Original Paper

The Protective Role of Vitamins (C&E), Selenium, Silymarin and Rehydran-N against Lead Toxicity under Heat Stress Conditions

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ABSTRACT

This study was done to investigate the use of vitamins C & E, selenium, silymarin and rehydration solution to ameliorate lead toxicity under heat stress conditions. Male albino rats were subdivided into four groups: the first was a control group, the second was exposed to heat stress (40 ± 2 °C) in a closed and controlled chamber, the third received 25 mg/100 g body weight lead acetate day by day and exposed to the same heat stress conditions, the fourth was exposed to the same lead and hyperthermia conditions and supplemented three times per week with 1 mg/100 g body weight of each of vitamins C & E, silymarin, and 0.01mg/100g B.W. of selenium, and a daily drink of rehydran-n solution. Blood samples were collected after 25 days of treatment. Lead was found to induce significant elevations in blood glucose, total protein, cholesterol, lead, ALT, AST, GGT, ALP and LDH levels under heat stress conditions. Hyperthermia induced apoptotic DNA fragmentation, which was aggravated under lead intoxication. A reduction in body weight was observed in heat stressed groups. Hepatomegally was observed in heat stressed animals, which was aggravated with lead intake. Under heat stress condition, randomly scattered hepatocytes showed acidophilic and apoptotic changes. Under heat and lead exposure, these changes were enhanced and showed midzonal distribution, in addition to marked periportal microvesicular steatosis. Treatment of rats with vitamins C & E, silymarin, selenium, and rehydran-n resulted in marked improvement in the biochemical, molecular, physiological, and histopathological parameters.

Key Words: Rats, Lead, Hyperthermia, Pathology, Apoptosis, Steatosis

Introduction

Usage of lead and its compounds spread in modern industry.[1] Lead exerts its toxic effects by enhancing peroxidative damage to membranes.[2] Investigations suggest that effects of lead may be due to its interference with calcium in the activation of protein kinase C (PKC) and or through production of reactive oxygen species (ROS).[3] Lead induces DNA strand breaks[4,5] that induce apoptotic DNA fragments reversibly with time.[6] **Honchel et al** showed that lead enhances lipopolysaccharide and tumor necrosis factor-induced liver injury.[7] Kupffer cells promote lead induced hepatocyte apoptosis via oxidative stress.[8]

Heat stress was reported to induce alterations in hematological,[9] physiological,[10,11] and biochemical[12] parameters. Heat stress exaggerates the toxic effect of lead on different body systems.[13]

Vitamin C has been reported as a chelating agent in treatment of lead toxicity.[14] It reduces the possibility of lead interacting with critical biomolecules and preventing its toxicity.[15] Vitamin E is an antioxidant that helps in capturing reactive oxygen and free radicals produced by toxins.[16] Silymarin is a mixture of antioxidants excreted from Silybum marianum. It serves as a free radical scavenger

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and a membrane stabilizer that prevents lipoperoxidation and the associated cell damage.[17] It has a protective effect against experimental hepatotoxicity by regulating liver cell physiology and improving the performance of hepatic enzymes and bile production.[18,19] Vitamin C and silymarin supplements have been used effectively in amelioration of lead toxicity.

The present study aimed to evaluate the effect of using vitamins (C & E), silymarin, selenium and rehydration solution supplements as a protective measures against lead toxicity under heat stress conditions.

Materials and Methods

Animals:

Twenty (20) male albino rats (Rattus norvigicus), purchased from animal house of Garuonis University, Libya, 10 weeks old, weighing about 120 ± 10 g were used as experimental animals. Animals were housed in groups in plastic cages and were maintained under standard controlled conditions. Laboratory balanced diet and water were initially provided.

Experimental design:

Animals were segregated into four groups:

1 Normal controls.
2 Heat stressed group: they were exposed to 40 ± 2°C in a closed and controlled chamber and water ad-libitum.
3 Lead exposed group (25 mg/100g body weight day by day) under the same heat stress conditions (40 ± 2°C) and water ad-libitum.
4 Lead exposed group (25 mg/100g body weight day by day) under the same heat stress conditions (40 ± 2°C) and supplemented with vitamin C (1mg/100g body weight), vitamin E (1mg/100g body weight), selenium (0.01 mg/100g body weight) and silymarin (1mg/100 g body weight) by gastric tube 3 times/week and daily free drink rehydran-n solution (4g glucose anhydrous, 0.51g tri-sodium citrate anhydrous, 0.70g sodium chloride and 0.30g potassium chloride in 200 ml distilled water).

