Antioxidant Status, Lipid Peroxidation and Testis-histoarchitecture Induced by Lead Nitrate and Mercury Chloride in Male Rats

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ABSTRACT

This study was done to evaluate the effects of lead nitrate and mercury chloride in testis tissues of Wistar rats. Lead nitrate and mercury chloride are widely used heavy metals in industry. Oral lead and mercury administrations to adult male rats at doses 45 mg/kg bw and 0.02 mg/kg bw, respectively for 4 weeks caused a significant increasing in MDA levels and antioxidant enzyme activities (SOD, CAT, GPx and GST). The MDA levels and activities of antioxidant enzymes was lower in rats that were administrated by lead nitrate than mercury chloride treated group. Light microscopic analyses revealed that lead nitrate and mercury chloride induced numerous histopathological changes in testis tissues of rats. Histopathological observations of the testis tissues showed that mercury chloride caused more harmful effects than lead nitrate, too. The results indicate that lead nitrate and mercury chloride have reproductive toxicity, in male rats at the tested doses. The effect which we observed applying the lead nitrate and mercury chloride together, was more greater than when we used them alone.

Key Words: Antioxidant enzymes, lead, mercury, oxidative stress, testis

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Human and Animal Health
INTRODUCTION

Humans and animals were exposed to environmental pollutants at different stages of life [1]. Environmental pollutants such as heavy metals are the main factors responsible for oxidative stress. Intoxication by heavy metals constitutes serious threats to human health [2]. Järup [3] indicated that heavy metals such as lead, cadmium and mercury may evoke behavioral disturbances, learning and concentration difficulties and diminished intellectual capacity. The highest lead intake in adults is through lungs and gastrointestinal tract [4]. Lead accumulates primarily in the bones, liver and kidney of animals. Depending on the level of exposure, lead can harm the nervous and immune systems, cause reproductive impairment and alter calcium homeostasis [5]. Mercury intoxication can result from inhalation, ingestion, or absorption through the skin. Previous studies demonstrated that mercury caused renal toxicity, neurotoxicity, reproductive toxicity and hematotoxic effects [6]. Mercury, may cause accidental and occupational exposures and serious damage in various organs in human and experimental animals [7].

Oxidative stress is induced by reactive oxygen species (ROS). Extreme levels of ROS can negatively impact on sperm quality, motility and increased sperm DNA damage. Therefore, equilibrium is required between the generation of ROS and antioxidant scavenging activity in the male reproductive organs [8]. Cells have developed defence mechanisms, including enzymatic systems, against oxidative damage. Enzymatic defence systems involve enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) [9]. SOD catalyzes the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen [10]. CAT catalyzes the reduction of H₂O₂ to water and oxygen and protects the cell against H₂O₂ induced oxidative damage [11]. The GPx and GST catalyse the reduction of H₂O₂ to H₂O and the conjugation of electrophilic substrates to the thiol group of GSH so it produces less toxic forms, respectively [12,13]. Male germ cells are more susceptible to oxidative stress than other cells, because they have higher polyunsaturated fatty acids in their membranes than somatic cells. Oxidative stress plays an important role in the etiology of defective sperm formation, function, sperm count profile and male infertility [14]. So, in this study, we investigated the possible adverse effects of lead nitrate and mercury chloride on the testis tissue of rats in terms of oxidative stress. For this purpose, rats were given lead nitrate and mercury chloride by oral gavage for 4 weeks, then malondialdehyde (MDA) levels, SOD, CAT, GPx and GST activities also histopathological changes of testis tissues were assessed.

MATERIALS AND METHODS

CHEMICALS

The heavy metals lead nitrate and mercury chloride, and all the other chemicals were purchased from Sigma Aldrich. Lead nitrate and mercury chloride were dissolved in distilled water [15,16].

ANIMALS

Sexually mature male Wistar rats were obtained from the Gazi University Laboratory Animals Growing and Experimental Research Center. The rats were acclimated to the laboratory environment (18-22 °C and a 12 h light/ dark cycle) for 10 days before use. All animals were housed in plastic cages and given standard laboratory chow and water ad libitum.

EXPERIMENTAL PROCEDURE

The animal treatment protocol was approved by the Gazi University Animal Experiments Local Ethics Committee (Protocol no: G.U.ET – 13.011). All animal experiments were performed according to the international guidelines for care and use of laboratory animals.

