THE COMPARATIVE ROLE OF ASCORBATE AND CHELATORS IN REVERSING OXIDATIVE STRESS, HEPATIC AND RENAL DYSFUNCTION IN SUB-ACUTE LEAD POISONING

B. O. ONUNKWOR*[1], R. N.UGBAJA[1] , D. A. OMONIYI[2] AND A. O. DOSUMU[1]

[1]Biochemistry Department, College of Biosciences, Federal University of Agriculture Abeokuta.
[2]College of Medicine, University of Ibadan

***Corresponding author:** benovinadict@gmail.com;   **Tel:** +23408068618104

ABSTRACT

Lead has been implicated in the induction of reactive species production, leading to organ dysfunctions. The ameliorative roles of ascorbate and chelators in acute lead poisoning were comparatively studied in thirty-five male Wistar rats (150-200g), segregated into 5 groups (n=7/Group): group 1 (administered normal saline), groups 2-5 were orally exposed to 75mg/kg body weight lead acetate (PbAc) daily for 14 days. Pre-therapy blood samples were collected to ascertain blood lead level (BLL) and catalase activity 24hours after the last PbAc exposure. Groups 3, 4, and 5 were then treated with 30mg/kg body weight D-penicillamine; 30mg/kg body weight succimer; and 500mg/kg body weight ascorbate respectively for 10 days, followed by the assay for indices of oxidative stress, hepatic and renal dysfunctions. Results obtained showed significantly elevated BLL in the four groups exposed to PbAc. which were significantly reversed about 2 folds in groups 3-5 after therapeutic interventions. Pre-therapy blood catalase activity of the PbAc treated groups was significantly ($p<0.05$) reduced by 39% when compared with the control group, however ascorbate significantly ($p<0.05$) increased catalase activity by 2 folds above the control; decreased plasma activities of alanine transaminase and aspartate transaminase, blood urea nitrogen and creatinine among the groups administered therapeutics. These findings indicate that ascorbate is more effective

Key words: antioxidant, blood lead level (BLL), chelating agents, oxidative stress, plumbism, xenobiotic

INTRODUCTION

Lead is a heavy metal found in the earth crust usually in compound forms as anglesite ($PbSO_4$), cerusite ($PbCO_3$), galena (PbS). Since ancient civilization, lead has been of widespread use and an insidious environmental toxin. It is sometimes known as a severe and aggressive contaminant `in human and animals' health status (Haleagrahara, *et. al.*, 2011; Moreira, *et. al.*, 2001; Nriagu, *et. al.*, 1997). Its ubiquity in the environment- the air, water, soil, food, among others makes it one of the leading metallic xenobiotic particularly in many developing countries (Koedrith and Young, 2011; Oteiza, *et. al.*, 2004; Sajitha, *et. al.*, 2010; Shalan, *et. al.*, 2005; Shehata, 2011).

Lead is relatively poorly absorbed into and excreted from the body, moving into soft tissues and bones where it remains deposited for a long time until therapeutic intervention (Balali-Mood, 2010; Moreira, *et. al.,* 2001). The Centres for Disease Control and Prevention, (CDC), World Health Organisation Occupational Safety and Health Administration (WHO-OSHA) redefined elevated blood lead levels (BLL) as that $\geq 10\mu g/dl$ in children and $40\mu g/dl$ in adults. A notable route of lead poisoning (plumbism) beyond this safe level for children is through ingestion, and most adults through occupational exposure (Payal, *et. al.,* 2009; Shehata, 2011; Sujatha, *et. al.,* 2011; Tkachenko and Kurhahyuk, 2011).

Widely known is the fact that heavy metals such as lead induce lipid peroxidation by the production of Reactive Oxygen Species (ROS) such as hydroxyl peroxide and superoxide radicals; and Reactive Nitrogen Species (RNS) such as nitric oxide, peroxynitrite and S-nitrosothiols (Chen, *et. al.,* 2003; Choudhary, *et. al.,* 2007; Flora, *et. al.,* 2004; Kumar, *et. al.,* 2011). Often, these radicals have rendered oxidative damage to DNA, proteins and lipids. ROS and RNS often result to the decreased activity of antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase (Ibrahim, *et. al.,* 2012; Sajitha, *et. al.,* 2010; Shalan, *et. al.,* 2005), which are also oxidative stress markers.

