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Original Research

Role of L-arginine Against Lead Toxicity in Liver of Rats with Different Resistance to Hypoxia

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Abstract

The aim of this work was to determine if the inhibition or stimulation of NO synthesis modulates liver damage induced by chronic lead intoxication. Lead nitrate (3.6 mg/kg, *per os*) was administered one time a day for 30 days to male Wistar rats with low and high resistance to hypoxia treated simultaneously with L-arginine (600 mg/kg, i.p.) or N^{ω}-nitro-L-arginine (L-NNA, 35 mg/kg, i.p.) 30 min. before lead exposure. L-Arginine treatment protected the liver of rats with low resistance to hypoxia partially by reducing lipid hydroperoxides level, the thiobarbituric acid reactive substances (TBARS) concentration, and altering the antioxidant defense system depletion induced by lead intoxication. Treatment of lead-exposed highly resistant rats by L-arginine did not reduce the TBARS level, but lowered the lipid hydroperoxides concentration. The increased glutathione antioxidant defense system in liver of L-NNA-treated rats reflects the antioxidant action of L-NNA for this animal group.

Keywords: lead intoxication, oxidative stress, antioxidant defense system, resistance to hypoxia, liver

Introduction

Exposure to lead often results in pathological conditions that are consistent with intracellular oxidative damage [1, 2]. Significant knowledge about the toxic properties of this element have been produced in recent years, and potential mechanisms of action have been investigated [1-5]. Although the mechanisms by which lead induces oxidative stress are not completely understood, evidence indicates that multiple mechanisms may be involved [1, 3].

The increase of lipid peroxidation in tissues has been implicated in lead-induced organ damage and dysfunction [2-4]. Lead toxicity includes dysfunction of central and peripheral nervous systems, the haemopoietic system, cardiovascular system, kidneys, liver, and reproductive systems [1, 2, 5]. These findings reported a possible involvement of oxidative stress in the pathophysiology of lead toxicity. Some known biochemical mechanisms of lead toxicity let us hypothesize that some of lead's effects on the component of antioxidant defense systems might cause impairment in oxidant and antioxidant balance of cells, resulting in oxidative damage [2].

Individual resistance to hypoxia provides an individual reaction of mitochondrial respiratory chain functioning [6, 7], mitochondrial ion transport [8], properties of mitochondrial enzymes, and energy metabolism [7, 9], monooxygenase system activity [10], biotransformation of xenobiotics, and drug-metabolizing system [11, 12]. Differences in the parameters of adenylate pool in hepatocytes of low- and highly-resistant-to-hypoxia rats indicate that energy metabolism is a mechanism involved in the formation of individual cell resistance to oxygen deficiency [9]. Individual constitutional resistance to hypoxia may be a serious criterion for an individual approach in pharmacotherapy of hypoxic states and diseases, as well as for prognosis and prevention of early and distant complications of irrational pharmacotherapy [12].

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Multiple data indicate that nitric oxide plays an essential signal role in many systems [13-16]. Physiological concentrations of released NO have been reported to cause a protective effect in various cell models [13, 16]. Our laboratory studies previously showed that pharmacological treatment with L-arginine-induced protection against acute stress [6], physical loading [17], and lead-induced oxidative stress in the blood of rats differing in individual resistance to oxygen deficiency [18]. Therefore, in the present study we investigated whether L-arginine can be used for protection of a rat's liver against lead-induced oxidative stress.

In the present study the main goal was to investigate the effects of L-arginine on altered oxidative stress parameters before lead-induced oxidative stress and to show that pharmacological treatment with L-arginine, a nitric oxide precursor, induces protection against lead intoxication in liver of rats with different resistance to hypoxia.

Materials and Methods

Animals and Experimental Design

Male Wistar rats (180-220 g) were used in the study. Rats were housed at a constant temperature of 20±2ºC. The animals (n=6 per group) had free access to food and water throughout the experiments. All procedures were done in accordance with guidelines for the care and use of animals in scientific research. Previously, animals were divided into two groups: rats with low resistance and high resistance to hypoxia. The individual constitutional resistance to hypoxia was evaluated as survival time (min.) in the altitude chamber 11,000 m above sea level. Survival time was measured after achieving altitude. Cessation of breathing served as the criterion for resistance to hypoxia.

