Comparison of the protective effects of garlic (*Allium sativum* L) extract, vitamin E and N acetyl cystein on testis structure and sperm quality in rats treated with lead acetate

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SUMMARY

The deterioration of male reproductive function is one of the major manifestations of lead exposure. For exploring the protective effects of some antioxidants (garlic extract, vitamin E and N acetyl cystein) on testicular lead toxicity, 25 healthy adult male rats were divided into 5 equal groups: the first group was reserved as the not exposed and not treated group (negative control) whereas the second group served as positive control (lead acetate administration in drinking water (1000 ppm) for 28 days), and rats from the 3 last groups were intoxicated with lead acetate but treated with aqueous garlic extract (500 mg/kg/day by gavage), vitamin E (350 ppm in the ration) and N acetyl cystein (800 ppm in the ration), respectively. Sperm quality, testis stereologic, morphometric and histological changes were assessed on day 28. Lead poisoning has significantly affected sperm quality (reduction in epididymal sperm count, in sperm motility and viability), reduced seminiferous tubules characteristics (histological proportions, epithelium height and volume density) and induced necrotic changes mainly in secondary spermatocytes. Sperm viability was significantly increased with garlic or vitamin E treatment and epididymal sperm count with N acetyl cystein treatment. Significant increases in the seminiferous tubule epithelium height and volume density as well as in the proportions of seminiferous tubules within testis (only with the vitamin E) were observed with antioxidant co-treatments. However, histological lesions were still present albeit diminished. These results show that testis lead toxicity was mediated by oxidative stress and that garlic extract may act as an antioxidant such as vitamin E and N acetyl cystein, partially preserving sperm quality and tubule histological organisation.

Key-words: Male rat, garlic, sperm quality, testis, stereology, morphometry, histology, spermatocyte, vitamin E, N-acetyl cystein

Introduction

Lead (Pb) is one of the well-known ubiquitous non-essential metals, poisoning the environment. Thus, it is obvious that lead exposure is implicated in serious health hazards in animals and humans [18]. The deterioration of male reproductive health is one of the major manifestations of occupational and/or environmental exposure to lead [21]. NAHA *et al.* [25] suggested that men working in lead based factories showed poor sperm production in terms of quality and density. BISWAS and GHOSH [6] demonstrated that lead exposure reduces the activities of testicular steroidogenic
enzymes in rats. Recent studies showed that lead inhibits activities of antioxidant enzymes, including glutathione peroxidase, catalase and superoxide dismutase [12].

Garlic (*Allium sativum* L.) is a versatile vegetable often used as ingredient in many dishes for flavour, aroma and taste enhancement [31]. It is a good source of dietary phytochemicals with proven antioxidant properties and ability to modulate the detoxification systems [26]. Some researchers isolated and identified several flavonoids and sulphur-containing compounds (diallyl sulphide, trisulphide and allyl-cystein) in garlic [23]. These are likely to play an important role in the widely demonstrated biological effects of garlic, which include anti-tumour [10], hypolipidemic, hypocholesterolemic, antiatherosclerotic, antioxidant [8] and immunomodulatory [7] effects.

Some previous investigations demonstrated that garlic organo-sulphur compounds prevent toxicity induced by cyanide, sodium nitrite [11], carbon tetrachloride [22], ethanol [19] and sodium arsenate [8]. The toxicity mechanism for all of them entails oxidative stress and impairment in the antioxidant defence systems. Based on this fact, the following discussion will provide the therapeutic potential of *A. sativum* in prevention of lead acetate induced toxicity on various systems. This study was carried out to investigate the possible protective properties of *A. sativum* extract on lead-induced toxicity in rat testis.

**Materials and Methods**

**AQUEOUS GARLIC EXTRACT, ANIMALS, PROTOCOL DESIGN**

Fresh garlic was collected in August 2010 from the University plant garden, Tabriz, Iran. It was recognized and authenticated by an agricultural engineer and the voucher kept in the herbarium of university. Peeled garlic cloves (5 g) were crushed mechanically in a mortar-pestle for 1 minute, together with 10 mL distilled water. The crushed material was carefully decanted by pressing through cheesecloth (yield was 500 mg/mL). The aqueous garlic extract (AGE) was freshly prepared for the experiment and diluted accordingly.