Lead poisoning interferes with important metabolic activities such as the DNA repair system, thus inducing carcinogenicity (Patil, *et. al.,* 2006; Ponce-Canchihuaman, *et. al.,* 2010), interferes with and suppress the Heme synthesis pathway by inhibiting the key enzymes including δ-aminolevulinic acid dehydratase and ferrochelatase, leading to anaemia in most cases (Ademuyiwa, *et. al.,* 2005; Costa, *et. al.,* 1997; Patrick, 2006; Shehata, 2011; Verma and Dubey, 2003). Impaired protein synthesis and inhibition of sulfhydryl group containing proteins, has been reported in lead poisoning, resulting in the compromise of many functional proteins (Devi and Banerger, 2007; Ponce-Canchihuaman, *et. al.,* 2010; Tkachenko and Kurhahyuk, 2011). Systemic damage to lipid metabolism has not been exempted by this xenobiotic, chronic toxicity of lead has been observed to induce hypertension and atherosclerosis in many subjects. Its cumulative adverse effects also extend to the nervous system, the immune system, and many vital organs (Erdogan, *et. al.,* 2005; Faix, *et. al.,* 2005; Haleagrahara, *et. al.,* 2011; Leonidis, *et. al.,* 2010). In the kidneys precisely, chronic exposure to lead have been reported to trigger interstitial nephritis and chronic renal failure, while acute exposure may give rise to proximal tubular dysfunctions resulting in glycosuria, hyperphosphaturia, and aminoaciduria (Allouche, *et. al.,* 2011; Muselin, *et. al.,* 2010; Oteiza, *et. al.,* 2004; Patra, *et. al.,* 2000; Payal, *et. al.,* 2009).

Several therapies had been introduced by health professionals to treat acute and chronic lead poisoning. Noteworthy is the age-long use of chelating agents such as BAL (British Anti Lewisite), though its use has long been withdrawn (Gurer and Ercal, 2000). Other chelating agents presently still in use include: Calcium disodium ethylenediamine tetraacetic acid (CaNa$_2$EDTA), meso-2,3-dimercaptosuccinic acid (DMSA or Succimer), and D-Penicillamine all of whose mechanism of action is based on their ability to mobilize and chelate heavy metals such as lead and consequently aid their excretion from the body. These have always been used in severe cases of plumbism as the first line

of treatment (Flora, 2009; Flora *et. al.*, 2004; Kalia and Flora, 2005). Unfortunately, other metals of the same valency as lead are often chelated as well.

Recent reports on several research findings on the use of natural antioxidants, such as vitamin C, vitamin E and α-lipoic acid as therapeutic interventions in lead poisoning has revealed safer, promising advantageous and or gainful effects (Abam, *et. al.*, 2008; Ademuyiwa, *et. al.*, 2005; Bashandy, 2006; Gurer and Ercal, 2000; Onunkwor, *et. al.*, 2004; Ugbaja, *et. al.*, 2013). They have been reported to reverse and/or restore lead induced anomalous activities/levels of oxidative stress markers such as superoxide dismutase (SOD), catalase and reduced glutathione (Faix, *et. al.*, 2005; Ibrahim, *et. al.*, 2012). Precisely, the effectiveness of ascorbate has been attributed to its ability to scavenge or quench free radicals. It also decreases the intestinal absorption of lead by reducing ferric iron to ferrous iron in the duodenum, thereby increasing the availability of iron which competes with lead for intestinal absorption. An increasing number of scientists are now advocating their use over conventional chelating agents as they generally do not have any known and or life threatening side effects (Kilikdar, *et. al.*, 2011; Koedrith and Young, 2011).

Based on the premise that most studies on this xenobiotic is based on chronic exposure with consequent meagre information on acute plumbism; and with a view to finding which of the available treatments have a better therapeutic effect in sub-acute cases, whether chelating agents or natural antioxidants, this research was undertaken.