Rats were randomly assigned to four groups. Group I – rats with low resistance (Group Ia, n=6) and high resistance to hypoxia (Group Ib, n=6) served as control and received daily injections of sterile normal saline solution for 30 days. Group II (Pb group) – rats with low resistance (Group IIa, n=6) and high resistance to hypoxia (Group IIb, n=6) received daily 3.6 mg lead nitrate/kg bw through an oral route for 30 days. Group III (L-arginine and Pb group) – rats with low resistance (Group IIIa, n=6) and high resistance to hypoxia (Group IIIb, n=6) also received daily 3.6 mg lead nitrate/kg bw for 30 days, and during these days animals were given L-arginine at a dose of 600 mg/kg bw through an intraperetonial route (i.p.) 30 min. before administration of lead nitrate. Group IV – rats with low resistance (Group IVa, n=6) and high resistance to hypoxia (Group IVb, n=6) (L-NNA and Pb group) were treated like group III, except that it received N^{ω}-nitro-L-arginine (L-NNA, 35 mg/kg bw) 30 min. before administration of lead nitrate.

Drugs and Solutions

The following drugs were used: L-arginine hydrochloride, N^{ω}-nitro-L-arginine, lead nitrate, thiobarbituric acid (TBA), oxidized and reduced glutathione (GSSG and GSH), NADPH, 5,5-dithiobis-2-nitrobensoic acid (Sigma Aldrich, USA). Ethylenediaminetetraacetic acid (EDTA), thrichloroacetic acid (TCA), quercetin, hydrogen peroxide, ammonium molybdate, sodium aside, t-butylhydroperoxide, heptan, and isopropanol obtained from Fluka (Buchs, Switzerland). L-arginine hydrochloride and N^{ω}-nitro-L-arginine were dissolved in isotonic solution and treated i.p. in a dose of 600 and 35 mg/kg bw, respectively. The pretreatment time for L-arginine and L-NNA was 30 min. before administration of lead nitrate. Lead nitrate is suspended in distilled water and administered *per os* in a dose of 3.6 mg/kg bw. All drugs were freshly prepared. All other reagents used were of analytical reagent grade.

Tissue Isolation

Livers were removed from rats after decapitation. One rat was used for each homogenate preparation. Briefly, livers were excised, weighed, and washed in ice-cold buffer. The minced tissue was rinsed clear of blood with cold isolation buffer and homogenized in a glass Potter-Elvehjem homogenizing vessel with a motor-driven Teflon pestle on ice. The isolation buffer contained 120 mM KCl, 2 mM K_2CO_3, 10 mM HEPES, and 1 mM EGTA; a pH of 7.2 was adjusted with KOH.

Biochemical Assay

Lipid Hydroperoxides Level Assay

The method was described by Kamyshnikov [19]. To 0.2 mL of liver homogenate was added 4 mL "heptan-isopropanol" mixture and vortexed vigorously. Then 1 mL of HCl (pH 2.0), and 2 mL of heptan reagent were added, vortexed, and centrifuged at 2,000 rpm for 5 min. The lipid hydroperoxides level was read spectrophotometrically at 233 nm and expressed as E233 per mg protein. In blank, mixture without homogenate was used. Protein determinations were performed according to the method described by Bradford [20].

TBARS Assay for Lipid Peroxidation

Thiobarbituric acid reactive substances (TBARS) were measured by the method of Kamyshnikov [19]. Briefly, to 2.1 mL of liver homogenate was added 1 mL 20% TCA and 1 ml of TBA reagent, boiled in a waterbath for 10 min. The reaction was stopped by placing the tubes in ice-cold water. Then mixture was centrifuged at 3,000 rpm for 10 min. The extinction of supernatant was read at excitation 540 nm. The μmol of MDA (malondialdehyde) was calculated by using $1.56 \cdot 10^5$ $mM^{-1} \cdot cm^{-1}$ as extinction coefficient and lipid peroxide levels in the liver were expressed in μmol of MDA per mg protein.

Superoxide Dismutase (SOD, E.C. 1.15.1.1)

SOD activity was measured spectrophotometrically by the Kostiuk et al. method using quercetin as a sub-

strate after suitable dilution [21]. The reaction mixture in a total volume of 1 mL consisted of 0.1 M sodium phosphate buffer (pH 7.8) and 0.08 mM EDTA in proportion 1:1. Briefly, 0.1 mL of tissue homogenate (dilution 1:1,000) was added to 2.3 mL of distilled water and 1 mL of reaction mixture with EDTA and sodium phosphate buffer. One activity unit was defined as the amount of enzyme necessary to produce a 50% inhibition of the quercetin (1.4 μM) reduction rates measured at 406 nm in 0 and 20th min. Activity is expressed in units of SOD per mg protein.