Duration of administration:

Animals of different groups were anaesthetized and rapidly dissected after 25 days of treatment.

Collection of serum samples:

Blood samples were collected from the abdominal vein in glass centrifuge tubes, then centrifuged for 15 min. at 1000 x g. Sera were separated and stored at -30°C in deep freezer till further biochemical measurements.

Preparation of tissues for microscopical and gel examinations:

After animal dissection, liver was removed, blotted on filter paper, and weighed. Representative specimens were labeled by code numbers, and immediately placed in 10% formalin. Portions of 10 mg were taken immediately for gel examinations and the remaining portions were stored at -30°C.

Histopathological methods:

The specimens were processed to paraffin as per standard procedure. Five-micron thick histological sections were prepared, and stained with hematoxyline and eosin. The stained sections were examined blindly (without knowing the code number key) under light microscopy. Microscopic examinations of liver sections were done systematically with comments on architecture; portal, periportal, or lobular inflammatory cell infiltrate and its degree; liver cell necrotic or degenerative changes; bile ducts and vascular changes.

Gel preparation and electrophoresis of lysate tissue:

Gels were prepared with 1.8 % electrophoretic grade agarose (BRL). The agarose was boiled in tris-borate EDTA buffer (1 x TBE buffer; 89 mM tris, 89 mM boric acid, 2 mM EDTA, pH 8.3). 0.5 µg/ml ethidium bromide was added to gel at 40°C. Gels were poured and allowed to solidify at room temperature for 1h before samples were loaded. 10 mg hepatic tissue was squeezed and lysed in 200 µl lysing buffer (50 mM NaCl, 1mM Na2 EDTA, 0.5% SDS, pH 8.3) for at least 30 min.

For electophoretic pattern of nucleic acids of tissue lysate, 20µl of lysate hepatic cells was loaded in well, 5 µl 6 x loading buffer was on the lysing tissue. Electrophoresis was performed for 2 hours at 50 V in gel buffer (1x TBE buffer). Gel was photographed using a Polaroid camera while the DNA and RNA was visualized using a 312 nm UV transilluminator.

Nucleic acids extraction and molecular assessment for apoptosis:

Nucleic acids extraction was based on salting out extraction method,[20] whereas protein was precipitated by saturated solution of NaCl (5M). 10mg hepatic tissue

was squeezed in eppendorf tube and was lysed by 600 µl lysing buffer (50 mM NaCl, 1mM Na_2 EDTA, 0.5 % SDS, pH 8.3), and was shaken gently. The mixture was kept overnight at 37°C. For protein precipitation, an amount of 200 µl of saturated NaCl was added to the samples and then shaken gently, and centrifuged at 12,000 rpm for 10 min. The supernatant was transferred to new eppendorf tube and the DNA was precipitated by 700-µl cold iso-propanol. The mixture was inverted several times till fine fibres of nucleic acids appeared, and centrifuged for 10 min at 12,000 rpm.

The supernatant was removed. For washing, an amount of 500 µl 70% ethyl alcohol was added and centrifuged for 8 min at 12,000 rpm. The supernatant was decanted or tipped and the tubes blotted on Whatman paper or clean tissue for 15 min. For apoptosis, once the tube was seen to be dry, the pellet was resuspended in 50 µl or appropriate volume of TE buffer (10 mM tris, 1mM EDTA, pH8), and supplemented with 5% glycerol for 30 min. The resuspended DNA with 6 x loading buffer was loaded directly on gel. So, the fine apoptotic bands could be detected even in the control sample.

Apoptosis analysis:
For apoptosis, the extracted DNA was gently resuspended with TE buffer supplemented with 5% glycerol, gently pipetted, and then mixed with 6 x loading buffer, and loaded directly on the gel.[21] The remaining DNA was kept at -20°C for another loading. Apoptotic bands appeared and located at 180 bp.