MATERIALS AND METHOD
Chemicals
Lead acetate (ACS reagent, grade >99% pure), diethyl ether, ammonium thiocyanate, ferric chloride hexahydrate and all other chemicals used were of analytic reagent grade.

Experimental Design
Thirty-five healthy male Wistar rats (150 - 200g), purchased from the Department of Anatomy, Faculty of Veterinary Medicine, University of Ibadan, Ibadan were used for this investigation. They were maintained at 25°C in a well ventilated animal house on a 12:12 hr light-dark cycle with free access to standard rodent laboratory chow and clean water *ad libitum.* All conditions of animal experimentation conformed with the NIH guidelines as outlined in NIH publication (National Institutes of Health, 2011). The rats were acclimatized for two weeks prior to experimental treatments.

They were then randomly and evenly distributed into five groups (n=7). group 1(positive control)- was administered normal saline (0.9% NaCl), groups 2-5 were exposed to 75mg/kg body weight (b. wt) lead acetate (PbAc) daily for 14 days. Prior to therapeutic interventions, blood samples were collected by tail incision to ascertain the blood lead level (BLL) and catalase activity 24hours after the last PbAc exposure. Groups 2 (negative control), 3, 4, and 5 were then treated with normal saline, 30mg/kg b .wt. D -Penicillamine, 30mg/kg b. wt. meso-2,3-dimercaptosuccinc acid (DMSA), and 500mg/kg b. wt. Ascorbate respectively in two divided therapies of 5days each up to a total of ten days with 5 days rest in between the two doses to allow for lead redistribution. All administration was via oral route. After therapeutic interventions, all the animals were fasted overnight, subjected to light anaesthesia and then sacrificed. Blood samples were collected via cardiac puncture.

Assay of Biochemical Parameters
Estimation of BLL

For the estimation of the BLL, 1ml of the whole blood samples were acid digested and the BLL was then determined by atomic absorption spectrometry (Thermo Scientific S Series Atomic Absorption Spectrophotometer (Model Type S4 AA System) as described by Ademuyiwa, et. al., (2005). Precisely 1ml of blood was digested with concentrated nitric acid and brought to a constant volume. Analysis on a certified Spex, element standard (Spex Industries Inc., Edison, New Jersey, USA) was done for external standardization along with samples. Procedure accuracy of the lead analyses was evaluated by spiking control made for each sample and the mean recovery rate obtained for all analyses.

Catalase activity assay

The catalase activity was measured by the method of Beers and Sizers (1952) in which the rate of decomposition of H_2O_2 was measured spectrophotometrically from changes in absorbance at 240nm. 2.0 ml of phosphate buffer diluted whole blood was added to cuvette. Reaction was initiated by the addition of 1.0 ml of freshly prepared 0.059M H_2O_2. One unit is equal to one micromole of hydrogen peroxide decomposed per minute under specified conditions at 25oC. Activity of catalase is expressed as units/ml.

Xanthine oxidase assay

The activity of Xanthine oxidase (XO) in the plasma was determined using the spectrophotometric method described in the Worthington Enzyme Manual (2008). The rate of formation of urate from hypoxanthine is determined by measuring increased absorbance at 290 nm. A unit of activity is that forming one micromole of urate per minute at 25°C.

From the blood sample, the plasma was separated by centrifugation, aliquoted into eppendorf tubes and frozen until analysis. The plasma concentration of alanine transaminase (ALT), aspartate transaminase (AST), blood urea nitrogen (BUN) and creatinine were determined spectrophotometrically using Cypress diagnostic kits.

Statistical Analysis

The results obtained were Mean ± Standard Error of Mean (SEM). One-way Analysis of Variance (ANOVA) test was performed to check the significance of differences among the groups followed by Duncan's post hoc test to compare the treated groups with the control group. Values of $p < 0.05$ were regarded as significant, using the Statistical Package for Social Sciences (SPSS) version 16.0.

RESULTS
Blood Lead Levels (BLL)

While the concentration of lead in the control remained insignificantly different throughout the period of experiment, marked changes were recorded in the BLL of the other four groups. The three groups administered therapeutics showed a significant decrease in the BLL by 33% on the average with the DSMA being most effective. The negative control group administered lead only, showed no significant change in the level of lead in the blood (Fig. 1).