A total of 25 healthy adult male albino Wistar rats weighing 210 ± 10 g, obtained from the University of Tabriz animal house was housed in individual stainless steel cages at 23 ± 1°C and exposed to 12 hours light/dark cycle. They had access to a standard rodent laboratory diet and drinking water *ad libitum* throughout the whole experimental period.

After one-week long acclimatization period, they were randomly divided into 5 equal groups (containing each 5 animals) according to the dietary treatments applied for 4 weeks: rats from the group 1 served as untreated controls and were fed with the standard diet; rats from the other 4 groups were orally treated with lead acetate (1000 ppm in drinking water) and animals in the groups 3, 4 and 5 were additionally treated with the aqueous garlic extract (500 mg/kg/day) by gavage, vitamin E (350 ppm mixed with the daily ration) and N-acetyl cystein (800 ppm mixed with the daily ration), respectively. The animals were observed daily for signs of toxicity. Food intake and body weights were recorded daily during the experimental period. At the end of experimental period (on the day 28), animals were given rest overnight and then on the next day, they were sacrificed under light ether anaesthesia.

**ASSESSMENT OF TESTICULAR FUNCTIONS**

**Epididymal sperm concentration and sperm motility:**

The epididymal sperm count and sperm progressive motility were evaluated by the following method [17]: Epididymal spermatozoa were obtained by mincing the epididymis with anatomical scissors in 5 ml of Ham's F12 medium and incubated at 32°C for 2 minutes. An aliquot of this solution was placed in Neubauer haemocytometer and motile sperms were counted by using light microscope at x 400 magnification. Non-motile sperm numbers were first determined, followed by counting total sperm. Sperm motility was expressed as a percent of motile sperm from the total sperm counted. Percentage of morphologically abnormal spermatozoa was determined by the method described by EVANS and MAXWELL [13]. According to this method, slides were prepared with Wells and Awa stains for morphological examination and 1% eosin B and 5% nigrosine in 3% sodium citrate dehydrate solution for live / dead ratio was used. A total of 400 sperm cells were counted on each slide under light microscope at x 400 magnification.

**Testicular stereology and histopathology:**

One of the testes of each animal was fixed in neutral buffered 10% formalin and then microscopic slides were made of them using haematoxylin and eosin staining.

Testicular stereology (semiferous tubules diameter, semiferous epithelium height, volumetric proportion of tubules and quantification of Sertoli and Leydig cells per gram of testis) was evaluated by light microscopy. To obtain the height and tubular diameter of the semiferous epithelium, 30 histological cross sections of the semiferous tubules were analyzed, contoured as circular as possible, and randomly selected at x 400 magnification per animal. The volume density of testicular components was determined with a 441- intersection grid, where 20 fields were analyzed for each animal, at x 400 magnification, totalling 8820 points of assessment. The percentage occupied by each element in testicular parenchyma and the total testicular volume were used to obtain the volume of testicular components (semiferous epithelium, lumen, Leydig cells). As the testicular density is very close to 1 (1.03-1.04), the testicular weight was considered to be equal to its volume.
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Cell quantification:

The calculation of the total length of the seminiferous tubules (LST) was conducted based on total volume of the seminiferous tubule (TSV) according to the formula provided by ATTAL and COUROT [3]: 

\[ \text{LST} = \frac{\text{TSV}}{\pi \times R^2} \]

In 20 cross sections of seminiferous tubules, the number of nucleoli of Sertoli cells was quantified. With the LST and number of nucleoli of Sertoli cells (S) per seminiferous tubule cross section, it was possible to calculate the total number of Sertoli cells (TNSC) per testis using the formula provided by HOUCHERAU-DE-REVIER and LINCOLN [16]: 

\[ \text{TNSC} = \frac{\text{LST} \times n^0}{\text{corrected from } S \text{ per transverse section/thickness section}} \]

To find TNSC/testis, the calculated value was divided by the testis weight. To quantify the total number of Leydig cells in testis, the method proposed by PAULA et al. [28] was used. With the volume of a Leydig cell and the total volume of these cells in testis, it was possible to obtain their total number per testis and per gram of testis.