Catalase (CAT, E.C. 1.11.1.6)

CAT activity was determined by measuring the decrease of H_2O_2 in the reaction mixture using a spectrophotometer at the wave length of 410 nm by the method of Koroliuk et al. [22]. The reaction was started by adding 0.1 mL of homogenate to 2 mL of 0.03% of H_2O_2 solution and 1 mL of 4% ammonium molybdate. One unit of catalase activity is defined as the amount of enzyme required to clear 1 μmol of H_2O_2 per min per mg protein.

Glutathione Reductase (GR, E.C. 1.6.4.2)

GR activity was assayed as described by Glatzle et al. by measuring the oxidation of $NADPH_2$ at 340 nm [23]. The reaction mixture consisted of 67 mM sodium phosphate buffer (pH 6.6), 7.5 mM GSSG, 0.1 mL of tissue homogenate, and 6 mM $NADPH_2$. The specific activity is expressed as μmol $NADPH_2$ per min per mg protein.

Glutathione peroxidase (GPX, E.C. 1.11.1.19)

The activity of GPX was measured spectrophotometrically following the method of Moin [24]. The assay mixture contains 0.1 M Tris-HCl buffor with 6 mM EDTA and 12 mM sodium aside (pH 8.9), 4.8 mM GSH, 20 mM t-butylhydroperoxide, and 0.01 M 5.5-dithiobis-2-nitrobensoic acid. The rate of GSH reduction was followed spectrophotometrically at 412 nm. Glutathione peroxidase activity was expressed as μmol GSH per min. per mg protein.

Statistical Analysis

For statistical analysis, ANOVA with the Tukey test was used to compare the groups [25]. In all cases, a difference was considered significant when p was <0.05. Student *t* tests with 95% confidence intervals (α=0.05) were applied to determine the significance of differences between groups low and highly resistant to hypoxia. Correlations between lipid hydroperoxides, TBARS levels, and enzyme activities at the set significance level were determined by the regression method. Interactions were established by the Pearson test for linear correlation. All statistical calculations were performed on separate data from each individual with STATISTICA version 8.0.

Results

Figs. 1 and 2 show the degree of liver lipid peroxidation for lead-exposed rats. The chronic administration of lead nitrate during 30 days increased lipid hydroperoxide levels by 169% ($p<0.05$) and by 225% ($p<0.05$) for rats with low and high resistance to hypoxia (Groups IIa and IIb, respectively). Moreover, differences between lipid peroxide levels in liver of lead-exposed rats with high resistance (Group IIb) was higher by 17.7% ($p<0.05$) compared to that in the low-resistant-to-hypoxia rats (Group IIa). Lead also increased TBARS level by 71.5% ($p<0.05$) and 48.3% ($p<0.05$). Administration of L-arginine to lead-exposed rats with low resistance to hypoxia (Groups IIIa) decreased lipid hydroperoxides and TBARS levels by 37.8% ($p<0.05$) and by 28.3% ($p<0.05$), respectively. Animals with low resistance to hypoxia receiving both L-NNA and lead nitrate (Groups IVa) showed higher TBARS values. For rats with high resistance to hypoxia (Groups IVb), L-NNA prevented an increase of lipid hydroperoxide levels and did not produce any effect on liver TBARS levels. TBARS levels of the lead-exposed group are taken as a reference.

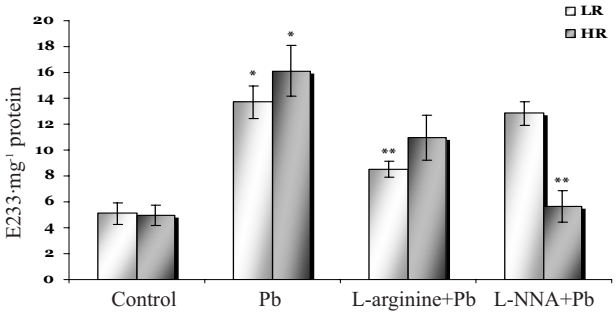

Fig. 1. Effect of L-arginine or N$^{\omega}$-nitro-L-arginine (L-NNA) on lead-induced lipid hydroperoxides levels (E233·mg^{-1} protein) in the liver of rats with low (LR) and high resistance to hypoxia (HR). Each bar represents the mean±SEM.
* significantly different from the control group, $p<0.05$;
** significantly different from the lead-exposed group, $p<0.05$.

