

Comparative Effect of Silymarin and D-Penicillamine on Lead Induced Hemotoxicity and Oxidative Stress in Rat

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ABSTRACT

Background: This study was performed to investigate the adverse effects of acute lead intoxication on hemogram, erythrocyte osmotic fragility and oxidant/antioxidant status and the probable ameliorating effect of silymarin in comparison to d-penicillamine.

Methods: Forty-eight albino rats were divided in 8 groups and received the following treatments in a 10 day experiment in Shahid Chamran University of Ahvaz, southwest Iran in 2015. Group 1: Normal saline as control; Group 2: 25 mg/kg lead acetate, intraperitoneally (IP) for the last 5 days; Group 3: 100 mg/kg D-penicillamine, IP for the last 5 days; Group 4: 200 mg/kg silymarin, orally for 10 days; Group 5, 6, 7 and 8: In addition to lead, they received D-penicillamine, for the last 5 days, silymarin for 10 days, a combination of silymarin for 10 days and D-penicillamine for the last 5 days, and silymarin for the last 5 days, respectively.

Results: Lead exposure induced a significant microcytic anemia accompanied by a significant elevation in total leukocyte, lymphocyte and neutrophil counts. Erythrocyte superoxide dismutase (SOD) and glutathion peroxidase (Gpx) activities were significantly increased along with a significant elevation of malondialdehyde (MDA) concentration in lead treated rats. Activities of SOD and Gpx were significantly alleviated by silymarin administration for 10 days while both D-penicillamine and silymarin could significantly reduce MDA concentration.

Conclusion: Acute lead exposure induced significant leukocytosis and anemia that was associated with increased activity of erythrocyte antioxidant enzymes and lipid peroxidation. Silymarin in contrast to D-penicillamine treatment was more effective in preventing lead-induced oxidative stress in erythrocytes.

Keywords: D-Penicillamine, Hemotoxicity, Lead Poisoning, Oxidative Stress, Silymarin.

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INTRODUCTION

Lead poisoning is the most widely studied occupational and environmental hazard to human and domestic animals [1]. This metal is widely employed in alloys, pigments, batteries and other industrial applications due to its characteristics including low melting point and high vapor pressure. This substantial lead consumption has resulted in environmental pollutions and various health hazards [2]. Blood serves as a major target for the toxic effects of inorganic lead besides being its route of transportation [3].

Hemoglobin synthesis is suppressed in Pb poisoning in consequence of the inhibition of enzymes that perform in this pathway. It also reduces the life span of circulating erythrocytes by destruction of cell membranes [4].

Several mechanisms have been proposed to explain the Pb-induced toxicity. One is induction

of oxidative stress through the generation of reactive oxygen species and depletion of the cellular antioxidant pool [5].

D-penicillamine has been used for several decades in chelation treatment of lead toxicity [6]. D-penicillamine administration can reduce blood lead level through complexes, which it forms with this heavy metal and increasing its urinary excretion [7]. However, D-penicillamine is associated with a number of side effects, which reduce its clinical utilization as a metal chelator. Nephrotic syndrome and a range of autoimmune reactions are among the adverse effects of this drug observed in a significant proportion of treated patients [6]. Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical disease due to safe use, their antioxidative properties and their possible roles in intra and extracellular defense against oxidative stress [8]. Lead induced

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toxicities were alleviated by administration of various antioxidants including vitamin C, E, green tea, pectin and flaxseed oil [4].

Silymarin is a standardized mixture of antioxidant flavonolignans (silybin and silibinin) extracted from the medicinal plant *Silybum marianum*. It is a free radical scavenger and a membrane stabilizer that prevents lipoperoxidation induced cell damage [9] and was proved to have a protective effect against experimental hepatotoxicity [10]. Moreover, alternative beneficial effects of silymarin comprising cardioprotective, antidiabetic, anti-inflammatory, and iron chelating were recently demonstrated [11-13].

To the best of our knowledge, there are no studies concerning the hemoprotective effect of silymarin against lead intoxication. Hence, the present study was carried out to investigate the adverse effect of acute lead intoxication on hemogram, erythrocyte osmotic fragility and erythrocyte oxidant/antioxidant status and the probable alleviating effect of silymarin in comparison to d-penicillamine against lead induced hemotoxicity.

