Curative Role of D-Penicillamine versus Combined Garlic and Silymarin Extracts on Lead-Induced Nephrotoxicity and Oxidative Stress in Adult Male Albino Rats

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Abstract

Background: Lead (Pb) poisoning is still an important global health problem, especially in developing countries. Lead has been extensively used for many years ago, and it will continue to be used in future.

Aim of work: This study was conducted to compare the curative role of D-penicillamine versus combined garlic and silymarin extracts for treating nephrotoxicity in adult male rats exposed to Pb for 3 months.

Material and methods: The study was conducted on 70 adult male albino rats divided into 7 groups each of 10 rats, where they received all chemicals by oral gavage. Groups I & II were the negative and positive control rats, respectively. Group III received 25 mg/kg/day D-penicillamine for 1 month (4th month). Group IV received combined garlic 20 mg/kg/day + silymarin 200 mg/kg/day for 1 month (4th month). Group V received 20 mg/kg/day Pb acetate for 3 months. Group VI received Pb acetate for 3 months followed by D-penicillamine for another 1 month, with the previously mentioned doses. Group VII received Pb acetate for 3 months followed by combined garlic & silymarin for another 1 month, with the previously mentioned doses. At the end of the experimental period (4 months), Pb-level in blood, urine & kidney tissue, kidney function tests (blood urea and serum creatinine) and serum malondialdehyde (MDA) & glutathione peroxidase (GSH-Px) were measured. Light microscopic examination of hematoxylin and eosin stained kidney tissue was performed to detect histopathological changes.

Results: Lead administration significantly elevated blood lead level (BLL), kidney lead level (KLL), urinary lead level (ULL) and malondialdehyde (MDA), while glutathione peroxidase (GSH-Px) showed significant decrease when compared with the negative control rats. In Pb treated group, histopathological examination of the kidney revealed extensive tubular damage by presence of necrotic epithelial cells, tubular degeneration, necrosis, cell swelling, mononuclear cell infiltration, and degenerated organelles. Kidney functions didn't show any significant differences among all groups. Treatment of rats by D-penicillamine or combined garlic & silymarin extracts resulted in significant decrease in Pb-levels and MDA with a significant increase in blood GSH-Px levels. Also, significant improvement of lead induced histopathological lesions in the kidneys was observed. Combined garlic and silymarin extract treatment showed more significant improvement than D-penicillamine treated Pb-administered groups, in most of the tested parameters.

Conclusion: Both D-penicillamine and combined garlic & silymarin extracts can reduce lead induced nephrotoxicity and oxidative stress. Moreover, garlic & silymarin curative effect is better than that of D-penicillamine.

Recommendations: Exposure of the population to lead must be controlled. Search for sensitive blood or urinary marker for early detection of kidney damage and further studies are needed to consider a combination of garlic and silymarin is effective in the treatment of lead-induced toxicities.

Keywords: Lead, D-penicillamine, garlic, silymarin, kidney.

Introduction

Lead (Pb) has been in use by human population for the past 5000 years. During this period lead production has been said to have increased from a mere 10 tons per annum to a staggering 1 million tons per year, a phenomenon that has accompanied industrial progress (Brown and Margolis, 2012). As a highly toxic heavy metal, the pollution and exposure risks of lead are of widespread concern for human health. It is well-documented to be one of the most dangerous and insidious poisons affecting humans and animals of all ages (Cao et al., 2015; Behmke et al., 2015; Godwill et al., 2015). A safe level of lead exposure has not been defined and it is widely accepted that even small quantities are harmful to human and other organisms (Mielke et al., 2013).
Nowadays, although the vast majority of its uses have disappeared, Pb is still present in many industrial activities such as car repair, manufacturing and recycling of batteries, lead paint removal, demolition, refining and smelter. Moreover it is used for maintenance of structures found in the open air as bridges or water towers, in solder of cans of food or beverages, glazed ceramic, and can be present in drinking water or in tobacco smoke as well as leaded gasoline. In addition environmental pollution by lead is persistent and widespread, affecting the population (Al-Rudainy, 2010; García-Lestón et al., 2010; Bi et al., 2015; Piade et al., 2015). Lead content in lipstick and other consumer products has become an increasing concern. In 2010, the United States Food and Drug Administration tested 400 lipstick samples and found a maximum Pb concentration of 7.19 part per million, which constitutes great risk for adults that chronically apply lipstick as well as instances where children might incidentally ingest lipstick products (Monnot et al., 2015).

The exposure routes for Pb are, mainly, through inhalation and ingestion, where adults absorb approximately 10% of ingested Pb through the gastrointestinal tract. Once absorbed, Pb is found in all tissues, but 90% of the load is deposited in bone, where it accumulates over the carrier’s lifetime and can act as an endogenous source of the metal (Padilha et al., 2011; Boisa et al., 2014).

It is well documented that lead can cause deleterious effects on the reproductive, haematological and cardiovascular systems, neurotoxicity, nephrotoxicity, genotoxicity and embryotoxicity in laboratory animals and humans (Anjum et al., 2011; Kayaalti et al., 2015; Skerfving et al., 2015; Szymańska-Chabowska et al., 2015). Lead exposure is an established cause of chronic kidney disease in adults and children, where adverse associations between blood Pb and kidney function have been observed (Lee & Kim, 2012).