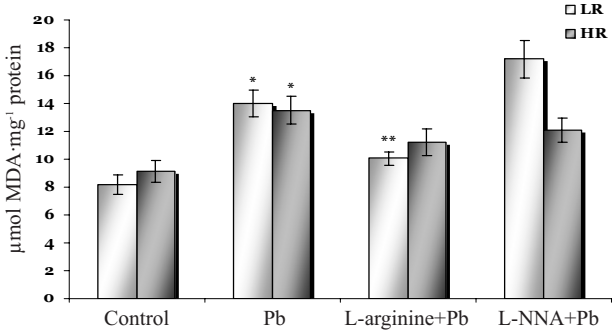

Fig. 2. Effect of L-arginine or N$^{\omega}$-nitro-L-arginine (L-NNA) on lead-induced lipid peroxide levels (μmol MDA·mg^{-1} protein) in the liver of rats with low (LR) and high resistance to hypoxia (HR). Each bar represents the mean±SEM.
* significantly different from the control group, $p<0.05$;
** significantly different from the lead-exposed group, $p<0.05$.

Table 1. Antioxidant enzyme activity in the liver of lead-exposed rats with low resistance to hypoxia given L-arginine or N^{ω}-nitro-L-arginine (L-NNA) treatment.

Antioxidant enzyme activity	Control (Group Ia)	Pb (Group IIa)	L-arginine + Pb (Group IIIa)	L-NNA + Pb (Group IVa)
SOD, U·mg^{-1} protein	312.35±18.29	205.56±24.38*	240.08±24.98	182.55±21.05**
CAT, μmol·min^{-1}·mg^{-1} protein	9.76±0.47	12.63±0.89*	12.82±0.72	13.31±0.70
GR, μmol·min^{-1}·mg^{-1} protein	134.01±7.86	78.45±8.22*	95.17±6.15	74.20±6.31
GPX, μmol·min^{-1}·mg^{-1} protein	39.85±2.98	28.19±2.11*	54.94±3.12**	30.48±3.35

* significantly different from the control group, P<0.05
** significantly different from the lead-exposed group, P<0.05

Table 2. Antioxidant enzyme activity in the liver of lead-exposed rats with high resistance to hypoxia given L-arginine or N^{ω}-nitro-L-arginine (L-NNA) treatment.

Antioxidant enzyme activity	Control (Group Ib)	Pb (Group IIb)	L-arginine + Pb (Group IIIb)	L-NNA + Pb (Group IVb)
SOD, U·mg^{-1} protein	448.24±31.15	244.96±25.75*	245.49±20.29	219.10±20.89
CAT, μmol·min^{-1}·mg^{-1} protein	9.91±0.54	13.04±0.51*	12.55±0.64	12.32±0.61
GR, μmol·min^{-1}·mg^{-1} protein	152.16±9.10	92.38±8.71*	98.31±5.91	128.15±7.50**
GPX, μmol·min^{-1}·mg^{-1} protein	42.74±2.19	31.37±2.37*	48.29±2.20**	67.56±3.88**

* significantly different from the control group, P<0.05
** significantly different from the lead-exposed group, P<0.05

SOD activity decreased by 34.2% (p<0.05) and 45.4% (p<0.05) in liver of rats with low and high resistance to hypoxia after lead intoxication (Groups IIa and IIb, respectively); L-arginine or L-NNA treatment did not further increase it significantly (Tables 1 and 2). Hepatic CAT activity increased significantly after lead intoxication by 29.4% (p<0.05) and 31.6% (p<0.05) for rats with low and high resistance to hypoxia (Groups IIa and IIb, respectively). Neither L-arginine, nor L-NNA modified CAT activity significantly. The activity was comparable to that in lead-exposed rats, given no treatment.