MATERIALS AND METHODS

Laboratory Animals

This study was performed in the clinical pathology laboratory, faculty of veterinary medicine, Shahid Chamran University of Ahvaz, southwest Iran from October to December 2015. Forty eight albino female rats (Wistar strain) weighing 180–200 g were housed in groups of 6, in plastic cages, in an air-conditioned room maintained at a temperature of 24 ± 2 °C and a relative humidity of 55 ± 5 %, with a 12 h light/dark illumination cycle. They were fed a commercial laboratory pelleted diet and tap water *ad libitum*.

All procedures were done in accordance with ethical guidelines for care and use of laboratory animals. The study was approved by the Experimental Animals Committee of Shahid Chamran University of Ahvaz, Iran.

Experimental Design

Animals were equally and randomly divided into 8 groups. All groups were subjected to a 10-day experiment as follows:

Group 1 (negative control): 0.5ml saline (0.9%NaCl), intraperitoneally (IP) for 10 days.

Group 2: 25 mg/kg lead acetate (Merck, Germany), IP for the last 5 days [14].

Group 3:100 mg/kg D-penicillamine(Sigma Chemical Co., USA),IP for the last 5 days [15].

Group 4: 200 mg/kg silymarin (Sigma Alderich, USA), orally by gavage for 10 days [16].

Group 5: 25 mg/kg lead acetate and 100 mg/kg D-penicillamine, IP for the last 5 days.

Group 6: 200 mg/kg silymarin orally by gavage for 10 days and 25 mg/kg lead acetate, IP for the last 5 days.

Group 7: 200 mg/kg silymarin orally by gavage for 10 days, 25 mg/kg lead acetate and 100 mg/kg D-penicillamine for the last 5 days.

Group 8: 200 mg/kg silymarin orally by gavage and 25 mg/kg lead acetate, IP for the last 5 days.

Blood Collection and Preparation of Hemolysate

At the end of experimental period, rats were anesthetized with chloroform (Merck, Germany) and blood samples were collected via cardiac puncture into EDTA containing tubes. The blood samples were used for hematologic and osmotic fragility assessment and the remaining were transformed to hemolysate in order to analyze oxidant/antioxidant status. To prepare hemolysate, erythrocytes were washed 4 times with 0.9% NaCl solution and mixed with cold redistilled water. The lysate was subsequently diluted with 0.01 mol/l phosphate buffer pH 7.0, so that a final dilution factor of 100 would be obtained. The prepared hemolysates were then stored at -70 °C until performing further analysis.

Hematological Assessment

Complete blood counts including, total erythrocyte count (RBC), hematocrite value (HCT), hemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and total white blood cells (WBC) were determined by the BC-2800Vet hematology analyzer (Mindray, China). Differential leukocyte counts were also estimated manually as described by Meyer and Harvey (2004) [17].

Saline Osmotic Fragility (of) Test

Osmotic fragility test was performed on erythrocytes from all blood samples. A 10% phosphate buffered NaCl stock solution was prepared [18]. A 1% salt working solution was then serially diluted with distilled water to make a series of 16 tubes of 0.85, 0.80, 0.75, 0.70, 0.65, 0.60, 0.55, 0.50, 0.45, 0.40, 0.35, 0.30, 0.25, 0.20,

0.10, and 0% salt solutions. Twenty microliters of whole blood was added to each tube and mixed. The tubes were incubated at 25 °C for 30 min, and then centrifuged at 450 g for 10 min. Optical density (OD) of each supernatant was read at 540 nm against distilled water using a spectrophotometer (Optima SP 3000 plus, Tokyo, Japan). Percentage hemolysis was calculated at each concentration of NaCl considering the 16th tube as 100% hemolysis. A graph of hemolysis percent against salt concentration was plotted. The concentration of NaCl causing 50% hemolysis was expressed as median corpuscular fragility (MCF).

Determination of Lipid Peroxidation and Antioxidant Status

The lipid peroxidation end product, malondialdehyde (MDA), was measured as thiobarbituric acid reactive substance in hemolysates [19].

The activities of antioxidant enzymes including superoxide dismutase (SOD) and glutathione peroxidase (GPX) were assayed using commercial assay kits according to the manufacturer's instructions (Randox Laboratories Ltd. G.B.).

Statistical Analysis

Analysis of variance (ANOVA) and Tukey's Post Hoc tests were employed to compare the data between groups using SPSS software (SPSS Inc., Chicago, IL, USA). All values were expressed as mean \pm standard error (SE) and $P < 0.05$ was considered as statistically significant.