STATISTICAL ANALYSIS

Data are expressed as means ± standard deviations (SD). Differences among the experimental groups were assessed by one-way ANOVA followed by Tukey test using SPSS-version 19 –Software. Values were considered statistically significant when \( p < 0.05 \).

Result

No clinical signs were recorded in rats exposed to lead poisoning. No significant differences in body weight, weight gains and food intake were evidenced between groups.

As shown in Table I, although the relative testis weights have not significantly differed among groups, the sperm quality was significantly altered in lead exposed rats: the epididymal sperm count, the sperm motility and the sperm viability were significantly depressed compared to the not exposed controls \(( p < 0.01)\). When rats were treated with

<table>
<thead>
<tr>
<th>Negative Control</th>
<th>Positive controls</th>
<th>+ AGE</th>
<th>+ Vit. E</th>
<th>+ NAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSI (10^6/mL)</td>
<td>0.57 ± 0.04</td>
<td>0.59 ± 0.04</td>
<td>0.61 ± 0.08</td>
<td>0.66 ± 0.03</td>
</tr>
<tr>
<td>ESC (10^6/mL)</td>
<td>33.8 ± 2.1\text{a}</td>
<td>19.3 ± 1.4\text{c}</td>
<td>25.2 ± 1.3\text{b}</td>
<td>24.1 ± 0.8\text{bc}</td>
</tr>
<tr>
<td>Sperm motility (%)</td>
<td>72.7 ± 1.5\text{c}</td>
<td>62.3 ± 0.7\text{c}</td>
<td>66.7 ± 0.8\text{b}</td>
<td>69.8 ± 1.8\text{b}</td>
</tr>
<tr>
<td>Sperm viability (%)</td>
<td>90.8 ± 1.0\text{c}</td>
<td>73.2 ± 1.2\text{c}</td>
<td>82.5 ± 1.5\text{b}</td>
<td>80.2 ± 1.3\text{b}</td>
</tr>
</tbody>
</table>

AGE: aqueous garlic extract; NAC: N-acetyl-cystein; TSI: testicular-somatic index given with the formula: testes weight/total body weight; ESC: Epididymal sperm count. Different superscripts a,b,c in the same row indicate significant differences \(( p < 0.05 \) or more) among groups.

Table I: Changes in sperm quality and testes weight in rats orally exposed to lead (1000 ppm in drinking water) and eventually orally treated with aqueous garlic extract (500 mg/kg/day), vitamin E (350 ppm mixed with the daily ration) and N-acetyl-cystein (NAC, 800 ppm mixed with the daily ration) for 28 days compared to rats not exposed to lead (negative controls). Results are expressed as means ± standard deviations.

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>PST (%)</td>
<td>87.7 ± 6.4\text{a}</td>
<td>76.3 ± 5.1\text{b}</td>
<td>80.4 ± 0.9\text{a}</td>
<td>81.6 ± 2.2\text{b}</td>
</tr>
<tr>
<td>STD (μm)</td>
<td>265.4 ± 20.6</td>
<td>255.3 ± 12.9</td>
<td>257.5 ± 11.7</td>
<td>261.3 ± 12.6</td>
</tr>
<tr>
<td>STEH (μm)</td>
<td>74.3 ± 3.6\text{a}</td>
<td>58.8 ± 2.1\text{b}</td>
<td>66.5 ± 3.5\text{b}</td>
<td>68.5 ± 3.7\text{b}</td>
</tr>
<tr>
<td>STEVD (%)</td>
<td>55.5 ± 4.1\text{a}</td>
<td>46.0 ± 1.0\text{b}</td>
<td>51.5 ± 2.4\text{b}</td>
<td>52.5 ± 1.4\text{b}</td>
</tr>
<tr>
<td>Sertoli cells (%)</td>
<td>1.3 ± 0.6\text{a}</td>
<td>1.5 ± 0.3\text{c}</td>
<td>1.5 ± 0.3\text{b}</td>
<td>1.5 ± 0.2\text{b}</td>
</tr>
<tr>
<td>Leydig cells (%)</td>
<td>1.1 ± 0.5</td>
<td>0.8 ± 0.3</td>
<td>0.8 ± 0.5</td>
<td>0.9 ± 0.7</td>
</tr>
</tbody>
</table>