Current treatment of Pb poisoning includes the use of chelating agents (Padilha et al., 2011). Penicillamine, a sulfhydryl containing amino-acid and a degradation product of penicillin, is a pharmaceutical chelating agent, where the D-isomer is used mainly as a chelating agent in heavy metal toxicity including Pb, mercury and copper poisoning (Flora and Pachauri, 2010).

Although several chelating agents have been shown to reduce Pb toxicity, some of them are burdened with undesirable side effects (Aaseth et al., 2015). Accordingly, Pb intoxication therapy is looking for the development of new therapeutic agents with different modes of action especially from the medicinal plants (Gargouri et al., 2013).

Recent studies have demonstrated and validated many medicinal properties attributed to garlic. Different types of garlic supplements like garlic powder (tablets), garlic extracts (capsules, tablets and liquid) and garlic oil (capsule) is commercially available; each being different in organosulfur compound profile. Extensive studies carried out on garlic have described the presence of two main classes of antioxidant machineries, namely flavonoids and sulfur-containing compounds as diallyl sulfide, trisulfide and allyl-cysteine (Zaidi et al., 2015). These are likely to play an important role in the widely demonstrated biological effects of garlic, which include antitumor, hypolipidemic, hypocholesterolemic, antiatherosclerotic, antioxidant and immunomodulatory effects (Schäfer and Kaschula, 2014; Mohammadi and Oshaghi, 2014).

Silymarin is a polyphenolic flavonoid derived from milk thistle (Silybum marianum). It contains a mixture of four flavonolignan isomers: silibinin (70–80%), silychristin (20%), silydianin (10%), and isosilybin (0.5%). Silibinin is therefore the major active constituent of silymarin and is responsible for its pharmacological activity. The results of several studies using animal models showed that Silymarin has anti-inflammatory, cytoprotective, neuroprotective and anticarcinogenic effects that suppress production of reactive oxygen species (ROS) and lipid peroxidation in blood and liver (Jain et al., 2011; Vaid et al., 2013; Oliveira et al., 2015; Yang et al., 2015).

The aim of the present study is to compare the curative role of D-penicillamine versus combined garlic and silymarin extracts on the nephrotoxic effects and oxidative stress of sub-chronic Pb administration in adult male albino rats.

**Material and methods**

This study is a part of a research project funded by the Project Management of Zagazig University.

**I-Material**

- **Chemicals**
  1) Lead-acetate [(C2H3O2)2Pb: Lead acetate in the form of white crystals manufactured by El Nasr Pharmaceutical Chemical Company.]
  2) D-Penicillamine: in the form of white crystalline powder obtained from Sigma-Aldrich Chemical Co.
  3) Aqueous garlic extract (AGE): was prepared at department of Pharmacognesy Faculty of Pharmacy- Zagazig University.
  4) Silymarin crude material: was prepared from the milk thistle plant at the Department of Pharmacognesy, Faculty of Pharmacy, Zagazig University.

- **Animals**

  Seventy adult male albino rats, of average weight between 180-200 gm, were obtained from the animal house of Faculty of Medicine, Zagazig University. Before commencing experimentation, the animals were subjected to 7 days period of acclimatization to be adapted to environment, to ascertain their physical well being and to exclude diseased animals. Food was offered in equal amounts to all rats in each cage and water was offered ad libitum in separate clean containers.
• **Ethical considerations of the study**
  
  The study was conducted according to the guide for care and use of laboratory animals.
  
  All ethically approved considerations for animal housing and handling were considered.
  
  The experimental protocol used followed the regulations for administration and for painless scarification of the experimental animals.
  
  The animals were acclimated in the animal house before the start of the study.

• **Kits**
  
  Bio-diagnostic kits for estimation of Pb-level, blood urea, serum creatinine, serum malondialdehyde (MDA) and glutathione peroxidase (GSH-Px) were used.

**II- Methods**

1) **Extraction of garlic (Figure-1)**

Garlic extract was prepared by homogenizing the required amount of garlic cloves in an appropriate volume of distilled water to prepare a concentration of 20 mg/ml. The homogenate was centrifuged at 3000 x g for 10 minutes to remove particulate matter and the supernatant fraction was used for the experiment (Shaarawy et al., 2009).

2) **Extraction of crude silymarin (Figure-1)**

Silybum marianum was extracted as described by Polyak et al. (2007). Briefly, fine powder of seeds was made in a blender. The seed powder were defatted in n-hexane 2–3 times and extracted with aqueous acetone. The extract was concentrated to remove acetone by using a rotary evaporator, and then washed by hexane again to remove hydrophobic impurities. The remaining concentrate was treated by 1% NaCl solution to remove water soluble impurities. The precipitate and solid obtained through drying were combined together to form crude silymarin. The crude silymarin was washed with aqueous ethanol and then dried completely to give the refined silymarin.

3) **Experimental design**

The rats were randomly divided into 7 groups each of 10 rats, where they received all treatments through oral gavage for 6 days/week:

- **Group I (negative control group)**
  
  Kept on normal diet and water for 4 months and used to estimate basic parameters.

- **Group II (positive control group):**
  
  Received 1 ml saline/rat/day for 4 months.