Lead intoxication decreased liver GR activity by 41.5% (p<0.05) and 39.3% (p<0.05) for rats with low and high resistance to hypoxia (Groups IIa and IIb, respectively). Treatment with L-arginine did not modify this value, while L-NNA treatment increased GR activity by 38.7% (p<0.05) for rats with high resistance to hypoxia (Group IVb). A significant decrease of liver GPX activity of rats with low (by 29.3%, p<0.05) and high resistance (by 26.6%, p<0.05) was recorded as lead-exposed rats compared with control rats. Treatment with L-arginine under lead intoxication increased GPX activity of rats with low (by 94.9%, p<0.05) and high (by 53.9%, p<0.05) resistance (Groups IIIa and IIIb, respectively) compared with lead group.

Several correlations between checked parameters were found (Fig. 3, 4). TBARS level of the liver from lead-exposed rats with low resistance (Group IIa) correlated inversely with SOD activity (r=-0.853, p=0.031). Activity of CAT correlated positively with level of lipid peroxidation products (r=0.885, p=0.019) from lead-exposed rats with high resistance to hypoxia (Group IIb) (Fig. 3). Thus, CAT activity was connected with SOD activity in the liver of lead-exposed rats with low resistance to hypoxia given L-arginine (Group IIIa) (r=0.820, p=0.046). The relationships between liver CAT and GR activities were inverse (r=-0.866, p=0.026) (Fig. 4).

Discussion

Our results suggest that antioxidant defense systems (for SOD activity, p<0.05) in liver of animals from control group is higher for animals with high resistance to hypoxia. This effect could be attributed to its ability to interact with ROS and other radicals to produce less toxic species during lead-induced oxidative stress.

Recent studies suggest that oxidative stress is one of the important mechanisms of toxic effects of lead [1-5]. We measured the TBARS and lipid hydroperoxides levels, a products indicative of the extent of lipid peroxidation (Figs. 1, 2). Results from the present study testify to enhanced TBARS and lipid hydroperoxide levels in lead-exposed rats. The lipid peroxide level in liver from rats of the lead-exposed group was significantly higher (p<0.05) than the respective control value. Our results are in agreement with previous studies and indicate that lead intoxication increases lipid peroxidation level [1-5].

Extensive lipid peroxidation has been proposed as the basis of lead toxicity [1-5]. A growing amount of evidence indicates that lead is able to produce ROS that results in lipid peroxidation, DNA damage, and depletion of antioxidant defense systems. Lead is known to have some toxic effects on membrane structure and functions [1, 2]. Patrick [2] demonstrated the propensity for lead to catalyze oxidative reactions and generate ROS. ROS inhibits the production of sulfhydryl antioxidants and enzyme reactions, impairing heme production, causing inflammation in vascular endothelial cells, damaging nucleic acids, inhibiting DNA repair, and initiating lipid peroxidation in cellular membranes. These wide-ranging effects of ROS generation have been postulated to be major contributors to lead-exposure related disease [2]. Yiin and Lin demonstrated possible toxic effects of lead on membrane components and identified a correlation between these effects and lead-induced oxidative damage [26]. Although several mechanisms have been proposed to explain the lead-induced toxicity [1, 2], no mechanisms have yet been determined. Liver, kidneys and brain have been considered as the target organs for the toxic effects of lead [27].

Treatment of rats with low resistance by L-arginine (Group IIIa) reversed the effects of lead on oxidative stress parameters, suggesting ROS as a possible contributor to the liver damage that occurred. The increases in lipid peroxidation levels in lead-exposed rats were accompanied by alterations in antioxidant defense systems, including decreased SOD, GR, and GPX activity and increased CAT activity. The correlative relationships between prooxidative parameters and activities of antioxidant enzymes also agree, as was expected if the peroxidation process is subsequent to the consumption of intracellular antioxidants (Figs. 3 and 4).

We previously suggested that L-arginine treatment restored the blood antioxidant defense system under lead intoxication, especially for rats with low resistance to hypoxia [18]. Induction of NO synthesis was proposed for this purpose; its effects on lead-induced oxidative damage were shown to be beneficial [18]. L-arginine, NO precursor, seems to be a good antioxidant couple because of their several properties [13]. Nitric oxide has been shown to inhibit the Fenton reaction by binding to ferrous iron, and thus preventing the formation of hydroxyl radical [28]. NO has been shown to reduce the ferryl heme formed from the

