Enzymatic toxicity induced by lead acetate and its possible reversal by α tocopherol in testicular tissue of Swiss albino mice

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Abstract
Objective: An important part of male infertility of unknown etiology may be attributed to various environmental and occupational exposures to toxic substances, such as lead. Lead is the known environmental contaminant adversely affecting the male reproductive system in human and experimental animals. The cytotoxic effects of lead on male reproductive system involve the production of reactive oxygen species (ROS) and oxidative damage in tissue. The higher quantities of ROS in testicular tissue detrimentally affected enzymatic activities so as to produce verities of biochemical abnormalities and finally reduced sperm count.

Methods: Adult male mice were divided into three groups: control, treated with lead acetated (1.25 mg/kg/day) and recovery group lead acetated (1.25 mg/kg/day) with vitamin E (2 mg/kg/day). Treatments were administered by daily gavage for 45 days. After treatment testis and epididymal weights were recorded and testis was used for the biochemical analysis (acid phosphatase (ACPase), alkaline phosphatase (ALKPase), and glutathione (GSH) and epididymal was used to count sperm.

Results: Administration of lead acetate at a dose of 1.25mg/kg body weight for 45 days decreased the activities of all above parameters. Lead exposure resulted in oxidative stress and this was well extrapolated from the increase in lipid peroxidation products (LPP). Coadministration of vitamin E (2 mg/kg/day) with lead acetate restored all the parameters cited above to near the control values.

Conclusions: This concludes that vitamin E has beneficial effects against lead acetate induced enzymatic toxicity in testicular tissue of mice.

Keywords: Albino mice, Enzymatic, Lead acetate, Testes, Vitamin E

1. Introduction
Incidence of poor male fertility among the population of reproductive age is increasing at an alarming rate and thus, a major concern is oriented towards the protection of male reproductive health [1,2]. The reason for reduced fertility is attributed to exposure to several chemicals released into nature. The preponderance of these pollutants, particularly toxic metals is mainly due to anthropogenic life style and rapid industrialization [3]. Lead is the most abundant toxic metal in the environment [4] Lead does not have any detectable beneficial biological role however on the contrary its detrimental effect on physiological, biochemical and behavioral dysfunctions have been documented in animals and humans by several investigators [5]. Lead is a male reproductive toxicant [6]. Toxicity is manifested in male reproductive function by deposition of lead in testes, epididymis, vas deferens, seminal vesicle and seminal ejaculate. Lead has an adverse effect on sperm count, sperm motility and retarded the activity of alive sperm [7]. Clinical and animal studies indicate that abnormalities of spermatogenesis result from toxic exposure [8]. The mechanism behind lead toxicity is the oxidative stress and it develops when there is an imbalance between the generation of reactive oxygen species and the scavenging capacity of antioxidants in the reproductive tract. Reactive oxygen species (ROS) have been shown to have an important role in the normal functioning of a reproductive system and in the pathogenesis of infertility [9]. Accumulated evidence has revealed that testicular enzymology which is basically characterized by steroidogenesis process, gets disrupted, at least in part, by oxidative stress mechanisms [10]. Studies in male rats have shown that lead intoxication disrupts testicular steroidogenesis by inhibiting the activities of testicular steroidogenic enzymes [11]. Enzymes are one of the major targets for metalloid action. Measurement of certain patterns of cellular enzymes under different conditions of treatments with various types of toxicants could provide good evidence.
for the cytotoxicity and hence the impairment of cell function [12]. Reproductive toxicity is the adverse effects of chemicals on gonadal structure and functions, alterations in fertility and impaired gamete function [13].

The treatment of lead poisoning, especially at sub clinical level is equally important. Most of the chelating agents tend to have adverse side effects and the benefits are usually transitory, since, blood lead can be rapidly replaced from the bone store [14]. Oxidative damage associated with the presence of lead has been illustrated as one possible mechanism involved in lead toxicity [15], which suggests that antioxidant might play a role in the treatment of lead poisoning [16]. Animals have protective mechanism in the form of antioxidant nutrients, vitamins and several enzymes. Antioxidant may play an important role in abating some hazardous effects of lead. The body consists of an elaborate antioxidant defense system that depends on dietary intake of antioxidant vitamins and minerals. Chow [17] reported vitamin E and occupies an important and unique position in the overall antioxidant defense. The antioxidant function of vitamin E is closely related to the status of many dietary components. Antioxidative properties of vitamin E is believed to prevent reproductive disease associated with oxidative stress [18]. Vitamin E interacts with oxidizing radicals and terminates the chain reaction of lipid peroxidations [19]. In many studies, vitamin E neutralizes lipid peroxidation and unsaturated membrane lipids because of its oxygen scavenging effect [20]. El-Tohamy et al [21] showed that vitamin E has a preventive and reducible role against the oxidative stress induced by a toxic substance in the testes. The effect of lead acetate on testicular enzymes and its mechanism of action on the male gonads have not been studied. Therefore, the aim of this work is to study the effects of lead on testicular tissue. It is about a direct toxicity on testicular cells or an indirect toxicity via abnormalities of the testicular enzymatic activities and their protection by vitamin E.