- **Group III (D-penicillamine group)**
  
  Received 1 ml saline/rat/day for 3 months, then D-penicillamine in a dose of 25 mg/kg dissolved in 1 ml saline for 1 month. D-penicillamine is administrated on empty stomach, then no meals are given unless passage of 1 hour after administration (Golalipour et al., 2007).

- **Group IV (combined garlic+silymarin group)**
  
  Received 1 ml saline/rat/day for 3 months, then a combination of 20 mg/Kg/day of garlic (Shaarawy et al., 2009) and 200mg/kg/day of silymarin (Soto et al., 2010) dissolved in 1 ml saline for 1 month.

- **Group V (Pb acetate group)**
  
  Received 1 ml saline/day for 1 month then, received 20 mg/kg/day lead acetate (Instito’ ris et al., 1999) dissolved in 1 ml saline, for 3 months.

- **Group VI (Pb acetate + D-penicillamine group)**
  
  Each rat received Pb acetate for 3 months followed by D-penicillamine for another 1 month, with the previously mentioned doses.

- **Group VII (Pb acetate + combined garlic & silymarin group)**
  
  Each rat received Pb acetate for 3 months followed by combined garlic & silymarin for another 1 month, with the previously mentioned doses.

4) **Urine Collection (Figure-2):**

At the end of the study (4 months) the animals were housed individually for 24 hours in metabolic cages and allowed to access water. Urine sample was collected from each rat by means of these metabolic cages (Kurien et al., 2004) enough cages were prepared.

5) **Blood collection:**

After urine collection, a volume of 2 ml venous blood samples were obtained from the retro-orbital plexus of anaesthetized animals (Johnson, 2007). These blood samples are collected in glass tubes, allowed to clot then centrifuged to collect the clear sera to perform all blood-related biochemical analysis.

6) **Kidney sampling:**

After blood collection the rats were sacrificed, both kidneys of each rat were divided into 2 parts, one part weighed 1 gm was wrapped with aluminum foil and embedded in liquid nitrogen for 1 hour then kept frozen in -80°C till used to asses lead concentration in kidney (Miller et al., 1987). The 2nd part was fixed in formalin 10 % solution for histopathological examination after staining with haematoxyline and eosin (H&E) according to Bancroft and Stevens (2002).

7) **Biochemical Studies:**

a) **Estimation of Lead Levels**

  Lead was estimated in the blood (BLLs), urine (ULLs) (µg/dL) and kidney tissues (KLLs) (µg/gm tissue) by graphite furnace atomic absorption spectrophotometry (Model 210 VGP, Buck Scientific USA) at the Central Laboratory, Faculty of Veterinary Medicine, Zagazig University, Lead loads were estimated at 217 nm wavelength, which was generated, from a lead cathode lamp, following the instrument’s manual instruction according to...
the method described by (Miller et al., 1987; AOAC, 1990).

b) Kidney function tests
Blood urea and serum creatinine were determined using the method proposed by Henry (1974).

c) Assessment of Blood Oxidative Stress:
I. Erythrocyte malondialdehyde (MDA) in blood
Lipid peroxidation was measured by estimating serum MDA according to the method proposed by Yoshioka et al. (1979).

II. Blood glutathione peroxidase (GSH-Px)
Glutathione peroxidase (GSH-Px) enzyme was determined in blood according to the method proposed by Pleban et al. (1982).

8) Statistical analysis
SPSS Software program was used. Mean values (X) ± Standard Deviation (SD) were calculated. Analysis of variance (ANOVA or F test), least significant difference (LSD) and Z-test for two samples proportion were performed. Differences were considered significant when P-value is less than 0.05.

Results
Death rate
Two rats died in group V, one rat in group VI and one rat in group VII.

Biochemical Results
No significant differences (p>0.05) were detected in all tested biochemical parameters among negative control (group I), positive control (group II), penicillamine-treated group (group III) and combined garlic & silymarin treated group (group IV) (table-1).

a) Lead Levels (Tables 2,3,4 &5)
For BLL, ULL & KLL, Significant elevations (p<0.05) were detected in all mean values of Pb-treated group (group V), D-penicillamine treated group after Pb (group VI) and combined garlic & silymarin treated group after Pb (group VII) when compared to negative control group (group I).

For BLL & KLL mean values, groups VI & VII showed significant decrease (p<0.05) compared to the corresponding value in groups V. Also, group VII showed significant decrease (p<0.05) in comparison with group VI.

For ULL mean values, groups VI & VII showed significant increase (p<0.05) compared to the corresponding value in group V. Moreover, group VII showed significant increase (p<0.05) in comparison with group VI.

b) Kidney Function Tests (Table-2)
No significant changes (P>0.05) were detected in the mean levels of blood urea & serum creatinine of groups V, VI and VII when compared to group I.

c) Blood Oxidative Stress:
i. Erythrocyte malondialdehyde (MDA) in blood (Tables 2 & 6)
Significant elevations (p<0.05) were detected in the mean MDA levels of group V and group VI compared to groups I and VII. Also, Significant elevation (p<0.05) was detected in the mean MDA levels of group V compared to group VI.