Catalase activity before and after therapeutic treatment

Prior to therapeutic interventionss, catalase activity in the whole blood decreased significantly in all the four groups orally exposed to lead acetate (95.95±4.31 U/mL, 86.36±5.01 U/mL, 98.99±7.69 U/mL and 88.47±13.65 U/mL) compared to that of the control

group (149.55±7.5 U/mL) at p<0.05. However, the administration of chelating agents and ascorbate to groups 3, 4, and 5 thereafter reversed the lead induced decreased activity significantly P<0.05 elevating blood catalase activity by two folds (189.57±11.79 U/mL, 197.99±10.60 U/mL and 216.95±30.13 U/mL respectively), as shown in Fig. 2. The order of most significant increase was ascorbate > penicillamine > succimer.
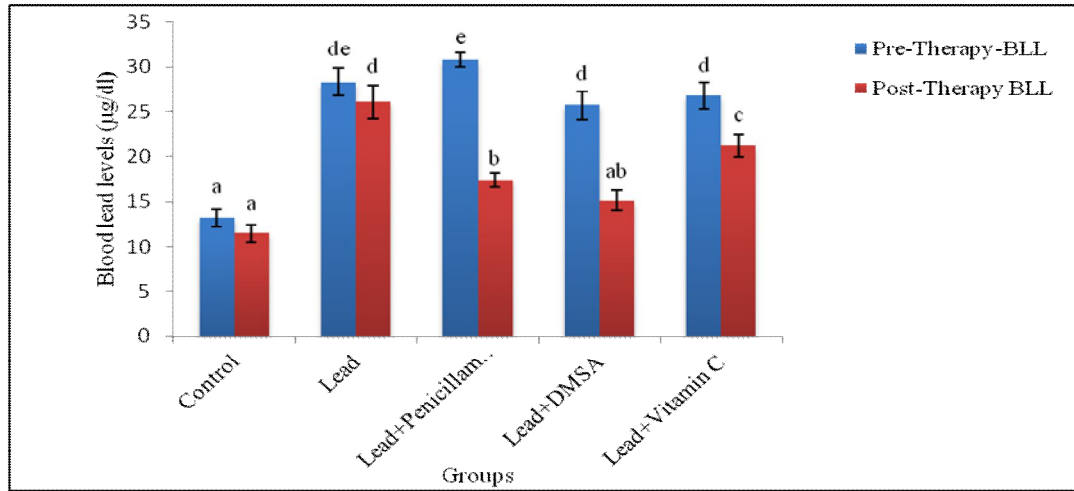

Fig. 1: Blood lead levels before and after therapeutic intervention. Bars not having any letter (a-e) in common are significantly different (p < 0.05).
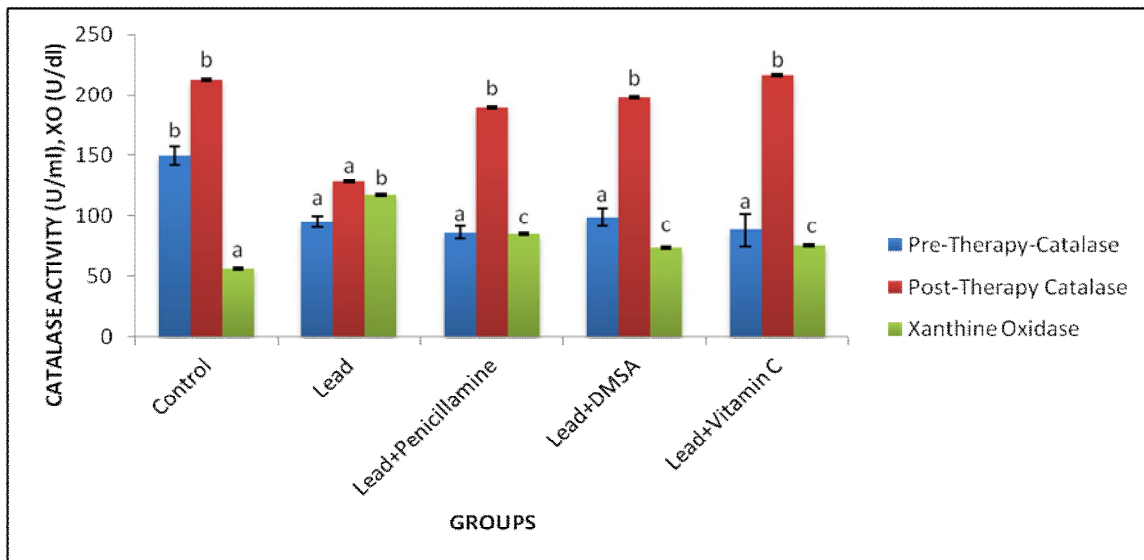