RESULTS

Hematological Assessment

Total leukocyte and lymphocyte counts were elevated in all lead exposed rats so that they

were significantly different in groups 2 (Lead), 5 (Lead + pen) and 8 (Lead + Sil. 5d) when compared to control group ($P < 0.05$) (Table 1). A significant rise was also observed in neutrophil count, consistent with total leukocytes, in all lead treated groups regardless of the type of treatment ($P < 0.05$).

Mean erythrocyte count, hemoglobin concentration and hematocrit were significantly decreased in groups 2, 5, 6, 7 and 8 (all lead exposed groups) in comparison to control group ($P < 0.05$) (Table 2). In addition, a significant decline was noticed in MCV values of all groups with lead exposure ($P < 0.05$). Mean MCH values were also reduced in the mentioned groups, although insignificantly ($P \geq 0.05$). There were no significant differences in MCHC, RDW and platelet count between groups.

Saline Osmotic Fragility (of) Analysis

There were no significant changes in percentage hemolysis in various salt concentrations between different groups (Figure 1). Median corpuscular fragility (MCF) was also unaffected by different treatments ($P \geq 0.05$) (Table 2).

Oxidant/Antioxidant Assessment

Erythrocyte SOD activity was significantly increased in group 2 (Lead), 5 (Lead + pen), 7 (Lead + Pen + Syl. 10d) and 8 (Lead + Sil. 5d) ($P < 0.05$) accompanied by a significant rise in Gpx activity of groups 2 and 5 ($P < 0.05$) (Table 3). Concentration of MDA was significantly elevated due to lead exposure in rats of group 2 while this effect was alleviated by D-penicillamine or silymarin treatments in groups 5, 6 (Lead + Sil. 10d), 7 and 8 ($P < 0.05$) (Table 3).

Table 1. Leukogram and platelet assessment results as mean \pm SE in different groups.

	Cont	Lead	pen	Sil (10d)	Lead + pen	Lead + Sil (10d)	Lead + Pen + Sil (10d)	Lead + Sil (5d)
WBC ($\times 10^3/\mu\text{l}$)	5.96 \pm 0.95	18.22 \pm 1.71*	5.7 \pm 1.19	7.88 \pm 2.66	19.84 \pm 5.7*	13.04 \pm 4.12	14.44 \pm 4.08	18.16 \pm 2.43*
Lym ($\times 10^3/\mu\text{l}$)	4.1 \pm 0.7	8.33 \pm 0.9*	3.65 \pm 0.9	4.76 \pm 1.27	9.24 \pm 2.22*	5.28 \pm 1.32	6.82 \pm 1.93	9.48 \pm 1.58*
Mon ($\times 10^3/\mu\text{l}$)	0.2 \pm 0.03	0.35 \pm 0.03	0.21 \pm 0.04	0.5 \pm 0.36	0.32 \pm 0.06	0.2 \pm 0.07	0.54 \pm 0.23	0.32 \pm 0.08
Neut ($\times 10^3/\mu\text{l}$)	1.66 \pm 0.23	9.6 \pm 1.13*	1.83 \pm 0.28	2.61 \pm 1.05	8.22 \pm 1.73*	7.85 \pm 2.8*	7.08 \pm 2.04*	8.36 \pm 1.14*
Plt ($\times 10^3/\mu\text{l}$)	452.16 \pm 29.09	430.28 \pm 52.92	489.0 \pm 39.83	476.83 \pm 19.57	368.33 \pm 78.51	356.16 \pm 57.81	547.66 \pm 20.81	362.25 \pm 83.97

* in each row indicates significant difference compared to control group.

Table 2. Erythrocyte assessment results as mean \pm SE in different groups.