Rats were randomly divided into four treatment groups, each with six rats. The first group served as a control and was administered 1ml/kg b.w (body weight) distilled water. The second group was dosed with lead nitrate dissolved in distilled water by gavage at the dose level of 45 mg/kg b.w (1/50 LD₅₀) [16,17]. The third group was treated with mercury chloride at the dose level of 0.02 mg/kg b.w (1/50 LD₅₀) [15]. The forth group were exposed to lead nitrate+mercury chloride (45 mg/kg b.w lead nitrate + 0.02 mg/kg b.w mercury chloride). The tested doses of lead nitrate and mercury chloride were administrered for 4 weeks.
At the end of the 4th week, the rats were weighed and sacrificed and the testis tissues were excised.

HISTOLOGICAL EXAMINATION
The organs were fixed in Bouin’s solution. Then samples were processed using a graded ethanol series and embedded in paraffin. Parafin sections 4-6 μ thick were stained with haematoxylin and eosin for histopathological examination and photographed using a light microscope (Olympus BX51, Tokyo, Japan) with an attached camera (Olympus E-330, Olympus Optical Co. Ltd., Japan). Ten slides were prepared from each testis. All sections were evaluated for the degree of separating of cells from basal region, edema in interstitial tissue, degenerative changes in seminiferous tubules and decreasing number of spermatogenic cells. The severity of changes was assessed for each slide by scoring using a scale of no (-), mild (+), moderate (++), and severe (+++) damage.

MEASUREMENT OF MDA LEVELS AND ANTIOXIDANT ENZYME ACTIVITIES
Testis MDA levels were assayed using the thiobarbituric acid test as described by Ohkawa et al. [18] at 532 nm. The level of MDA is defined as nmol/mgprotein.

The SOD activity was estimated in tissue homogenate by the method of Marklund and Marklund [19] at 440 nm. Testis GST activity was determined according to the procedure of Habig et al. [20] at 340 nm. CAT and GPx activities were assayed by the method of Aebi [21], Paglia and Valentine [22] respectively at 240 nm. The activities of GST and GPx were defined as nmol/mgprotein, CAT activity was defined as μmol/mgprotein, SOD activity was defined as U/mgprotein.

STATISTICAL ANALYSIS
The data are expressed as the mean ± SD and were analyzed by a one-way analysis of variance (ANOVA) test followed by Tukey. The level of significance was set at P < 0.05. The program SPSS for Windows 20.0 was used to analyze the data. Dependence of various data sets was characterized by calculating their Pearson’s correlation. The closer the coefficient is to either -1 or 1 the stronger the correlation between the variables.

RESULTS

Malondialdehyde (MDA) Levels
When the lead nitrate and mercury chloride treated groups were compared with the control group at the end of 4th week, there were significantly increasing in the MDA levels in the testis tissues. The MDA levels were statistically significantly higher in the mercury chloride treated group compared to lead nitrate treated group. Treated with combination of lead nitrate and mercury chloride caused more harmful effects than use of them alone (P < 0.05, Fig. 1).

Figure 1: Effects of subacute treatment of lead nitrate and mercury chloride on MDA levels in the testis tissues of rats. Each bar represents mean±SD of six animals in each group. Significance at P<0.05.

aComparison of control and other groups.
bComparison of lead nitrate group and other groups.
cComparison of mercury chloride group and other groups.

Superoxide Dismutase (SOD) Activity
Compared to the control group, there were statistically significantly increased in the SOD activity in the lead nitrate and mercury chloride treated groups at the end of the experimental period. In addition to this, SOD activity was higher in mercury chloride treated group compared with the lead nitrate treated group. In combination with lead nitrate and mercury chloride caused more damages than use of them alone (P < 0.05, Fig. 2).
**Catalase (CAT) Activity**
A significant increasing in CAT activity was observed in the lead nitrate and mercury chloride treated groups compared with the control group. Also significant increasing were observed in the mercury chloride treated group compared with the lead nitrate treated group in testis tissues. Lead nitrate+mercury chloride group has the highest CAT activity between all groups (P < 0.05, Fig. 3).

**Glutathione Peroxidase (GPx) Activity**
The lead nitrate and mercury chloride groups showed significantly increasing in the GPx activity compared with the control group. We observed more increasing in mercury chloride treated group than lead nitrate treated group in testis tissues. The highest GPx activity was observed in lead nitrate + mercury chloride group among all the groups (P < 0.05, Fig. 4).

