	29.33	29.42	41.33
			8.5	32.33	30.42	28.83	27.08	27.25	41.33
		Temp	30 ^{o}C	37.58	35.67	34.08	32.33	32.50	41.33
			37 ^{o}C	22.08	21.58	21.25	20.92	20.67	41.33
			45 ^{o}C	26.25	25.75	25.42	25.08	24.83	41.33
2	30			34.50	32.67	31.00	29.33	29.42	41.33
3	60			32.33	30.42	28.83	27.08	27.25	41.33
4	90			30.25	28.67	26.83	25.25	25.33	41.33

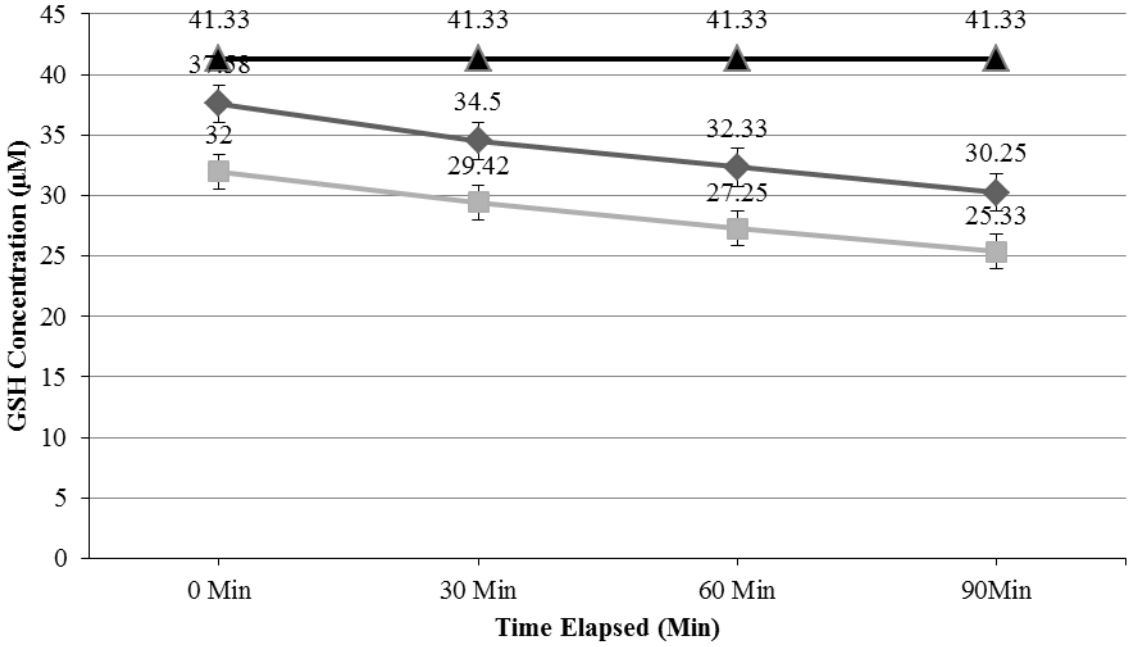

Figure 7. Effect of LAA concentration on the lymphocytes–GSH content with time incubation period (0-90min). ▲, Lymphocytes-GSH control (1 ml 0.9% NaCl+1 ml of lymphocytes). ♦, LAA (10 µM); ■, LAA (50 µM). Results are the mean ±SE of three experiments.

that lead interacts with glutathione that exists in lymphocytes and thus forms lead-GSH conjugates which might be the mechanisms of uptake inhibition and decreased toxicity of lead in the cells. This hypothesis supports the idea that the concentration of the glutathione in lymphocytes was found lowered upon addition of lead compounds to the solutions of lymphocytes as compared to control solution of lymphocytes. This appears that lead may be taken up by the cells as lead-GSH conjugate. The formation of conjugate (Pb-SG) is a process of detoxification of heavy metals. This process of conjugation may contribute to decrease lead induced toxicity. Most experimental research has indicated that after exposure of animals to lead, there is an increased amount of GSSG with a parallel drop of GSH in different organs (Sivaprasad et al., 2003; Droge et al., 1994). Our results are also consistent with the earlier study that incubation of human blood in the presence of lead compounds (concentration 100- 400 µg/dl) causes a drop of GSH concentration (Hunaiti and Soud, 2000). Our study postulate a new concept in toxicology that will help us in understanding numerous areas of chemical induced injuries. This mechanism of action will also add further to our understanding that might help in the development of a better therapeutic approach in managing to chemical exposures in certain situations. This study also supports the pharmacological role of glutathione in lead induced toxicity.

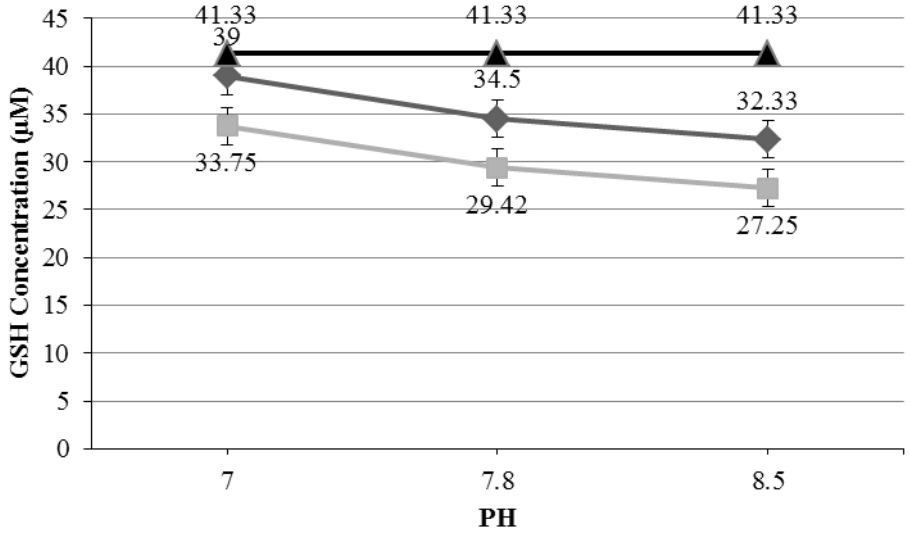

Figure 8. Effect of PH on the lymphocytes–GSH content due to LAA. ▲, Lymphocytes-GSH control (1 ml 0.9% NaCl+1 ml of lymphocytes). ♦, LAA (10 µM); ■, LAA (50 µM). Results are the mean ±SE of three experiments.