	Cont	Lead	pen	Sil (10d)	Lead + pen	Lead + Sil (10d)	Lead + Pen + Sil (10d)	Lead + Sil (5d)
RBC ($\times 10^6/\mu\text{l}$)	7.51 \pm 0.36	5.95 \pm 0.16*	7.3 \pm 0.25	7.54 \pm 0.34	6.39 \pm 0.74*	6.8 \pm 0.27*	5.76 \pm 0.23*	6.02 \pm 0.24*
Hb (g/dl)	14 \pm 0.68	10.45 \pm 0.3*	12.83 \pm 0.62	13.65 \pm 0.58	10.53 \pm 1.46*	11.23 \pm 0.66*	10.2 \pm 0.53*	10.03 \pm 0.38*
HCT (%)	49.58 \pm 2.36	35.5 \pm 0.48*	46.48 \pm 1.93	49.53 \pm 2.18	37.45 \pm 5.11*	40.13 \pm 2.19*	35.95 \pm 1.83*	35.88 \pm 1.28*
MCV (fl)	66.13 \pm 1.62	60.58 \pm 0.65*	63.63 \pm 0.74	65.8 \pm 0.81	58.11 \pm 0.95*	60.97 \pm 0.59*	60.38 \pm 0.86*	59.7 \pm 0.43*
MCH (pg)	18.58 \pm 0.39	17.11 \pm 0.18*	17.46 \pm 0.26	18.08 \pm 0.14	16.28 \pm 0.37*	16.93 \pm 0.25	17.03 \pm 0.18*	16.61 \pm 0.06*
MCHC (%)	28.18 \pm 0.19	28.15 \pm 0.17	27.51 \pm 0.24	27.5 \pm 0.19	28.08 \pm 0.47	27.31 \pm 0.08	28.56 \pm 0.15	27.90 \pm 0.17
RDW	12.68 \pm 0.41	12.9 \pm 0.18	11.33 \pm 0.34	11.4 \pm 0.33	12.43 \pm 0.37	12.21 \pm 0.27	12.3 \pm 0.27	11.58 \pm 0.27
MCF (%)	0.505 \pm 0.005	0.504 \pm 0.015	0.523 \pm 0.009	0.529 \pm 0.004	0.516 \pm 0.011	0.531 \pm 0.007	0.491 \pm 0.014	0.516 \pm 0.010

* in each row indicates significant difference compared to control group.

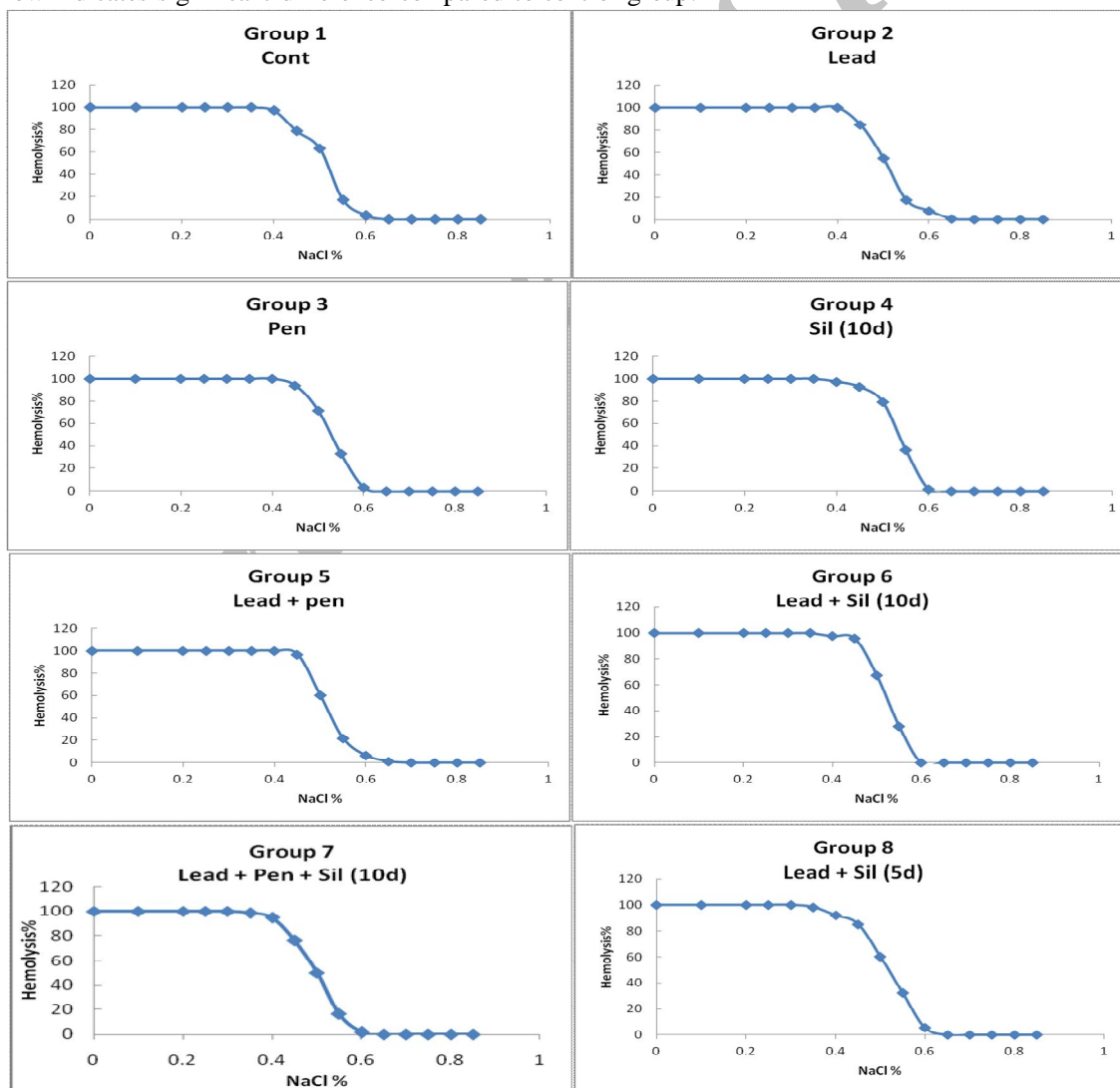
**Figure 1.** Mean erythrocyte Osmotic Fragility (OF) plots in different groups.