Fig. 2: Blood catalase and xanthine oxidase activities before and after therapeutic intervention. Bars not having any letter (a-c) in common are significantly different (p < 0.05).
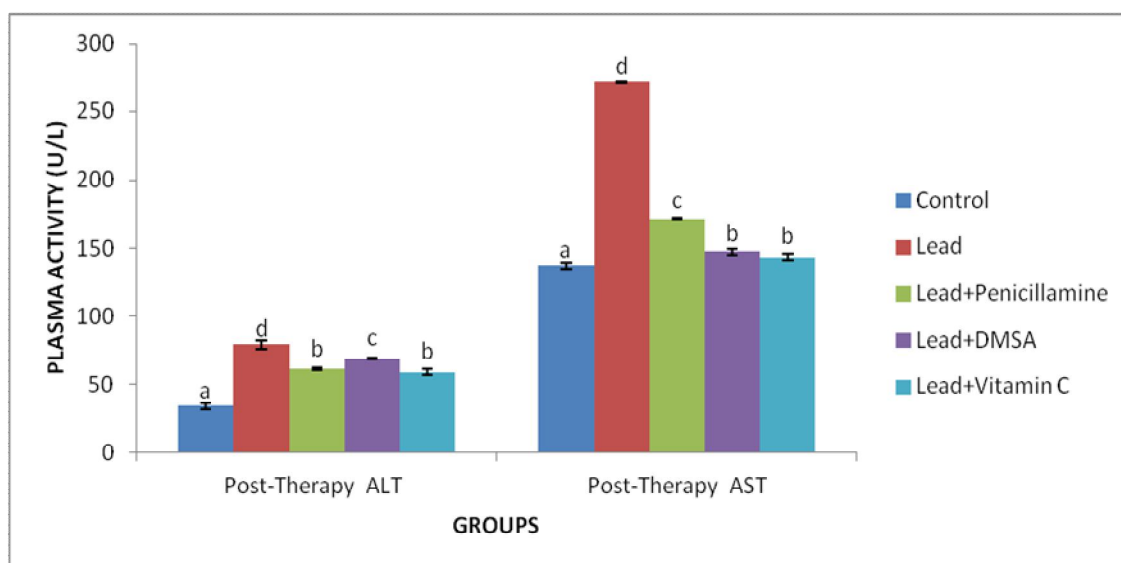
Fig. 3: Plasma ALT and AST activities after therapeutic intervention. Bars not having any letter (a-d) in common are significantly different ($p < 0.05$).
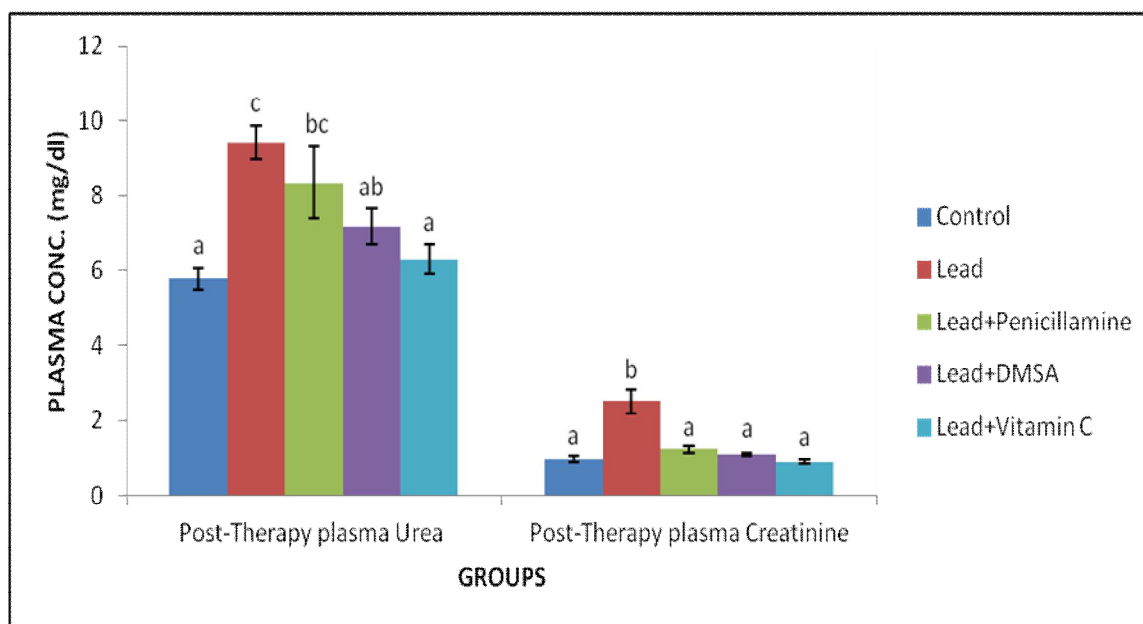