AGE: aqueous garlic extract; NAC: N-acetyl-cystein; PST: proportions of seminiferous tubules expressed from the total testis mass; STD: seminiferous tubule diameter; STEH: seminiferous tubule epithelium height; STEVD: seminiferous tubule epithelium volume density. Different superscripts a,b,c in the same row indicate significant differences \(( p < 0.05 \) or more) among groups.

Table II: Stereologic and morphometric values of testis tissue in rats orally exposed to lead (1000 ppm in drinking water) and eventually orally treated with aqueous garlic extract (500 mg/kg/day), vitamin E (350 ppm mixed with the daily ration) and N-acetyl-cystein (NAC, 800 ppm mixed with the daily ration) for 28 days compared to rats not exposed to lead (negative controls). Results are expressed as means ± standard deviations.
garlic, vitamin E or N acetyl-cysteine, the sperm quality was slightly improved but remained globally lower than in negative controls (not exposed to lead). In garlic treated rats, the sperm viability was significantly increased compared to rats only exposed to lead \( (p < 0.01) \) but still remained significantly lower than the control value \( (p < 0.05) \) and the epididymal sperm count and the sperm motility, albeit numerically increased, were not significantly different to values observed in lead exposed rats. In rats treated with vitamin E, while the epididymal sperm count remained low, the sperm motility has significantly increased compared to poisoned rats \( (p < 0.05) \) and reached values closely related to the control value and the sperm viability was also significantly higher than in positive controls \( (p < 0.01) \) but has remained depressed compared to the not exposed rats \( (p < 0.05) \). With N acetyl dietary supplementation, sperm motility and viability remained significantly low compared to the negative controls \( (p < 0.05) \) and did not significantly differ from values recorded in poisoned rats and only the epididymal sperm count has significantly increased \( (p < 0.05) \).

Stereologic and morphometric criteria measured in testis tissue were summarized in Table II. It was observed that lead caused marked decreases in all measured parameters although differences in seminiferous tubule diameter and in Leydig cell percentages between the poisoned rats and the negative controls were not significant because of high value dispersion. Oral treatments with garlic, vitamin E and N acetyl cysteine have significantly improved stereologic and morphometric testis parameters compared to the lead exposed rats (except for the proportions of seminiferous tubules with garlic and N acetyl cysteine treatments) \( (p < 0.05) \) but have not completely restored the cellular organization of testis (differences with the negative controls were significant: \( p < 0.05) \).

Hyperaemia and oedema in interstitial tissue (figure 1), decrease in germinal epithelial cell number, single cell necrosis, partial necrosis in some tubules with karyorrhexis specially in secondary spermatocytes (figure 2), tissue debris in some tubules (figure 3), vacuolar degeneration (figure 4) and germinal epithelium detachment from basement membrane (figure 5) were observed. Secondary spermatocytes were the most vulnerable cells among all cell classes. These lesions were most prominent in rats only exposed to lead but were also observed in exposed animals treated with garlic, vitamin E or N acetyl cysteine.