Non-significant change (p>0.05) was detected in the mean MDA level of group VII when compared to the control group I.

ii. Blood glutathione peroxidase (GSH-Px), (Tables 2 &7)
Significant decreases (p< 0.05) were detected in the mean GSH-Px levels of groups V, VI and VII compared to group I.

Significant increase (p<0.05) was detected in GSH-Px mean levels of groups VI & VII when compared to group V. However, non-significant change (p>0.05) was detected in GSH-Px mean level of group VII when compared to group VI.

Histopathological Results (Tables 8,9 &10)
Examination of H&E stained kidney sections of groups I, II, III and IV showed normal renal corpuscles consisting of Bowman’s capsule with an outer covering layer of simple squamous epithelium and an inner wall formed of flattened epithelial cells. The glomerulus enclosed within the Bowman’s capsule showed normal architectural pattern and the proximal convoluted tubules were numerous with round outlines (Figures 3 a, b & c).

In group V, Pb treatment caused extensive tubular damage, different degrees of cloudy swelling of epithelial cells of renal tubules, cell swelling and vacuolation, necrosis, mononuclear cell infiltration, congestion, homogenous acidophilic materials and contraction of the glomeruli with expansion of Bowman’s space (Figure 3 d, e & f). All mentioned pathological changes when statistically analyzed by Z-test, showed significant increase in comparison with the control group.

In group VI, treatment of Pb-administered rats with D-penicillamine displayed relieve of the renal pathological changes caused by lead except for congestion and moderate degree of inflammatory cells infiltration (Figure 3 g), which showed significant increase compared to the control group and group VII but showed non-significant change when compared with group V.

In group VII, treatment of Pb-administered rats with combined garlic & silymarin improved the histopathological changes which were observed in the lead treated group but, displayed mild degree of congestion which showed non-significant change when compared to control group, group V and group VI (Figure 3 h).
Table 1: One way ANOVA for statistical comparison of BLL, KLL, ULL, blood urea, serum creatinine, MDA and GSH-Px among negative control (Group I), positive control (Group II), D-penicillamine treated (Group III) and combined garlic & silymarin treated group (Group IV) during the whole period of the study.

<table>
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<td>BLL µg/dL</td>
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<td>10.61±1.94</td>
<td>9.98±2.65</td>
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<td>KLL µg/gm tissue</td>
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<td>0.891±0.041</td>
<td>0.887±0.049</td>
<td>0.884±0.050</td>
<td>0.856±0.088</td>
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<td>ULL µg/dL</td>
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<td>0.48±0.14</td>
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<td>0.217</td>
<td>0.884</td>
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<td>Blood urea</td>
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<td>27.21±3.28</td>
<td>27.14±3.28</td>
<td>26.79±2.597</td>
<td>27.26±3.17</td>
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<td>Serum creatinine</td>
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<td>0.93±0.211</td>
<td>0.95±0.193</td>
<td>0.94±0.184</td>
<td>0.94±0.207</td>
<td>0.025</td>
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<td>MDA µmol/L</td>
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<td>0.49±0.053</td>
<td>0.46±0.042</td>
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<td>GSH-Px U/L</td>
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<td>0.46±0.042</td>
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<td>2.035</td>
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M: mean value, SD: standard deviation, n: number of rats in each group.

Table 2: One way ANOVA for statistical comparison of BLL, KLL, ULL, blood urea, serum creatinine, MDA and GSH-Px among negative control (Group I), lead treated group (Group V), lead + D-penicillamine treated (Group VI) and lead + combined garlic & silymarin treated group (Group VII) during the whole period of the study.

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<td>BLL µg/dL</td>
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<td>11.13±1.14</td>
<td>29.51±7.4</td>
<td>21.44±3.6</td>
<td>395.647</td>
<td>0.000*</td>
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<td>KLL µg/gm tissue</td>
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<td>8.91±0.041</td>
<td>11.15±2.185</td>
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<td>121.597</td>
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<td>ULL µg/dL</td>
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<td>19.53±4.35</td>
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<td>Blood urea</td>
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<td>Serum creatinine</td>
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<td>MDA µmol/L</td>
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M: mean value, SD: standard deviation, n: number of rats in each group, *: significant change p>0.05.

Table 3: Least significant difference among negative control (Group I), lead treated group (Group V), lead + D-penicillamine treated (Group VI) and lead + combined garlic & silymarin treated group (Group VII) regarding BLL.

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<td>I</td>
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M: mean value, SD: standard deviation, n: number of rats in each group, *: significant change p<0.05.

Table 4: Least significant difference among negative control (Group I), lead treated group (Group V), lead + D-penicillamine treated (Group VI) and lead + combined garlic & silymarin treated group (Group VII) regarding KLL.

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M: mean value, SD: standard deviation, n: number of rats in each group, *: significant change p<0.05.
Table-5: Least significant difference among negative control (Group I), lead treated group (Group V), lead + D-penicillamine treated (Group VI) and lead + combined garlic & silymarin treated group (Group VII) regarding ULL.

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<td>-</td>
<td>-</td>
<td>0.0005*</td>
<td>-</td>
</tr>
</tbody>
</table>

M: mean value, SD: standard deviation, n: number of rats in each group, *: significant change p<0.05.