Fig. 4: Plasma urea and creatinine levels after therapeutic intervention. Bars not having any letter (a-c) in common are significantly different ($p < 0.05$).

Xanthine oxidase (XO) activity in the plasma

All four groups treated with lead had a significantly high level of plasma XO (1.18 ± 0.01, 0.85 ± 0.02, 0.74 ± 0.03 and 0.76 ± 0.01 U/dl respectively) at $p < 0.05$ when compared with the control group (0.56 ± 0.01 U/dl); Fig. 2. The therapeutics administered had no significant effect on the plasma level of XO ($p > 0.05$).

Alanine transaminase (ALT) and Aspartate transaminase (AST) activities in the plasma

The activities of ALT and AST in the plasma were observed to be significantly higher ($p < 0.05$) in the group administered lead only (79.17 ± 3.30 U/L and 271.67 ± 0.71 U/L respectively) when compared with the control group (33.71 ± 2.68 U/L and 137.22 ± 1.87 U/L respectively) and the three groups thereafter administered with therapeutic agents. However, of the groups administered therapeutics, the group administered ascorbate had the least activities of these liver marker enzymes (59.08 ± 2.55 U/L and 143.42 ± 2.42 U/L respectively) in the plasma, Fig. 3.

Blood urea nitrogen (BUN) and creatinine concentration in the plasma

The assay results obtained for the rats' kidney function followed a similar trend as observed for the liver marker enzymes; the concentrations of urea and creatinine in the plasma were significantly lower ($p < 0.05$) in the groups administered with the therapeutics. The group administered ascorbate had the least urea and creatinine concentration in the plasma (6.31 ± 0.38 mg/dl and 0.89 ± 0.06 mg/dl respectively), when compared with the lead only group: 9.43 ± 0.45 mg/dl and 2.51 ± 0.31 mg/dl respectively, as indicated in Fig. 4.

DISCUSSION

Sub-acute lead poisoning has been found to present more severe clinical symptoms in the population, hence this study evaluated the relative effectiveness of chelating agents and natural antioxidants in cases of plumbism using D-Penicillamine, DMSA and Ascorbate.

Oral route of administering lead acetate was preferred in this study because of earlier reports that the accumulation of lead in vital organs, like the liver and kidney, was significant upon ingestion (Muselin, *et. al.*, 2010). This was confirmed in this study; there was a significant increase ($p < 0.05$) in the BLL of all groups administered lead acetate compared to the positive control group. Lead ingestion is common among children because most of their toys contain a form of lead coating (Nriagu, *et. al.*, 1997; Stravreva-Veselinovska and Ziranovic, 2010; Zhang, *et. al.*, 2012). The significant presence of this metal in such vital organs may be as a result of the body's self-effort to excrete the poison since the liver is especially known for its role in detoxification, and the kidney in excretion (Patra, *et. al.*, 2000).