**Discussion**

The results of the present research showed that the lead exposure in drinking water caused significant reductions in epididymal sperm concentration, suppression of sperm progress motility, and live/dead sperms ratio. Many studies on reproductive system of male animals have documented lead as a toxicant for testicular tissue and functions \[4, 29\] such as significant reductions in the number of spermatozoa.
within the epididymis in mice received lead acetate (0.25% and 0.50%) in drinking water [34]. One possible explanation is that these compounds may be toxic to testicular histological structure. Second explanation is that lead may have a direct influence on sperm quality. Moreover, the decrease in sperm motility can be due to indirect effects of lead, like increase of ROS (reactive oxygen substances) generation in sperm cells. By causing lipid oxidation, ROS alter the integrity and the fluidity of cellular membrane structures, particularly of cell membrane which is essential for sperm motility, structural integrity, and ultimately for sperm viability [1, 5, 35]. This finding is in agreement with other reports who reported that administration of lead acetate resulted in an obvious decline in sperm quality [20]. In another study, where lead acetate (10 mg/kg of body weight) was administered to rats once a week for 6 and 9 weeks, a decrease in sperm counts and absolute concentration of motile sperms was described in the latter period [17].

In the current study, treatments with garlic or vitamin E resulted in a significant improvement in sperm parameters in rats exposed to lead. In this regard, NUUTILA et al. [26] hypothesized that constituents of garlic extract and vitamin E inhibited lipid peroxidation and preventing reduced glutathione depletion. The postulated roles of garlic organo-sulphur compounds in prevention of lead toxicity can be explained by firstly their ability in free radical scavenging and secondly the prevention of GSH depletion [30]. Moreover, it can be suggested that the ameliorative potential of garlic was probably due to the combined effects both on metal absorption and excretion from the body. HANAFY and SOLTAN demonstrated that vitamin E sustained the sperm motility in rats exposed to lead more efficiently than in those exposed to cobalt or mercury [15]. Vitamin E could also improve the sperm motility in humans: treatment of astheno-spermic patients with oral vitamin E significantly decreased the lipid peroxidation in sperm and improved sperm motility [32]. Vitamin E allows free radicals to abstract a hydrogen atom from the antioxidant molecule rather than from polyunsaturated fatty acids, thus breaking the chain of free radical reactions, resulting in a marked decrease in the reactivity of free radicals [27]. Therefore, protective effect of vitamin E supplementation for the organism from toxic agents and free radical damages is a time consuming process.

Some studies indicate that the length of seminiferous tubules of testis is directly proportional to sperm production [24]. AL-OMAR et al. [2], HAMADOUCHE et al. [14] and CORPAS et al. [9] have reported that lead causes decrease in seminiferous tubules diameter in adult rats. Although the seminiferous tubule diameter was not significantly affected in the present study, the presented histological analysis indicated statistically significant decreases in seminiferous epithelium height and volume density, leading to significant reduction in the proportions of seminiferous tubules inside testis in lead poisoned rats. By contrast, oral treatment with antioxidants (vitamin E, garlic and N acetyl cystein at a lesser extend) succeeded in partly counteracting the deleterious effects of lead on seminiferous tubule dimensions by markedly increasing the tubule epithelium height and volume density and less evidently (for garlic and N acetyl cystein) the proportions of seminiferous tubules in testis.

Lead exposure produced pronounced testicular damages evidenced by histological alternations in testis include degeneration of seminiferous tubules, germ cell necrosis and vacuolar degeneration especially in secondary spermatocytes [33]. These findings correspond with the observations that showed lead acts as a spermicidal agent in the case of high exposure [33], leading to a dose-related suppression of spermatogenesis and serum testosterone concentration in the young adult male rats [14]. According to the presented results, secondary spermatocytes were the most affected germ cells in the treatment groups. Oral administrations of antioxidants (garlic, vitamin E and N acetyl cystein) have partly alleviated lead induced histological changes in testis but have not totally prevented them.
As a conclusion, it is evident that oral treatments with antioxidants such as the aqueous *Allium sativum* extract, vitamin E or N acetyl cysteine at a lesser extend, partially protected spermatogenesis and sustained sperm quality in animals chronically intoxicated with lead acetate by limiting oxidative stress but did not completely prevent necrotic histological alterations in tests.

**Acknowledgment**

The authors are thankful to the authorities of University of Tabriz for providing support to this study.

**References**


**Revue Méd. Vét., 2013, 164, 1, 27-33**
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