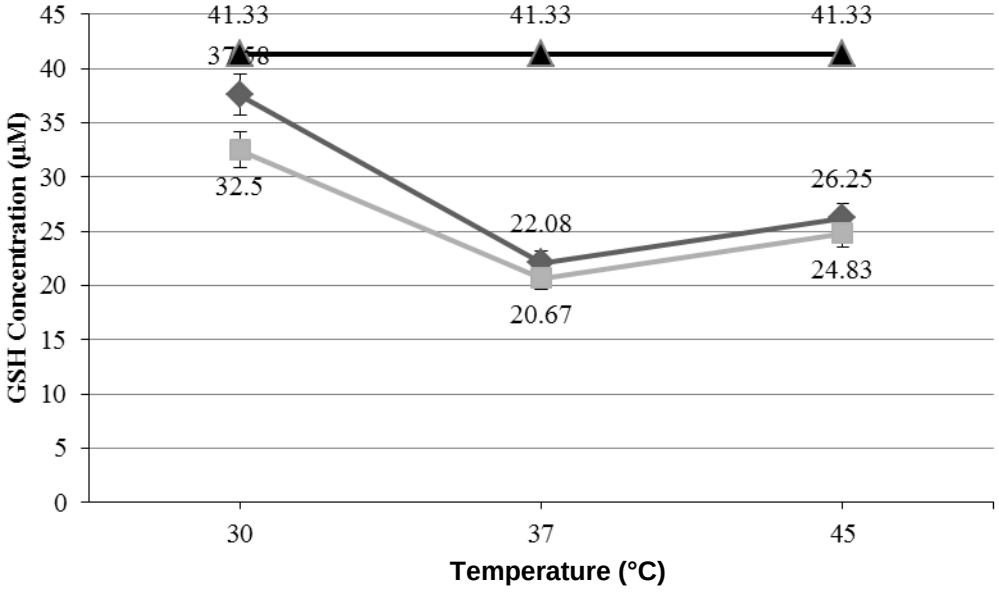

Figure 9. Effect of PH on the lymphocytes–GSH content due to LAA. ▲, Lymphocytes-GSH Control (1ml 0.9% NaCl+1ml of lymphocytes); ♦, LAA (10 µM); ■, LAA (50 µM). Results are the mean ±SE of three experiments.

Upon addition of inorganic and organic compounds of lead metal to the solutions of lymphocytes caused the decrease in concentration of glutathione. Addition of various concentrations in the range of (10-50 µm) lead acetate and LAA caused the reduction in the concentration of glutathione in a dose dependent manner. The concentration of glutathione was further decreased upon time incubation of lymphocytes with inorganic and organic compounds of lead metal. pH, 8.5 has been found to be most favorable for the reaction which might be due to the pKa (9.5-11) value of GSH. Temperature has less effect on the reaction of lead compounds with GSH of lymphocytes, although, the physiological temperature that is, 37°C provided favorable environment for such reactions. Our results are in agreement with Hunaiti and Soud (2000) where incubation of human blood in the presence of lead compounds (concentration 100- 400 µg/dl) caused a drop of GSH concentration. The

concentration of glutathione was measured spectrophotometerically by Ellman's reagent. This method of determination of GSH is based on the reductive cleavage of DTNB by -SH groups to produce intense yellow colour due the production of Ellman's anion, with an absorbance maximum at 412 nm. Glutathione concentration in µM was determined by comparing the absorbance/concentration of the unknown sample to the absorbance/concentration curve obtained with known concentration of reduced glutathione.

The chemical reaction in the background of interaction of lead compounds (e.g., lead acetate) with lymphocytes-GSH, and the subsequent interaction of Ellman's reagent (ESSE) with the unbound lymphocytes-GSH can be proposed as follows:

$Pb + 2GSH \longrightarrow Pb(SG)_2$ (Pb-GSH conjugate)

$ESSE + GSH \longrightarrow ESSG$ (mixed disulfide) $+ ES^-$ (Ellman's anion)

Conclusion

The addition of the lead compounds including lead acetate and lead acetylacetonate to the lymphocytes of blood were taken up by these cells through glutathione and most probably formed lead-glutathione conjugates (Pb-SG). The interaction and coordination of lead metal to glutathione and the uptake of lead metal by glutathione in the cells may be considered as toxicological and pharmacological approach, respectively. Finally, the mechanism of this process of conjugation in these cells is a physiological approach which accounts for the toxicological and pharmacological action of lead and glutathione, respectively.

ABBREVIATIONS

GSH, Reduced glutathione; **AIDS,** acquired immunodeficiency syndrome; **ROS,** reactive oxygen species; **GSSG,** oxidized glutathione; **DTNB,** 5-5, dithiobis 2-nitrobenzoic acid; **LAA,** lead acetyl acetonate; **MPA,** metapfosphoric acid; **PBS,** phosphate buffer saline; **HPLC,** high performance liquid chromatography.

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