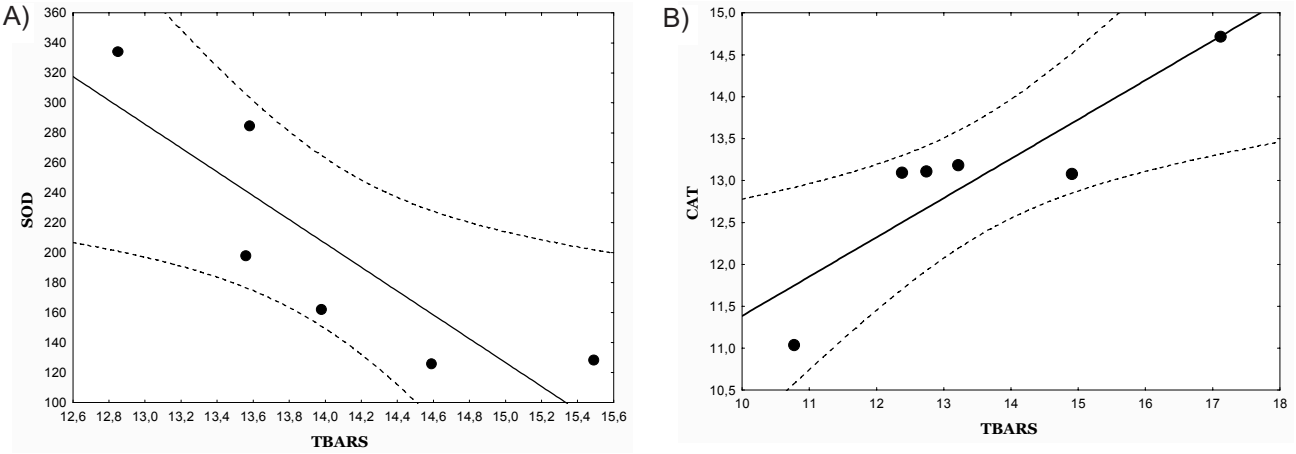

Fig. 3. Dependencies between TBARS level and SOD activity (A), TBARS level and CAT activity (B) in the liver of lead-exposed rats with low (A) and high resistance to hypoxia (B).

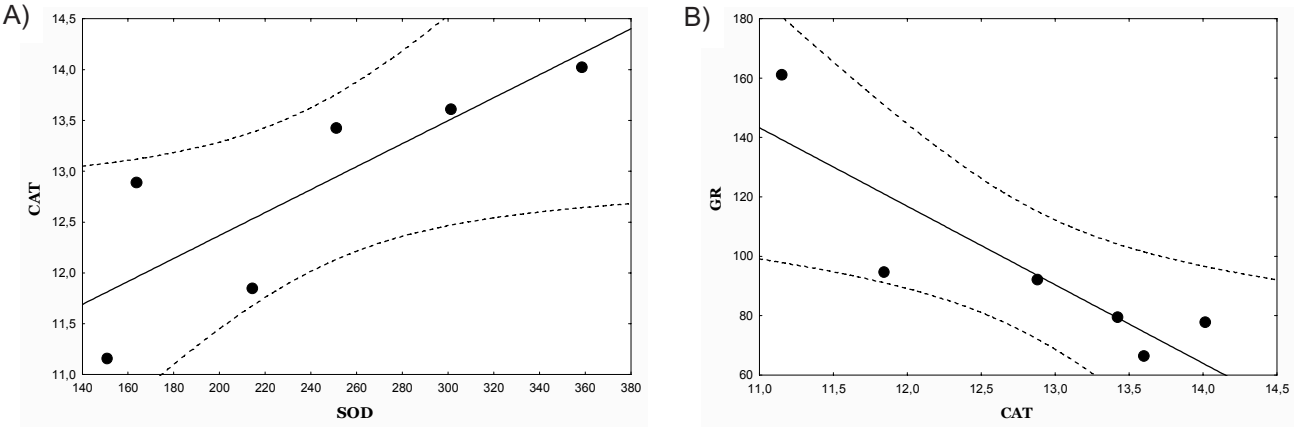

Fig. 4. Dependencies between SOD and CAT activities (A), CAT and GR activities (B) in the liver of lead-exposed rats with low resistance to hypoxia treated by L-arginine.

interaction of hemoglobin with peroxides, and so prevent lipid oxidation by this mechanism. NO may interfere with the detoxication of hydroperoxides [13].