Table-6: Least significant difference among negative control (Group I), lead treated group (Group V), lead + D-penicillamine treated (Group VI) and lead + combined garlic & silymarin treated group (Group VII) regarding MDA level.

<table>
<thead>
<tr>
<th>Groups</th>
<th>I (M±SD)</th>
<th>V (M±SD)</th>
<th>VI (M±SD)</th>
<th>VII (M±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=10</td>
<td>n=8</td>
<td>n=9</td>
<td>n=9</td>
</tr>
<tr>
<td></td>
<td>0.491±0.053</td>
<td>2.01±0.412</td>
<td>0.748±0.041</td>
<td>0.485±0.057</td>
</tr>
<tr>
<td>I</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.8149</td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000*</td>
<td>-</td>
</tr>
<tr>
<td>VI</td>
<td>-</td>
<td>-</td>
<td>0.000*</td>
<td>-</td>
</tr>
</tbody>
</table>

M: mean value, SD: standard deviation, n: number of rats in each group, *: significant change p<0.05.

Table-7: Least significant difference among negative control (Group I), lead treated group (Group V), lead + D-penicillamine treated (Group VI) and lead + combined garlic & silymarin treated group (Group VII) regarding GSH-Px level.

<table>
<thead>
<tr>
<th>Groups</th>
<th>I (M±SD)</th>
<th>V (M±SD)</th>
<th>VI (M±SD)</th>
<th>VII (M±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=10</td>
<td>n=8</td>
<td>n=9</td>
<td>n=9</td>
</tr>
<tr>
<td></td>
<td>0.484±0.047</td>
<td>0.190±0.04</td>
<td>0.391±0.11</td>
<td>0.363±0.07</td>
</tr>
<tr>
<td>I</td>
<td>0.000*</td>
<td>0.0258*</td>
<td>0.0003*</td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>0.0002*</td>
<td>0.000*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VI</td>
<td>-</td>
<td>0.5285</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

M: mean value, SD: standard deviation, n: number of rats in each group, *: significant change p<0.05.

Table-8: statistical Z-test for two samples proportion comparing histopathological changes of the kidney in lead treated group versus negative control group.

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Group</th>
<th>I N2/N1</th>
<th>V N2/ N1</th>
<th>Proportion</th>
<th>Z value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloudy swelling of tubular epith.</td>
<td>0/10 6/8</td>
<td>0.75</td>
<td>3.3541</td>
<td>0.0008*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell vaculation</td>
<td>0/10 6/8</td>
<td>0.75</td>
<td>3.3541</td>
<td>0.0008*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell necrosis</td>
<td>0/10 4/8</td>
<td>0.50</td>
<td>3.5355</td>
<td>0.0118*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mononuclear cell infiltration</td>
<td>0/10 7/8</td>
<td>0.875</td>
<td>3.7839</td>
<td>0.0002*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vascular congestion</td>
<td>0/10 7/8</td>
<td>0.875</td>
<td>3.7839</td>
<td>0.0002*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wide bowman's space</td>
<td>0/10 6/8</td>
<td>0.75</td>
<td>3.3541</td>
<td>0.0008*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N1: total number of rats in each group, N2: total number of rats in each group, *: significant change at p<0.05.

Table-9: statistical Z-test for two samples proportion comparing histopathological changes of the kidney in lead + D-penicillamine treated group (group VI) versus negative control group (group I).

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Group</th>
<th>I N2/N1</th>
<th>VI N2/ N1</th>
<th>Proportion</th>
<th>Z value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloudy swelling of tubular epith.</td>
<td>0/10 0/9</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Cell vaculation</td>
<td>0/10 0/9</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Cell necrosis</td>
<td>0/10 0/9</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Mononuclear cell infiltration</td>
<td>0/10 4/9</td>
<td>0.444</td>
<td>2.3727</td>
<td>0.01778*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vascular congestion</td>
<td>0/10 5/9</td>
<td>0.5556</td>
<td>2.7458</td>
<td>0.00596*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wide bowman’s space</td>
<td>0/10 0/9</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

N1: total number of rats in each group, N2: total number of rats in each group, *: significant change at p<0.05.
Table -10: statistical Z-test for two samples proportion comparing histopathological changes of the kidney in lead treated group (group V) versus lead + D-penicillamine treated group (group VI).

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Group</th>
<th>N2/N1</th>
<th>proportion</th>
<th>N2/ N1</th>
<th>Proportion</th>
<th>Z value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloudy swelling of tubular epith.</td>
<td>V</td>
<td>6/8</td>
<td>0.75</td>
<td>0/9</td>
<td>0</td>
<td>3.2298</td>
<td>0.00124*</td>
</tr>
<tr>
<td>Cell vaculation</td>
<td>V</td>
<td>6/8</td>
<td>0.75</td>
<td>0/9</td>
<td>0</td>
<td>3.2298</td>
<td>0.00124*</td>
</tr>
<tr>
<td>Cell necrosis</td>
<td>VI</td>
<td>4/8</td>
<td>0.50</td>
<td>0/9</td>
<td>0</td>
<td>2.4258</td>
<td>0.0151*</td>
</tr>
<tr>
<td>Mononuclear cell infiltration</td>
<td>VI</td>
<td>7/8</td>
<td>0.875</td>
<td>4/9</td>
<td>0.444</td>
<td>1.8542</td>
<td>0.0643</td>
</tr>
<tr>
<td>Vascular congestion</td>
<td>VI</td>
<td>7/8</td>
<td>0.875</td>
<td>5/9</td>
<td>0.556</td>
<td>1.4428</td>
<td>0.1499</td>
</tr>
<tr>
<td>Wide bowman’s space</td>
<td>VI</td>
<td>6/8</td>
<td>0.75</td>
<td>0/9</td>
<td>0</td>
<td>3.2298</td>
<td>0.00124*</td>
</tr>
</tbody>
</table>

N1: total number of rats in each group, N2: total number of rats in each group, *: significant change at p<0.05.