Much widely known effect of lead toxicity is oxidative stress. Oxidative stress is a broad term referring to several changes *in vivo* such as the presence of free reactive oxygen or nitrogen species, decrease in the count or activity of antioxidants and antioxidant enzymes, increased lipid peroxidation, among others (Raafat, *et. al.*, 2009). One of the major concepts in the mechanism of heavy metal toxicity is attributed to its ability to generate reactive oxygen species which cause peroxidation of lipids (Draper *et al.*, 1993; Ademuyiwa, *et. al.*, 2005; Bashandy, 2006; Harishekar and Kiran, 2011).

Catalase is an important antioxidant enzyme which helps in decomposing the reactive hydrogen peroxide to less deleterious water and oxygen (Kilikdar, *et. al.*, 2011). In this study, lead toxicity significantly reduced the activity of catalase, therefore indicating that free radicals are generated in lead poisoning. The plausible reason for decreased catalase activity is that as more free reactive oxygen species, especially H_2O_2, were generated, more molecules of catalase were involved in peroxide breakdown, leading to a transient exhaustion of the catalase pool (Gurer, *et. al.*, 1999; Sujatha, *et. al.*, 2011; Verma and Dubey, 2003). Therapeutic intervention however reversed this trend in the order ascorbate > penicillamine > succimer. Several mechanisms have also been proposed to explain this reversal; but the much emphasized is that chelating agents and antioxidants alike have the ability to chelate lead out of the system, that is they form an easily excretable complex with lead (Haleagrahara, *et. al.*, 2011). Others attribute the reversal to the fact that most antioxidants work synergistically with enzymes such as catalase that are involved in reversal of oxidative stress. The values obtained when related with lead induced lipid peroxidation-though reported elsewhere, were consistent with that of catalase (Ugbaja, *et. al.*, 2013). This confirms that lead poisoning induces oxidative stress and brings about a consequent increase in lipid peroxidation and a decrease in the activity of catalase (Tkachenko and Kurhahyuk, 2011).

For catalase activity, the degree of effectiveness for each therapeutic administered differed significantly. Catalase activity in the therapeutic treated groups increased significantly ($p<0.05$), and the group administered ascorbate had the most positive numerical difference in catalase activity. Comparing the results obtained on the oxidative stress markers, it becomes clear that: first, ascorbate remains a very potent antioxidant against oxidative stress, in the event of lead poisoning. Ascorbate is able to prevent oxidative stress better probably because it elicits mechanisms that can prevent the generation of the free radicals before they accumulate (Raafat, *et. al.*, 2009). In other words, ascorbate is more of a scavenger than a chelator. Secondly, it is probable that chelating agents, like D-Penicillamine may be involved in some latent co-generation of free radicals (reactive nitrogen species) when administered beyond a certain dosage (Kilikdar, *et. al.*, 2011).

Xanthine oxidase is a pro-oxidative enzyme that measures the rate of conversion of hypoxanthine to xanthine and then to uric acid (Zhang, *et. al.*, 2010). An increased activity of this enzyme is considered to indicate increased rate of oxidative stress due to formation of superoxide anion free radicals (Kilikdar, *et. al.*, 2011; Zhang, *et. al.*, 2010). Therefore, lead as a potential initiator of free radical generation mechanism should significantly increase the plasma level of xanthine oxidase. The result obtained from the group administered lead only when compared with the control group proves this. The inability of the therapeutic agents to reduce plasma XO levels may imply that there is still a significant free radical generation in some body compartments. It could also be that there is a rather slow response of XO to therapeutics once elevated, more so it is nucleotide related.