**Glutathione S-transferase (GST) Activity**
GST activity was statistically significantly increased in the lead nitrate and mercury chloride treated groups compared to the control group. Mercury chloride increased the GST activity more than lead nitrate. A significant increasing in GST activity were observed in the lead nitrate+mercury chloride group compared with the all groups. (P < 0.05, Fig. 5).

**Correlation Coefficients**
Correlation coefficients illustrate quantitative measure of some type of correlation and dependence, meaning statistical relationships between two or more variables or observed data values. Correlation coefficients ($R_P$) between MDA concentration and other parameters were shown in Table 1. The closer the coefficient is to either -1 or 1 the stronger the correlation between the variables. Strong positive correlation was observed between MDA values and activities of antioxidant enzymes.

**Table 1:** Correlation coefficients ($R_P$) between MDA concentration and other parameters. $R_P > 0.9$ values are in bold, $0.9 > R_P > 0.8$ values are in italics.

<table>
<thead>
<tr>
<th>Antioxidant Enzyme</th>
<th>Correlation Coefficient ($R_P$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>0.982*</td>
</tr>
<tr>
<td>CAT</td>
<td>0.993*</td>
</tr>
</tbody>
</table>

*Correlation coefficients illustrate quantitative measure of some type of correlation and dependence, meaning statistical relationships between two or more variables or observed data values. Strong positive correlation was observed between MDA values and activities of antioxidant enzymes.
Effects of lead and mercury in rat testis


<table>
<thead>
<tr>
<th>GPx</th>
<th>0.914*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST</td>
<td>0.876a</td>
</tr>
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</table>

a Significant correlation at p < 0.05.

Histological Examination
The histopathological assessments performed on testicular sections from the control and treatment groups are presented in Figures 6, 7, 8 and 9. The testicular architecture of the control animals was normal, as characterized by complete spermatogenesis (Fig. 6). Distinct histopathological abnormalities were observed in the testis tissues of lead nitrate and mercury chloride treated rats (Figs. 7 and 8). An increment in the separating of cells from basal region, edema in interstitial tissue, degenerative changes in seminiferous tubules and decreasing number of spermatogenic cells were observed in the testis tissues in lead nitrate + mercury chloride treated group (Fig. 9). Table 2 shows the percentages of treated rats that showed abnormalities observed in the testis tissues.

Figure 6: Testicular sections of control rats showing seminiferous tubules (S) and interstitial tissue (I), x200, H&E

Figure 7: Testicular sections of lead nitrate treated rats showing separating of cells from basal region (→), edema in interstitial tissue (*), degenerative changes in seminiferous tubules (▲) and decreasing number of spermatogenic cells (>), x200, H&E

Figure 8: Testicular sections of mercury chloride treated rats showing separating of cells from basal region (→), edema in interstitial tissue (*), degenerative changes in seminiferous tubules (▲) and decreasing number of spermatogenic cells (>), x200, H&E

Figure 9: Testicular sections of lead nitrate+mercury chloride treated rats showing separating of cells from basal region (→), edema in interstitial tissue (*), degenerative changes in seminiferous tubules (▲) and decreasing number of spermatogenic cells (>), x200, H&E
Table 2: Grading of the histopathological changes in the testis sections

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Lead nitrate</th>
<th>Mercury chloride</th>
<th>Lead nitrate + Mercury chloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>separating of cells from basal region</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>edema in interstitial tissue</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>degenerative changes in seminiferous tubules</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>decreasing number of spermatogenic cells</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

Scoring was done as follows: (-) none, (+) mild, (++) moderate, (+++) severe

DISCUSSION

There are many studies which have indicated that lead and mercury exposure could cause biochemical and physiological dysfunctions in experimental animals and humans \([23,24]\). Recently, oxidative stress has become the focus of interest as a potential cause of male infertility \([8]\). Reactive oxygen species (ROS) are the subject of intense research because of their effects on cellular pathogenesis \([25]\). ROS are supposed to play one of the key roles in the development of testis toxicity which is a sporadic and challenging issue for pharmaceutical drug development \([26,27]\). So, we studied the harmful effects of lead nitrate and mercury chloride on testis tissues in terms of oxidative stress.