2. Materials and methods
2.1 Animals and treatments
Healthy adult male swiss albino mice (Mus musculus) weighing 35 to 40 g were used for the experiment. Animals (80 to 90 days) were maintained under standard laboratory condition and provided balance diet and water ad libitum daily. Animals were divided into control, experimental and recovery groups. The control group was given vehicle only. The experimental groups were given lead acetate (1.25 mg/kg) daily for 45 days by gavage (0.2 ml/animal). The recovery groups received lead acetate (1.25 mg/kg) and vitamin E (2 mg/kg) for the same period by the same route. The treatment duration of 45 days was selected as the length of the complete spermatogenic epididymal maturation cycle in mice. The doses selected were based on previous work in our laboratory [22].

2.2 Organ weights
Animals were sacrificed after their respective treatments and their testis and epididymidis weights were recorded with an automatic balance (AND GX-600, Japan).

2.3. Measurement of testicular enzymes
2.3.1 Acid phosphatase (ACPase)
Enzyme activity in the testis was estimated by the method of Bessey et al. [23]. A known amount of tissue was homogenized in a desired volume of cold distilled water. To 0.2 ml of homogenate, 0.6 ml of substrate buffer (pH 4.8) was added. The blank contained 0.2 ml water instead of homogenate. The tubes were incubated for 30 min at 37°C following which 4 ml of NaOH was added to each tube. The resultant yellow colour was measured in a Spectronic-103 colorimeter and expressed as µmole p-nitrophenol/100 mg fresh tissue weight/30min.

2.3.2 Alkaline phosphatase (ALKase)
Enzyme activity in the testis was estimated by the method of Bessey et al. [23] using alkaline buffer (pH 10.5). The units were as for ACPase, above.

2.3.3 Glutathione (GSH)
The concentration of glutathione in the testis was assayed by the method of Grunert and Philips [24]. A known amount of tissue was homogenized in 3 ml of 3% HPO3 and 1 ml distilled water and saturated with salt solution (1.5 g NaCl crystals). The solution was centrifuged at 800 g for 5 min. A 2 ml aliquot of supernatant was added to the sample tube containing 6 ml of saturated NaCl solution and allowed to stand for 10 min at 20°C. The blank tube was run with 2 ml of 2% HPO3 instead of the supernatant aliquot. Sodium nitroprusside and sodium cyanide, 1 ml each, was added to the blank sample tubes respectively. The coloured complexes developed was measured at 520 nm on a colorimeter against the blank tube. The units were µg glutathione/100 mg fresh tissue weight.

2.3.4 Lipid peroxidation determination (LPO)
Lipid peroxidation in testicular tissue was evaluated by the method of Ohkawa et al. [25]. To the sample tube containing 0.1 ml distilled water, 0.75ml of 20% acetic acid, 0.2 ml sodium dodecyl sulphate, 0.75 ml of 1% thiobarbituric acid (TBA) and 0.1 ml distilled water was added instead of homogenate. The solutions were mixed and heated in a water bath for 60 min at 95°C. The tubes were cooled immediately and 2 ml of 10% trichloroacetic acid (TCA) was added, mixed and centrifuged at 1000 g for 15 min. The O.D. of the supernatant was read at 532 nm. LPO was expressed as nmole melonyldialdehyde (MDA) formed/100mg tissue weight.

2.4 Sperms count and detection of sperms abnormality
Animals of all groups were sacrificed after their respective treatment and sperm samples were collected from the vas deferens, and appropriately processed for the study of sperm counting and sperm abnormality testes as recommended earlier [26]. The sperm suspension was prepared and centrifuged at 1000 rpm for 1 min. Sperm smears were drawn
on clean and grease free slides, dried overnight and then stained with 10% Giemsa diluted with sorresons buffer (pH 7.0) for one hour and subsequently observed. Morphologically abnormal sperm were recorded as per Wyrobek and Bruce [27]. Percentage of sperm abnormality was calculated by scanning abnormal sperm. The same suspension was used for sperm counting using a haemocytometer.