The accumulation of heavy metals such as lead has been known to be a notable cause of liver, kidney and brain damage (Hamadouche, *et. al.*, 2009; Moreira, *et. al.*, 2001; Stravreva-Veselinovska and Ziranovic,

2010; Tkachenko and Kurhahyuk, 2011). When the liver is damaged, it often results to a marked increase in the activities of the enzymes that are more confined within the hepatic cells. Such enzymes include alanine transaminase (ALT) and aspartate transaminase (AST), and so are marker enzymes to estimate the extent of damage in the liver. Lead is known to promote oxidative damage in liver by enhancing peroxidation of membrane lipids, a deleterious process carried out by free radicals most likely released by the auto-oxidation of accumulated amino levulinic acid due to inhibition of amino levulinic acid dehydratase (Balali-Mood, *et. al.*, 2010; Bashandy, 2006; Costa, *et. al.*, 1997; Gurer, *et. al.*, 1999; Patrick, 2006). Some reports attribute the liver damage peculiar to plumbism to the fact that lead binds to plasma proteins and is conducted to liver, where it causes alterations in high number of enzymes. It can also perturb protein biosynthesis in hepatocytes, including the structural proteins (Shalan, *et. al.*, 2005).

Over-production of free radicals following lead exposure initiates from mitochondria and spreads out quickly to all cellular and tissue components, causing dysfunction in cellular energy metabolism, membrane transportation and finally cell death. Another potent source of free radicals in the liver can be kupffer cells (Chen, *et. al.*, 2003). In response to oxidative stress induced by lead, endogenous antioxidants are consumed resulting in continuously decreasing tissue and cellular anti-oxidative capacity that leads to progressive oxidative injury (Bashandy, 2006; Patra, *et. al.*, 2011; Shehata, 2011).

Upon administration of therapeutics, the chelating agents showed a poor therapeutic response. D-Penicillamine and succimer were unable to restore normal AST levels in the blood significantly (p<0.05). This may be because AST is not so specific for liver function, and so its relatively persistent high levels in the plasma may be an indicator that some other organ, asides liver, is still being adversely affected in the body.

These heavy metals also affect the major organ of excretion- the kidneys. They affect the renal efficiency in the removal of potentially toxic metabolites such as urea, thus making the metabolites to be found in concentrations far higher than normal *in vivo* (Ponce-Canchihuaman, *et. al.*, 2010). In the kidneys precisely, chronic exposure to lead have been reported to trigger interstitial nephritis and chronic renal failure, while acute exposure may give rise to proximal tubular dysfunction with glycosuria, hyperphosphaturia, and aminoaciduria (Allouche, *et. al.*, 2011; Muselin, *et. al.*, 2010).

Kidney function was acutely affected by lead poisoning as well, but the use of chelating agents could not effectively correct the resulting kidney dysfunction. This is consistent with some report that chelators, though can reduce the BLL, they do not reverse most impairment caused by lead on systems and organs especially in children (Shannon, *et. al.*, 2005). The group administered ascorbate however showed a remarkable therapeutic effect by significantly restoring the plasma level of both the liver and kidney marker enzymes- ALT, AST, BUN and creatinine to near that of the control group. It implies therefore that there could be a positive link between ascorbate's scavenging property for free radicals and its ability to regulate blood plasma parameters about the normal range (Erdogan, *et. al.*, 2005; Raafat, *et. al.*, 2009).

Ascorbate has proved most effective in mobilizing lead from the liver and kidney as observed in this study. Though this may not be generalization in *vivo*, more studies must be done to ascertain the effectiveness of ascorbate in mobilizing lead from other vital tissues and organs like the bones. This is necessary on the premise that a large percentage of this metal on exposure becomes stored in the bones. This may throw more light on the mechanism behind calcium loss from the bones partly due to the presence of divalent ions like lead and how it can be alleviated.

CONCLUSION

This experiment has again indicated that lead poisoning inevitably leads to oxidative stress, and the dysfunction of vital organs such as the liver and kidney. Ascorbate has proved most efficacious in managing the effects of sub-acute lead poisoning such as oxidative stress and organ dysfunction. Since most antioxidants work synergistically with one another, ascorbate might have proved effective because of some other antioxidants present in the body of the animal subjects. Therefore, more study must be done to confirm ascorbate's independent efficacy, else the use of co-administration to mimic antioxidants' synergistic interaction that occur *in vivo*.

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