Biochemical analysis:
Serum total protein, cholesterol, triglycerides, glucose, ALT, AST, GGT, ALP and LDH levels were determined automatically using Integra 800 auto-analyzer, National Liver Institute, Menoufiya University. Lead was determined in serum samples by atomic absorption spectrophotometry using G.B.C. 902, double beam atomic absorption spectrophotometer (A.D.S. instrument), Faculty of Agriculture, Menoufiya University.

Statistical analysis:
Data were represented in tabular form as mean ± standard deviation. Students t-test was used for evaluating statistical significance for results according to **Hine and Wetherill**.[22]

Results

Body weight:
Decreased significantly by 33.83% after heat stress for 25 days and by 41.61% after lead treatment under hyperthermia. Vitamin supplementation reduced the effect of heat and lead on body weight.

Hepatosomatic index:
Heat stress induced elevation in hepatosomatic index (liver (g)/body weight(g) ratio) by 15.68%; however lead under hyperthermia aggravated this effect to 20.41%. On the other hand treatment with antioxidants with rehydration ameliorated these effects.

Biochemistry:
Heat stress resulted in significant increase in serum glucose, cholesterol, triglycerides, ALT and LDH levels. On the other hand, serum ALP activity decreased significantly. However serum total protein, lead, AST and GGT levels were not affected.

Lead induced significant increase in serum glucose, total protein, lead, ALT, AST, GGT, ALP and LDH levels under heat stress conditions. However serum cholesterol decreased significantly and triglycerides were not significantly affected.

Treatment of lead toxicity under heat stress conditions with rehydran-n solution and vitamins (C & E), silymarin, and selenium supplements improved all biochemical parameters under investigation, especially total protein, AST, GGT and ALP levels.

Molecular findings:
Heat stress induced fine apoptotic bands indicating high degree of liver cell destruction. Lead induced aggravation of apoptosis in liver cells under heat stress conditions. Treatment with vitamins (C & E), silymarin, and rehydration solution ameliorated these effects.

Histopathology:
Under heat stress conditions, randomly scattered hepatocytes showed acidophilic and apoptotic changes.
Rats exposed to lead and heat stress showed marked acidophilic and apoptotic changes in zone 2 (mid acinar zone) with microvesicular steatosis in zone 1 (periportal areas).

Vitamins C and E and other supplements resulted in significant reduction of both lead, and to some extent heat stress manifestations.

Discussion

Heat is a stressor that evokes several physiological reactions in humans and animals.[23] Many biochemical and physiological systems of the body are affected by exposure to heat, such as enzymatic, metabolic, cardiovascular, respiratory, haematopoietic, endocrinal and immunological systems, as well as blood and body fluid composition.[24-33] Lead intake causes toxic effects on different body systems.[34] Hyperthermia exaggerates the toxic effects of lead. There is evidence that combined supplementation with vitamin C and silymarin ameliorates the toxic effects of lead under mild conditions.

The present study showed that heat stress resulted in significant elevations in blood glucose, cholesterol, triglycerides, ALT and LDH levels.

Mertsching showed that the decrease of insulin release in heat stressed animals decreases the rate of glucose utilization in tissues which lead to increased blood glucose level.[35] Heat stress causes disturbances in liver function tests in hamsters,[36] rats,[37] and humans.[38] In humans and rats exposed to hyperthermia, autophagic vacuolation, and dilatation of both Golgi apparatus and endoplasmic reticulum, together with elevation of serum GOT, GPT and LDH activities, have been reported.

Our results indicated that lead induced significant elevations of blood glucose, total protein, lead, ALT, AST, GGT, ALP and LDH levels under heat stress conditions. These results are in agreement with those reported by earlier investigators (vide supra).