2.5. Statistics analysis
For all biochemical estimation a minimum of 10 to 12 replicates were used for each parameter and tissue. The data were statistically analyzed using ANOVA followed by Scheffe’s test for multiple pair wise comparisons [28]. A significant level of p ≤ 0.05 was accepted.

3. Results

3.1 Organ weights
The weights of testis (P <0.01) and epididymis (P <0.01) were significantly lower in mice treated with LA for 45 days compared to control, while vit.E + LA group did not show any significant difference in organ weights compared to control (Table 1).

3.2 Sperm Abnormality and Sperm Count
The analysis of sperm samples from LA treated mice depicted various types of sperm shape morphologies, which included hook-less, lance shaped, banana- shaped, flower like, balloon shaped (amorphous sperm). In addition double headed sperms were found with two normal and sometimes abnormal heads joined to a common-mid-piece. Furthermore, certain sperms were clearly double tailed. Occurrence of abnormal sperm population (P ≤ 0.001) and sperm counting profile significantly increases (P ≤ 0.01) in LA treated mice as compared to control. In contrast, LA injected mice supplemented with Vit.E encountered significantly decreased percentage of sperm abnormality and sperm count compared to LA treated mice (Table 1).

3.3 Assessment of biochemical changes
To determine the testicular damage caused by LA and the protective effect of vitamin E, the activities of some testicular enzymes (ACPase and ALKPase) were used as biomarkers of the testis. After 45 days of LA administration, several changes of the parameters have been observed to indicate the occurrence of testicular injuries by comparing to control group. In this investigation, a highly significant (P<0.001) decrease in ACPase, and ALKPase activities were observed after LA intoxication. LA treatment also resulted in a reduction (P<0.01) in glutathione levels. These parameters did not differ from control values when vit. E was coadministered with LA. Results of the present study emphasizes a sharp and significant rise (P ≤ 0.001) in LPO content in the testicular tissue of LA treated mice compared to vehicle control group. In the LA + Vit.E group, the levels of LPO in the studied tissues were significantly reduced compared to LA-groups, but the parameter did not come down to the control level (Table 2).

### Table 1: Organ weights (mg), sperm count (million /ml) and sperm abnormalities (%) in control and experimental animals.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Treated (LA) for 45 days</th>
<th>Treated (LA+Vit.E) for 45 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testes weight (mg)</td>
<td>123.5±4.2</td>
<td>87.5±6.2 **</td>
<td>119.1±1.0**</td>
</tr>
<tr>
<td>Epididymis weight (mg)</td>
<td>19.5±0.7</td>
<td>10.3±1.3 **</td>
<td>18.2±0.6 NS</td>
</tr>
<tr>
<td>Sperm count (million /ml)</td>
<td>42.5±1.04</td>
<td>15.7±0.81 **</td>
<td>38.07±1.19 **</td>
</tr>
<tr>
<td>Sperm abnormality (%)</td>
<td>2.37±0.29</td>
<td>9.5±1.01 ***</td>
<td>6.3± 0.08*</td>
</tr>
</tbody>
</table>

All values are expressed + SEM, Significant level, NS= Non significant, *= (P<0.05), **= (P<0.01), ***= (P<0.001) compared with control, treated (LA) and control treated (LA+Vit.E).

### Table 2: Testicular biochemical parameters of control and experimental animals

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Treated (LA) for 45 days</th>
<th>Treated (LA+Vit.E) for 45 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase (µmoles p-nitrophenol released/100 mg/30min)</td>
<td>12.44±4.37</td>
<td>5.40±0.59 **</td>
<td>10.90±0.20 NS</td>
</tr>
<tr>
<td>Alkaline phosphatase (µmoles p-nitrophenol released/100 mg/30min)</td>
<td>11.13±0.38</td>
<td>4.61±0.34 **</td>
<td>10.79±0.29 NS</td>
</tr>
<tr>
<td>Glutathione (µg/100mg)</td>
<td>101.45±2.68</td>
<td>66.28±3.06 **</td>
<td>90.87±1.86 *</td>
</tr>
<tr>
<td>Lipid peroxidation (nmole)</td>
<td>14.33±2.079</td>
<td>29.63±0.23 **</td>
<td>19.57±0.21 *</td>
</tr>
<tr>
<td>MDA formed/100mg</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All values are expressed ± SEM, Significant level, NS= Non significant, *= (P<0.05), **= (P<0.01), ***= (P<0.001) compared with control, treated (LA) and control treated (LA+Vit.E).