Figure (1) A photograph showing garlic extract (arrow) and silymarin crude material (S).

Figure (2) A photograph showing metabolic cage for urine collection.
Figure (3): Photomicrographs of adult male albino rat's kidney: [a] from negative control group showing normal renal medulla, [b] from group III (D-penicillamine group) showing normal renal cortex, [c] from group IV (garlic & sylimarin group) showing normal histological features, [d] from group V (lead acetate group) showing disorganization, different degrees of cloudy swelling of epithelial cells of renal tubules (cs), vaculation (v), cellular necrosis (n) & inflammatory cells infiltration (i), [e] from group V (lead acetate group) showing wide bowman's space (wb), sloughing of glomerular and tubular epithelium (s) & congestion of the cortex (c), [f] from group V (lead acetate) showing congestion (c), inflammatory cells infiltration (i) and homogenous acidophilic materials (h), [g] from group VI (lead acetate and D-penicillamine group) showing congestion (c) & moderate cellular infiltration (i) [h] from group VII (lead acetate and garlic + sylimarin group) showing some congestion (c) (H&E X 400).

Discussion

Lead belongs to the group of metals, which do not play a significant role in metabolic processes, but interfere with a variety of body functions. It is one of the most toxic environmental pollutants; even minimal doses of lead can cause serious dysfunctions of cellular metabolism prior to constitutional symptoms (Kaczor et al., 2013; Li et al., 2015). Matović et al. (2015) stated that particularly the kidneys are among the most prominent target organs after long-term exposure to lead.
In the present study, administration of lead acetate to rats in a dose of 20 mg/kg/day for 3 months produced significant increase of BLL, KLL & ULL when compared to the control groups. These significant changes can be explained by Flora et al. (2006) stated that the absorbed Pb is conjugated in the liver and passed to the kidney, where a small quantity is excreted in urine and the rest accumulates in various body organs and affects many biological activities at the molecular, cellular and inter-cellular levels. Zheng et al. (2011) mentioned that determination of Pb in urine is considered to reflect Pb that has diffused from plasma and is excreted through the kidney. In addition, Grandjean and Herz (2015) stated that past and cumulated Pb exposure and its risk are most often monitored by Pb in blood and urine.

For the BLL, it showed significant increases that exceeded 111 μg/dL in the current study. Zhang et al. (2015) reported that as a result of environmental pollution, the normal reference range for blood Pb is <10 μg/dL. Many reviews stated that BLL as low as 2 μg/dL can cause adverse health effects to multiple organ systems including renal one (Fadrowski et al., 2010; Liu et al., 2012).

Kidney lead level in this study significantly increased when compared to the level in the control rats. This can be explained by Guimarães et al. (2012); who stated that among the soft tissues, liver and kidneys show substantially higher lead concentrations. These organs play a vital part in the detoxification and metabolism of toxic substances. Sabath and Robles-Osorio (2013) stated that the kidney serves as a major organ of Pb excretion and as a site of accumulation, as well as target organ of Pb toxicity and are under risk of damage due to the oxidative reaction of lead.

The previous biochemical finding of accumulated lead in the kidney is supported by the histopathological changes observed in rat kidneys of the current study. This is similar to the study of Guimarães et al. (2012) who found that lead intoxication caused proximal tubular dysfunction or irreversible nephropathy, where these changes depended on the exposure regimens.

The sub-acute intra-peritoneal administration of 10mg/kg/day of lead acetate for 15 days in the study of Minerva et al. (2013) induced toxic levels of lead in the blood and caused renal toxicity (pathological and functional). Histological changes observed in kidneys of treated rats included tubular necrosis, epithelial degeneration, tubular dilatation, and swelling. Proximal tubules lost their characteristic appearance and appeared irregular, deformed, and some epithelial cells of proximal tubules lacked brush borders.

The observed kidney findings are in agreement also with the findings of many other studies (Liu et al.; Wang et al.; Abdel Moneim et al., 2010; Binkowski et al., 2013). Moreover, Solidum (2014) reported that Lead is hard to biodegrade, once accumulated in the biologic system it leads to system deficits. The non significant change in blood urea and serum creatinine which observed after lead administration in the present study passes in agreement with cohort study of Weaver et al. (2009) in which higher lead dose was associated with lower serum creatinine in exposed lead workers. This can be explained according to Onopiuk et al. (2015) who stated that, common indicators of renal function lack necessary sensitivity and specificity. Moreover Wasung et al. (2015) stated that serum creatinine is limited as a marker of renal dysfunction and may be inaccurate in several situations and new sensitive biomarkers are needed in order to facilitate early diagnosis, guide interventions and monitor kidney disease progression. From the pathological point of view Galarreta et al. (2014) mentioned that renal parenchymal injury may begin early long before sufficient glomeruli and tubules have been destroyed to provoke a decline in glomerular filtration rate.