Table 3. Oxidant/antioxidant assessment results as mean \pm SE in different groups.

	Cont	Lead	pen	Sil (10d)	Lead + pen	Lead + Sil (10d)	Lead + Pen + Sil (10d)	Lead + Sil (5d)
SOD	2884.0 \pm	3805.9 \pm	3143.6 \pm	2971.4 \pm	4021.9 \pm	3227.0 \pm	4359.1 \pm	3991.5 \pm
(U/gHb)	145.70 a*	106.25 b	141.29 ab	145.75 a	393.59 b	169.22 ab	373.35 b	149.99 b
Gpx	144.45 \pm	251.88 \pm	117.92 \pm	81.36 \pm 14.32	201.35 \pm	83.36 \pm 15.57	80.77 \pm	177.61 \pm
(U/gHb)	13.33 ab	27.63 a	24.75 b	b	29.10 a	b	12.62 b	23.83 ab
MDA	11.4 \pm 1.96	29.73 \pm 7.53	19.29 \pm	17.06 \pm 2.28	20.17 \pm	25.16 \pm 1.66	23.49 \pm	25.24 \pm
(nmol/gHb)	a	b	0.71 ab	ab	1.01 ab	ab	3.47 ab	9.38 ab

*Different letters in each row indicate significant difference between groups.

DISCUSSION

The results of leukogram assessment revealed that acute lead exposure induced a significant elevation in total leukocyte, lymphocyte and neutrophil counts in rats. These effects partially subsided by treatment with silymarin for 10 days or a combination of d-penicillamine and silymarin.

Lead intoxication has been shown to stimulate a striking leukocytosis, due to neutrophilia and monocytosis, probably because of possible inflammations and immune response caused by this heavy metal in mice and rats [20-21]. In addition, occupational long-term exposures to lead were demonstrated to induce an increase in total leukocyte, neutrophil and monocyte counts, in association with elevated blood lead level [22-23].

On the other hand, there is evidence of antiinflammatory properties of silymarin [24], to which the beneficial impact of this herbal drug on leukocyte counts in the present study can be attributed.

In the present study, a microcytic hypochromic anemia was consistently observed in all lead exposed rats that were not affected by any of the treatments.

Heme synthesis is significantly suppressed by lead through inhibition of three major enzymes involved in this pathway, including δ -aminolevulinic acid dehydratase (ALAD), aminolevulinic acid synthetase (ALAS), and ferrochelatase [25-26] while its effect on ALAD is more profound. In addition, lead is capable to induce oxidative damage to erythrocyte proteins and membrane at high concentrations which plays an important role in erythrocyte destruction. Hence, the mechanic and osmotic fragilities induced by lead in erythrocytes could be reduced by various antioxidants [5].