4. Discussion
Lead represents a dangerous environmental and industrial pollutant. Several studies in experimental animals have brought the evidence that oxidative stress is implicated in the toxicity of lead. Administration of lead at the examined dose for 45 days caused a reduction in the relative weights of the testes and epididymal when compared to the control. The weight of the testes is largely dependent on the mass of the differentiated spermatogenic cells and spermatogenic arrest and inhibition of steroid biosynthesis of Leydig cell [29]. A significantly reduced sperm count in lead treated mice taken at
45 days after the treatment clearly shows the elimination of germ cells at different stages of development and points to damaging ROS generated through lead catalysis. In fact, oxidative damage to polyunsaturated fatty acids of cell membranes has long been considered to result in the impairment of membrane fluidity and permeability leading to the efficient damage of germ cells, spermatozoa and mature sperm [30]. Hence, the observed sperm count decreased in the lead treated mice may be due to direct interaction of ROS with the sperm cell membrane. The present finding is also consistent with previous studies [22]. Sperm abnormalities due to chemical mutagens are well documented [31].

In light of this, the present chemical, lead acetate has been considered as a potent mutagen causing formation of abnormal male germ cells population at higher doses [26]. However, the observation of the present study with a low dose of lead acetate contradicts this view. It is further stated that various chemical mutagens alter specific gene loci in chromosomes producing morphologically deformed sperm populations [32]. Recent studies, however, indicate that various species of ROS generated through metal catalysis potentially interact with gene strands causing mutations, thereby inducing changes in sperm morphology [33-34]. Therefore, the present mechanism of abnormal production of sperm and reduced sperm count may be an oxidative stress dependent phenomenon induced by lead catalysis. Oral administration of lead acetate for 45 days to adult male mice resulted increased lipid peroxidation potential in the testes of the treated mice. Since, in general, elevated LPO is considered as an indicator of oxidative stress [35-36], the significantly increased LPO in the lead treated mice compared to the untreated controls demonstrates increased oxidative stress generated in the present experiment. The higher membrane lipid content of testes is presumed to make them more vulnerable to oxidative stress [37]. In addition, Leydig cell, mitochondria and microsomes of testes are known to contribute significantly to an increased production of ROS. Moreover, lead ions are capable of generating and promoting free radical reaction to different extent in different tissues [38].

The present study demonstrated that the activities of ACPase, ALKPase and glutathione in the testicular tissue were significantly declined in LA-group comparing with controls. Several studies reported alterations in antioxidant enzyme activities such as SOD, catalase and glutathione peroxidase (GPX) and changes in the concentrations of some non-enzymatic antioxidant molecules, such as glutathione (GSH) in lead exposed animals [39] and workers [40]. These findings suggest a possible involvement of oxidative stress in the pathophysiology of lead toxicity.

Batra et al. [41] observed a dose dependent reduction in the activity of two major enzymes in the testis, Alkaline phosphatase and Na-K ATPase, in lead exposed animals which is another probable mechanism of lead induced reproductive toxicity. Our studies indicate that lead causes disturbances in metabolism of reproductive organs by alterations of biochemical parameters due to oxidative stress. Antioxidants provide a defense mechanism through 3 levels of protection-prevention, interception and repair. In a normal situation, the cellular antioxidant mechanisms present in almost all tissues and their secretions are likely to quench those reactive oxygen species (ROS) and protect against oxidative damage [42]. In the present study, it was observed that vitamin E is a potent antioxidant or free radical scavenger which reduces the lead toxicity in Swiss albino mice testes. The beneficial effects of vit.E can be attributed to the antioxidant effects of this vitamin; it is scavenger of oxygen-free radicals which are toxic byproducts of many metabolic processes [43].

Oda and El-Maddawy [44] reported that the beneficial effect of vit. E is mostly due to its antioxidant properties. Vit. E protects critical cellular structures against damage caused by oxygen-free radicals and reactive products of lipid peroxidation [45]. Moreover, vit. E is essential in maintaining the physiological integrity of testis, epididymis and accessory glands [46]. Traditionally, vitamin E is called an anti-sterility vitamin [47] and is associated with normal function of the male reproductive system [48]. Since vitamin E plays an important protective role against oxidative stress by reducing malondialdehyde level and improving the oxidative defense system activity in testicular cells [49], it is reasonable to assume that the positive effects of vitamin E observed in the present study resulted from its antioxidant property.

From the current results, it can be concluded that concurrent administration of vitamin E to LA treated animals ameliorated the induced weight and testicular enzymes damage. This is consistent with a vital role of vitamin E in antioxidant systems that protect against LA damage, possibly by preventing oxidative damage to testes. The present studies suggest therapeutic effects of vitamin E to minimize the testicular enzymatic toxicity of LA exposure.

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