Regarding the mechanism of the histopathological changes occurred in the kidney, it can be explained by many studies showed that lead-induced kidney injury is associated with a production of ROS, thus inducing oxidative stress; excito-toxicity and DNA damage (Dai et al., 2010; Liu et al., 2012; gargouri et al., 2013). The fact that the kidney organ is rich in mitochondria indicates that it is highly vulnerable to damage caused by oxidative stress (Sureshbabu et al., 2015).

Similar mechanism of toxicity has been proposed by Baranowska-Bosiacka et al. (2012) for the effect of Pb-exposure on blood, Li et al. (2015) for the gonadotoxic and spermatoxic effects of lead and by Karamian et al. (2015) during investigation of the neurotoxicity of lead in albino rats, all were through the generation of excessive amount of ROS and alteration of antioxidant defense systems in animals, therefore it has been suggested that Pb-induced oxidative stress contributes to the pathogenesis of lead poisoning by disrupting the pro-/antioxidant. This proposed mechanism can explain the significant alterations in the levels of erythrocyte MDA and blood GSH-Px levels which occurred in the current study and passes in agree with El-Sayed et al. (2015); Roy et al. (2015).

In the present study, lead-exposed rats treated with D-penicillamine for 1 month showed significant drop in BLL and KLL with significant increase in ULL when compared to lead- treated rats. Also, D-penicillamine led to decrease in MDA and increase in GSA-Px levels with great improvement in the histopathological structure of the kidney tissue. These findings are in agree with Blanusa et al. (2005); Kianoush et al. (2012); Sisombath et al. (2014) reported that D-penicillamine which has the ability to bind lead and facilitate its excretion from the body is the old-established well-known antidote for the treatment of lead poisoning.

However the consequence of long-term D-penicillamine treatment is associated with numerous side effects such as seizures, myasthenia gravis, polymyositis, systemic lupus erythematosus, degenerative elastosis, membranous glomerulonephritis and pemphigus (Roy et al., 2011; Poulas et al., 2012; Koraishy et al., 2013; Rahimi et
cant drop in BLL and KLL; 25 age on cellular.

In the present study, lead-exposed rats treated with a combination of garlic and silymarin for 1 month produced significant drop in BLL and KLL when compared to lead- treated rats. At the same time, the combination of garlic and silymarin showed more significant improvement in BLL and KLL than D- penicillamine treated group. These findings were supported by increased ULL and decreased MDA level in the combined garlic and silymarin treated group compared to D-penicillamine treated group. Also, this combination led to significant improvement in the histopathological structure of the kidney tissue better than D-penicillamine has done. The observed curative effect of combined garlic and silymarin on lead induced nephrotoxicity and oxidative stress is supported by Ramirez-Garcia et al. (2015) who stated that various renal damages induced by lead poisoning can be reduced by antioxidant agents and Sabiu et al. (2015) stated that timely intervention with exogenous antioxidants augments the cellular defense system to prevent free radicals oxidative damage on cellular macromolecules.

Regarding the effect of garlic on lead-induced nephrotoxicity, it has been known that garlic, is a source of many different sulphuric compounds, which partially share metabolic pathways with phytochelatins, employing cysteine as a basic precursor for the synthesis of phytochelatin, thiosulphinate and sulphoxide (Soudek et al., 2011). Aslani et al. (2010) showed that blood and tissue lead concentrations in mice are reduced when they are treated simultaneously with oral allicin and DMSA, where they proposed that allicin acts as a chelating agent in the treatment of lead poisoning. Therefore, it is presumable that garlic ingredients (allicin or allin) through their biologically active agents, such as thiosulfinate or amino functional groups, act through similar mechanisms as those of chelators, like penicillamine, to facilitate the excretion of lead from the body. In the study of Kianoush et al. (2012) who compared the therapeutic effects of garlic and D-penicillamine in patients with chronic occupational lead poisoning, they found out that garlic was safer clinically and as effective as D-penicillamine.

In the present study the concomitant administration of silymarin extract to that of garlic caused a combined antioxidant effect led to improvement in the stress condition evoked by lead administrated to the rats. This was reflected in the significant decrease in MDA and increase in GSA-Px levels. Silymarin administration has been proved by Mannem (2014) to cause a recovered level of GSH when compared to lead-treated rats. Many studies proved that silymarin has the potential to attenuate the effect of nephrotoxic chemicals due to its anti-inflammatroy, antioxidant and anti-apoptotic properties (Dashti-Khavidaki et al., 2012; Hashmi et al., 2013; Gad and El-Maddawy, 2014; Hamza and Al-Harbi, 2015). Sabiu et al. (2015) mentioned that in drug- and chemically-induced oxidative stress, silymarin has been reported as the primary therapeutic modality of choice.