Hyperthermia was reported to induce RBCs hemolysis,[39] which may be responsible for increased serum total protein. **Latner** showed that the increase in serum GGT is an indicator of liver fibrosis and correlates with the development of hepatobiliary diseases indicating toxic liver damage.[40]

Eissa et al reported increased serum alkaline phosphatase activity in workers subjected to lead.[41] This was interpreted to changes in membrane permeability[42] that lead to hypoxia of hepatocytes[43] and increased peroxidation.[44] The significant increase in serum ALT and AST activities in lead-intoxicated rats under heat stress conditions reflects the damage of cells and alterations in cell permeability.[45] In accordance with our findings, **Skoczynska et al** showed a decrease in plasma cholesterol in rats treated with small doses of lead.[46]

Recent studies have shown that lead induces hepatic DNA damage.[47-49] In the present study lead aggravated apoptotic DNA fragmentation induced by heat stress. **Pagliara et al** showed that lead induced hepatic hyperplasia followed by apoptosis mediated by oxidative stress in kupffer cells.[50]

The results of this study showed decreased body weight in both heat stressed groups. Decrease in body weight due to hyperthermia may be attributed to alterations of the availability of enzyme substrates and hormones, and to the decrease of food intake and excessive water loss[51-54] It may be related to increased catabolism and tissue destruction under heat stress conditions.

The loss of body weight is generally considered to be a physiological index of lead intoxication. **Gerber et al**[55] and **Abou-El Maged et al**[56] attributed the decrease of body weight to strong affinity of lead for -SH, amine, carboxylic and phosphate ligand groups of biological membranes and protein. This leads to enzyme inhibition and disruption of numerous metabolic pathways including those of oxidative phophorylation[57] causing general inhibition of metabolism, besides other factors such as malabsorption of food materials, depression of appetite, and reduction of food consumption.[58] Further, some authors attribute the growth retardation to hormonal imbalance due to lead toxicity.[59-61]

Our results show that heat aggravates hepatomegaly, and lead pronounces this effect. This may be due to the diffuse wide spread affection of zone 1 (periportal areas) by microvesicular steatosis, and the mild mononuclear inflammatory cell infiltration.

Zone 1 is upstream in the blood flowing to the liver acinus, and zone 3 is downstream. Therefore zone 1 hepatocytes are exposed to the highest concentration of lead compared to zone 3 hepatocytes. The periportal distribution of lead-induced microvesicular steatosis indicates a direct hepatotoxic effect of lead, and not through metabolic byproducts of lead.

It has been shown that lead nitrate induces a synchronized wave of hepatocyte proliferation in rat liver.[62] **Pagliara et al** showed that lead induces liver hyperplasia followed by apoptosis mediated by oxidative stress in Kupffer cells.[63]

Vitamin E is a known antioxidant that protect against toxicity through its free radical scavenging capacity; therefore, it can prevent lipid peroxidation and fix the biomembranes.[64] It can also prevent membrane phospholipid degradation through phospholipidases linkage.[65] Vitamin C has been reported to reduce the possibility of lead interacting with critical biomolecules and thus preventing its toxicity. It can be used as a chelator for lead to decrease the risk of its toxic effects.[66] The preventive activity of vitamin C may be related to its antioxidant efficacy that inhibits lipid peroxidation enhanced by lead. **Blankenship et al** showed that vitamin C protected cells from undergoing apoptosis.[67]

Silymarin has anti-inflammatory activities mediated by alteration of Kupffer cell function.[68] It has been reported that silymarin improved liver function tests related to hepatocellular necrosis and/or increased membrane permeability.[69] Its protective effect is attributed to its antioxidant and free radicals scavenging properties. Silymarin has been shown to reverse histopathological changes of CCl4, such as necrosis, fatty change, ballooning degeneration, and inflammatory infiltration of lymphocytes around the central vein. In agreement of our results silymarin was found to reduce hepatic collagen accumulation by 35% in rats with secondary biliary cirrhosis.[70] **Saravanan et al** showed that vitamin C or/and silymarin were hepatoprotective and have antioxidant effect against ethanol intoxication.[71] The hepatoprotective effect of silymarin may be attributed to its ability to scavenge oxygen free radicals, and inhibition of liver microsome lipid peroxidation.

The increase of water intake is an important means in amelioration of heat stress effects. Rehydran-n solution has high water content in addition to sodium, potassium, and glucose that improve the osmotic fragility of cellular membranes and hence may ameliorate heat stress.

In conclusion, combined supplementation with vitamins C, E, selenium, silymarin, and rehydran-n solution markedly reduce the hepatotoxic effects of lead under heat stress conditions, and can be used as a protective measure in such circumstances.

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