The measure of MDA could be useful diagnostic tool for estimation of oxidative stress. MDA is one of the products of peroxidized polyunsaturated fatty acids. So, increased MDA level is an important indicator of lipid peroxidation \([9,28,29]\). Previous studies show that heavy metals increased MDA levels in several rat tissues \([24,30,31]\). In our study, MDA levels also increased in the lead nitrate and mercury chloride treated groups. So, it might be due to lipid peroxidation effects of lead and mercury. Our result reveals that lead nitrate-mercury chloride treated rats had the highest concentration of MDA in the testicular tissues which indicates the generation of LPO and subsequently loss of membrane structure and function. Our results are thus in agreement with the findings of Xia et al. \([28]\), Apaydın et al. \([32]\) who reported an increase in testicular MDA levels in lead-treated rats relative to the control group. Xenobiotics exposure induced the lipid peroxidation and changed the activities of the antioxidant enzymes; SOD, CAT, GPx and GST. These scavenger enzymes are considered as the part of first line defense against ROS \([33]\). As shown in numerous studies, increased concentrations of MDA correlate with changing of enzyme activities \([31]\). Activities of SOD, GPx, CAT and GST are proven indicators of oxidative stress \([34,35]\). Also, these antioxidant enzymes are potential targets for heavy metal toxicity \([36,37]\). So we determined these parameters for understanding the toxicity of lead nitrate and mercury chloride. In our study the activities of CAT, SOD, GPx, and GST significantly enhanced in testis in lead nitrate and mercury chloride treated rats compared with normal rats. This finding is supported with the data of Apaydin et al. \([32]\). Mercury chloride showed more toxicity than lead nitrate except GST activity. In combination with lead nitrate and mercury chloride caused more damages than use of them alone. Oxidative damage of proteins by lead nitrate and mercury chloride exposure may lead to the structural alteration and functional inactivation of many enzymes and cell signaling receptors. Antioxidant enzyme activity changes reported in the present study may be due to the production of ROS. Because, Table 1 shows that MDA values and activities of antioxidant enzymes had strong positive correlation (For SOD \(R_P: 0.982\), for CAT \(R_P: 0.993\), for GPx \(R_P: 0.914\) and for GST \(R_P: 0.876\)). Also, previous investigations have shown that lead and...
mercurymay affect the cellular antioxidant defense and alter activities of antioxidant enzymes by inhibiting functional sulfhydryl groups, because they have high affinity for sulfhydryl groups in these enzymes resulting in toxic effects \cite{5,36}.

In previous studies, it was reported that heavy metals increased level of lipid peroxidation and also caused many histopathological alterations in several tissues like liver, kidney and brain \cite{31,36}. Heavy metals can pass through the blood testis barrier and induces testicular damage including degeneration of the spermatogenic and Leydig cells \cite{30}. Heavy metals may also affect male reproductive function \cite{14,38}. It has been demonstrated that mercury caused necrosis and disintegration of spermatocytes from basal membrane in testis tissues \cite{39} and lead caused necrosis in seminifer tubules, degenerative changes and edema in interstitial tissue \cite{25}. Lead nitrate and mercury chloride induced severe testicular toxicity as shown in the histopathological results, which associated with marked changes of biochemical results. In the present study, our investigation demonstrated that exposure to lead nitrate and mercury chloride induced histopathological changes of testis in concentrations of 1/50 LD₅₀. The histomorphological alterations such as separating of cells from basal region, edema in interstitial tissue, degenerative changes in seminiferous tubules and decreasing number of spermatogenic cells were shown of the testis of lead nitrate and mercury chloride treated groups. The results showed the histological changes were more abundant when compared mercury chloride treated rats with lead nitrate exposed rats. Demir et al \cite{9} indicated in their study that, an imbalance between ROS production and cellular antioxidant defences has been reported to occur in several pathological conditions. These pathological alterations which we obtained in this study in lead nitrate and mercury chloride treated groups may be due to increased ROS production which caused oxidative stress. In addition, light microscopic findings support the results of the MDA and antioxidant enzyme activity assays.

CONCLUSION

The present study demonstrated that oxidative stress generated by lead nitrate and mercury chloride resulted in increasing of morphological alterations with the increasing in MDA levels and SOD, CAT, GPx and GST activities. Thus, present study indicates that a low doses of lead nitrate and mercury chloride cause testicular toxicity in male rats. It may be related to oxidative effects of mercuric chloride and lead nitrate on testis cell membrane and also testis tissues. More increasing in SOD, CAT, GST, GPx activities, MDA levels and histopathological changes were determined in mercury chloride group than lead nitrate group. Also we can say according to the data of this paper, treated with combination of lead nitrate and mercury chloride caused more harmful effects than use of them alone.

Conflict of Interest

The authors declare that they have no conflict of interest.

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