The extracts of the flowers and leaves of *Silybum marianum* have been used for centuries to

treat liver disorders while it is accepted as a safe herbal product with anti-oxidant properties and no health hazard or significant side effect [27-28]. Despite a long history of its use and the large number of people who consume this substance, no conclusive data on its clinical efficacy in prevention or recovery of chemical hemotoxicity can be identified.

In the present study, none of the treatments (d-penicillamine nor silymarin) were effective in preventing or overcoming anemia in dose and duration applied in the present study. It might be due to the high level of lead exposure or the method through which d-penicillamine or silymarin were administered.

Erythrocyte osmotic fragility was not affected by any of the treatments in the current study. In other words, acute lead exposure did not alter erythrocyte resistance to hypoosmotic environments. In contrast, a decline in erythrocyte osmotic fragility was reported in workers occupationally exposed to lead with a dose-response relationship between blood lead level and osmotic fragility [29-30]. This tendency was assumed to be related to the alterations of RBC membranes, including an increase in membrane cholesterol following lead exposure. The absence of significant changes in erythrocyte osmotic fragility in the present study could be due to the acute lead intoxication in which the destruction in erythrocyte membrane and oxidative stress most likely resulted in direct removal of the damaged erythrocytes rather than remaining in circulation and altering the osmotic fragility.

In the present experiment, lead exposure resulted in increased activity of erythrocyte SOD and Gpx enzymes. Blood levels of the antioxidant enzymes SOD and catalase (CAT) has been elevated or suppressed by lead depending mainly on the level and duration of exposure. Higher levels of exposure to Pb in experimental or cross-

sectional studies are reported to cause increases in the activity of these enzymes as a consequence of elevated reactive oxygen species production [3, 31-32]. However, the activities of GPx, CAT and SOD as major antioxidant enzymes in the erythrocytes significantly crashed usually at chronic exposure with lower amounts of lead [33]. Lead has the ability to bind to the sulfhydryl group of enzymes of the antioxidative defense system, as well as replacing divalent bioelements that serve as their important co-factors, resulting in their inactivation [3, 33-34].

None of the treatments was able to adverse the lead induced SOD elevation in the current study except silymarin pretreatment, while Gpx activity was successfully reduced by silymarin administration for 10 days or a combination of d-penicillamine and silymarin. This reflects that silymarin can prevent the action of free radicals and oxidative stress in erythrocytes following lead intoxication. However, once the oxidative damage is produced by lead, silymarin is almost unable to reverse its consequences. Silymarin ability to act as cellular antioxidant has been attributed to its various beneficial effects including free radical scavenging, reduction of membrane lipid peroxidation and enhancement of GSH reserves [24].

MDA concentration, as a marker of lipid peroxidation, was significantly increased in the lead exposed rats. However, it was effectively alleviated in response to d-penicillamine or silymarin treatments. Similar results were reported by other researchers who observed a positive correlation between erythrocyte MDA levels and blood Pb levels [5, 33-35].

The MDA level was perfectly reduced by silymarin, as well as d-penicillamine in the present study, indicating a significant protection against the oxidative damage produced by lead. This finding seems to be compatible with Oda and El-Ashmawy's [36] in which silymarin returned MDA and GSH to the normal values, improved serum biochemical and histopathological changes of kidney and liver following acute mercury intoxication. Furthermore, simultaneous treatment with vitamin-E could prevent lipid peroxidation following 15 and 30 days of lead exposure in rats [5]. There is also evidence of protective effect of vitamin C and silymarin against toxic effects of lead on rat liver tissue in a previous study [8].

D-penicillamine in the present dose and duration of treatment was not much effective in

reducing the hematologic consequences of lead intoxication. These findings are in accordance with another study where D-penicillamine administration did not play an important role in treatment of hematologic effects of lead in subchronic intoxication in rats [37]. Eventhough this experiment was only conducted on acute lead toxicity in a limited period of time; the hematologic and oxidative consequences of intoxication, as well as comparative effects of different treatments were clearly noticeable.

CONCLUSION

Acute lead exposure induced significant leukocytosis and anemia that was associated with increased activity of erythrocyte antioxidant enzymes and lipid peroxidation. Silymarin pretreatment in contrast to d-penicillamine could alleviate lead induced oxidative stress in erythrocytes more effectively. Further studies are required to determine the possible effects of silymarin in prevention or treatment of lead induced hemotoxicity in different administration methods and in combination with other medications.

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