Therefore, it is plausible to assume that L-arginine inhibit the lipid peroxidation process that is known to be triggered by ROS. Further evidence of the efficacy of L-arginine in relieving lead-induced oxidative stress includes the increase in GPX activity in lead-exposed animals. Previous studies reportedly minimize reperfusion injury in a low flow, reflow model of liver perfusion in rats after induction of NO synthesis [29, 30]. The beneficial protective role of nitric oxide in immunological liver damage in mice was documented by Wang and Liu [31]. The present results are in agreement with these reports, indicating that L-arginine possesses important protective properties against lead-induced oxidative stress.

Recent studies have noted that NO is not a strong oxidant and cannot extract bis allylic hydrogen from an unsaturated fatty acid to begin the peroxidation chain reaction [32]. However, in combination with other agents, nitric oxide can both promote and inhibit the initiation of lipid peroxidation. On the other hand, in addition to inhibiting the initiation of lipid peroxidation, nitric oxide may also enhance this process. The reaction between nitric oxide and superoxide generates peroxynitrite. The reaction between nitric oxide and molecular oxygen generates nitrogen dioxide, in combination with radical-radical combination products, dinitrogen trioxide and dinitrogen tetroxide [33]. It has been known for some time that nitrogen dioxide is able to initiate lipid peroxidation. In light of this, the present study was undertaken to determine the beneficial effects of L-arginine on lead-induced oxidative stress in liver of rats differing in individual resistance to oxygen deficiency.

Several studies have reported alterations in antioxidant enzyme activities such as SOD, CAT, GR, and GPX, and changes in the concentrations of some antioxidant molecules, such as glutathione (GSH) in lead-exposed animals and workers [2-5]. An antioxidant defense system is a potential target for lead toxicity because antioxidant enzymes depend on various essential trace elements for their proper molecular structure and activity [34].

SOD destroys the free radical superoxide by converting it to peroxide that can in turn be destroyed by CAT or GPX reactions [35]. SOD converts superoxide to hydrogen peroxide and molecular oxygen. Another function of SOD is to protect dehydratases against inactivation by the ROS [36]. The depression in SOD activity in our study may result in cellular injury by superoxide radicals and inactivation of enzymes by interaction with oxygen radicals. This situation may reflect the highly correlative link between TBARS level and SOD activity in the liver from lead-exposed rats ($r=-0.853$, $p=0.031$).

CAT is one of the most efficient enzymes known. CAT reacts with H_2O_2 to form water and molecular oxygen; and with H donors using peroxide in a kind of peroxidase activity: H_2O_2 is enzymically catabolized in aerobic organism by catalase and several peroxidases. In animals, CAT and GPX detoxify H_2O_2. CAT protects cells from hydrogen peroxide generated within them. Even though CAT is not essential for some cell types under normal conditions, it plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response [37].

GR possesses a disulfide at its active site [38], which was suggested as a target for lead, resulting in the inhibition of the enzyme [39-41]. This inhibition leads to decreased GSH-GSSG ratios that render cells more susceptible to oxidative damage. Glutathione disulfide is reduced to GSH by GR using NADPH as a substrate [38]. The selenium-containing peroxidases, being the more important example glutathione peroxidase catalyze the reduction of a variety of hydroperoxides (ROOH and H_2O_2) using GSH, thereby protecting mammalian cells against oxidative damage. GPX, CAT, and SOD are metalloproteins and accomplish their antioxidant functions by enzymatically detoxifying peroxides, H_2O_2 and O_2, respectively [38-41]. In our study the activities of GR and GPX were significantly decreased in the liver of lead-exposed rats in respect to the control group. These results suggest that both the glutathione-mediated antioxidant defense system plays a critical role in intracellular antioxidant defense against lead-induced oxidative stress. The increase in CAT activity in lead-exposed animals may indicate further consumption of NADPH as a substrate for GR. L-arginine treatment of animals receiving lead for 30 days returned GPX activity to control levels that can be explained by the decreased need for NADPH.

Conclusions

1. Lead intoxication causes oxidative stress by inducing the generation of lipid peroxidation, and reducing the liver antioxidant defense system.
2. L-arginine administration resulted in decreased lipid peroxidation and reversed alterations in antioxidant defense system components in liver from rats with low resistance to hypoxia.
3. Treatment of lead-exposed rats with high resistance by L-arginine did not reduce TBARS levels, but lowered lipid hydroperoxide concentrations.
4. Therefore, it can be concluded that the increased glutathione antioxidant defense system (GR and GPX activity) in L-NNA-treated rats with high resistance reflect the antioxidant action of NO synthesis inhibition by L-NNA for this group of animals.

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