To explain the better curative effect offered by the combined garlic & silymarin extracts, research findings have suggested that administration of various antioxidants can prevent or subdue various toxic effects of lead and generation of oxidative stress in particular, where an antioxidant can prevent lead toxicity in three ways (Antonio-Garcia & Massó-González, 2008):
1. By inactivating the generated ROS at molecular level, thereby terminating the radical chain reaction (chain breaking).
2. By maintaining the lead in a redox state, which leads to its incompetency to reduce molecular oxygen.
3. By chelating the lead ion and preventing further formation of ROS.

Conclusions
This study demonstrates that a combination of garlic and silymarin has potent curative effects against Pb-induced nephrotoxicity by modulating the antioxidant pathway in rat kidney through inhibiting ROS generation and increasing kidney GSH level. Treatment with combination of garlic and silymarin also decreased the kidney Pb concentrations in rats and increased urinary excretion of lead. Moreover, Combination of garlic and silymarin offered better curative effect than D-penicillamine.

Recommendations
It is recommended to control the population exposure to the lead and find a sensitive blood or urinary marker for early detection of kidney damage. In addition further studies are needed for the use of a combination of garlic and silymarin and effectively replace D-penicillamine in the treatment of lead toxicities.

Acknowledgment
Sincere appreciation and deep gratitude to Professor Dr Iman A. El-Khishin the Project Manager and to the team members of the Project. Special thanks and sincere appreciation to Professor Dr Samia Salah Hafez, Prof. of Pharmacognesys, Faculty of Pharmacy- Zagazig University, for her accurate and kind help and support in performing garlic & sylimarin extraction. Great thanks to Dr Mona Gomaa, Lecturer of Histology, Faculty of Medicine- Zagazig University for her great help in reading histopathological results.

References


الدواء العلاجي للدبي-بيسيمالامين مقابل خلاصة الثوم والسيليمارين معا على التسمم الكلوي والإجهاد الناتجسي نتيجة للرصاص في ذكور الجرذان البيضاء البالغة

أمل محمد عبد الخالق و نيرمين عطية إبراهيم

� مازال التسمم بالرصاص يمثل مشكلة صحية عالمية خاصة في البلدان النامية. لقد استخدم الرصاص على نطاق واسع منذ سنوات بعيدة وسوف يستمر استخدامه في المستقبل.

يهدف هذا البحث إلى مقارنة الدور العلاجي للدبي-بيسيمالامين مقابل خلاصة الثوم والسيليمارين معا على التسمم الكلوي والإجهاد الناتجسي قادرين في ذكور الجرذان البيضاء البالغة بعد تعريضهما للرصاص لمدة ثلاثة أشهر.

وقد أجريت هذه الدراسة على سبعين من ذكور الجرذان البيضاء البالغة تقسيمهم إلى سبع مجموعات متساوية العدد وتم إعطاء الكيمائيات محل الدراسة عن طريق أنبوبة فم.

الكيمائيات المختلفة:
- المجموعة الأولى: مجموعة ضابطة سالبة
- المجموعة الثانية: ضابطة موجبة أعطيت ملي واحد من محلول الملح الفسيولوجي لمدة أربع شهور.
- المجموعة الثالثة: أعطيت الدبي-بيسيمالامين بجرعة يومية 25 مجم لكل كيلوجرام من وزن الجسم لمدة شهر.
- المجموعة الرابعة: أعطيت خلاصة الثوم والسيليمارين معا بجرعة يومية قدرها 20 مجم لكل كيلوجرام من وزن الجسم بالإضافة إلى 200 مجم لكل كيلوجرام من وزن الجسم من السيليمارين لمدة شهر.
- المجموعة الخامسة: أعطيت خلاصة الرصاص بجرعة يومية قدرها 20 مجم لكل كيلوجرام من وزن الجسم لمدة ثلاثة أشهر.
- المجموعة السادسة: أعطيت الدبي-بيسيمالامين بالإضافة إلى خلاصة الثوم والسيليمارين معا لمدة شهر.
- المجموعة السابعة: أعطيت خلاصة الرصاص بالإضافة إلى خلاصة الثوم والسيليمارين معا لمدة شهر.

و في نهاية فترة الدراسة، و هي أربعة أشهر في قياس نسبة الرصاص في الدم والبول والكلى، كما تم قياس نسبة الوريدية والكريتيتين والكولونيديد والجولتيلبون بروكسيداز. كذلك تم تحليل النسيج الكلوي.

وقد أظهر النتائج أن تعرض الجرذان للرصاص أدى إلى زيادة ذات دلالة إحصائية في نسبة الرصاص في الدم والبول والكلى. أما الوريدية والكريتيتين فلم تسجل زيادة كبيرة، و مما حدى إلى زيادة ذات دلالة إحصائية في نسبة المالونديالدهيد في النسيج الكلوي. كما أظهرت النتائج أن الدور العلاجي لخلاصة الثوم والسيليمارين معا أفضل من الدور العلاجي للدبي-بيسيمالامين.

و بناء على النتائج المستخلصة من هذه الدراسة يوصى بالتحكم في التعرض للرصاص والبحث عن التحاليل في الدم أو البول، و توصى بإجراء تجارب أخرى على إمكانية استخدام خلاصة الثوم والسيليمارين معا كعلاج فعال للأثار السمية للرصاص.

1 - قسم الطب الشرعي والسموم الإكلينيكية – كلية الطب – جامعة الزقازيق.