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Increased lipid peroxidation and ascorbic acid utilization in testis and epididymis of rats chronically exposed to lead

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Abstract

The hypothesis has been recently presented that lead may exert its negative effect at least partially through the increase of reactive oxygen species (ROS) level in tissues. However, little is known about the influence of lead intoxication on equilibrium between generation and elimination of ROS in the male reproductive system. Sexually mature male Wistar rats were given *ad libitum* 1% of aqueous solution of lead acetate (PbAc) for 9 months. Significantly higher lead concentrations were found in blood [median 7.03 (Q25–Q75: 2.99–7.65) *versus* 0.18 (0.12–0.99) μg dl^{-1}, $P < 0.01$], caput epididymis [median 5.51 (Q25–Q75: 4.31–7.83) *versus* 0.51 (0.11–0.80) μg g^{-1} d.m., $P < 0.001$], cauda epididymis [median 5.88 (Q25–Q75: 4.06–8.37) *versus* 0.61 (0.2 – 1.08) μg g^{-1} d.m., $P < 0.001$] and testis [median 1.81 (Q25–Q75: 0.94–2.31) *versus* 0.17 (0.03–0.3) μg g^{-1} d.m., $P < 0.01$] of lead-intoxicated rats when compared to the control. The concentration of ascorbyl radical, generated *in vitro* from L-ascorbic acid (present in tissues *in vivo*) was measured by means of Electron Paramagnetic Resonance (EPR) spectroscopy. The EPR signal of ascorbyl radical in caput epididymis, cauda epididymis, testis and liver of lead acetate-treated animals revealed a significant decrease by 53%, 45%, 40% and 69% *versus* control tissues, respectively. Plasma L-ascorbic acid content measured by high performance liquid chromatography (HPLC) method and total antioxidant status (TAS) measured by means of spectrophotometry were also significantly lower in the intoxicated *versus* control animals (28% and 21%, respectively). In the group exposed to lead the concentration of lipid peroxide in homogenates of the reproductive system organs was significantly elevated *versus* control group. It can be assumed that the lower EPR signal was caused by decreased tissue concentrations of L-ascorbic acid. The latter may have resulted from consumption of ascorbic acid for scavenging of ROS excess in tissues of animals chronically exposed to lead.

Introduction

L-Ascorbic acid is known to be an important antioxidant in the human organism. It mainly acts in the aqueous phase. However the cooperation with vitamin E between the aqueous and lipid phase is possible (Irshad & Chaudhuri 2002). Thus, it indirectly protects the lipid phase against

attack of ROS. In systemic fluids ascorbic acid is almost completely dissociated, and as an ascorbic anion takes part in the reduction of free radicals. Relatively less reactive ascorbate free radical ($A^{\bullet -}$) is generated as a result. In our study however, the amount of ascorbyl free radical ($A^{\bullet}H$) that was generated *in vitro* from the L-ascorbic acid (present *in vivo* in tissues) was measured (Gonet 1994; Buettner & Jurkiewicz 1995). According to Roginsky and Stegmann (1994) the concentration of ascorbyl free radical is proportional to the tissue concentrations of L-ascorbic acid (reduced form only) and therefore may be used for indirect measurements of L-ascorbic acid concentrations.

Lead is a toxic element for human and animal organisms (Adonaylo & Oteiza 1999; Ye *et al*. 1999; Vaglenov *et al*. 2001). Its negative impact on reproduction is well known (Thoreux-Manlay *et al*. 1995; Alexander *et al*. 1996; Gandley *et al*. 1999). Workers exposed to lead are oligo– (Alexander *et al*. 1996; Bonde *et al*. 2002) and astenozoospermic (Cullen *et al*. 1984). However, the mechanism of its activity in the male reproductive system is not fully understood.

A study in rats exposed to the high doses of Pb (50 or 200 mg kg^{-1} of body mass) for 3 months revealed an adverse influence on spermatogenesis and histological lesions within the epididymis of these animals (Batra *et al*. 2001). Some authors reported lead-induced decreased sperm count and motility as well as morphological abnormalities of spermatozoa in animals (Hsu *et al*. 1997, 1998b; Marchelwicz et al. 1993).

Some authors point to the possibility of increased ROS formation under the influence of lead (Hsu *et al*. 1997, 1998a; Yang *et al*. 1999; Ye *et al*. 1999; Upasani *et al*. 2001). Some authors have proved that elevated ROS decrease sperm motility. The effect is associated with the intensification of lipid peroxidation and with the decrease of polyunsaturated fatty acids content in sperm membranes (Riley & Behrman 1991; Fisher & Aitken 1997).

Therefore it seemed interesting to examine the changes in amount of lipid peroxides and L-ascorbic acid in organs of reproductive system of male rats intoxicated with lead acetate for 9 months. The level of utilized L-ascorbic acid may show indirectly the intensification of free radical reactions in the tissue and define the amount of generated ROS (Riley & Behrman 1991; Gonet 1994). This information may have a significant importance because of the fact that spermatozoa mature in caput and are stored in cauda epididymis.

Male infertility has recently been more often diagnosed (Aitken 1999; Iammarrone *et al*. 2003). The exposure of sperm to lead during the process of its maturation and its storage in epididymis may be one of the reasons for this phenomenon.

Materials and methods

Our experiments were approved by the Local Ethical Committee. Studies were performed on sexually mature male Wistar rats, weighing 300–350 g each. The rats were kept in a room with controlled temperature, under LD 12/12 regime. The experimental animals ($n = 8$) were allowed to drink freely an 1% aqueous solution of lead acetate $Pb(CH_3COO)_2 \cdot 3H_2O$ (PbAc) for 9 months, while the controls ($n = 8$) drank distilled water. After the time of treatment animals were sacrificed under Thiopental anaesthesia (120 mg·kg^{-1} b.w., i.p., Biochemie GmbH, Austria).

Lead concentration in blood and tissues

Lead content was measured in the testis, caput and cauda epididymis, liver and blood according to Behari (1981). Tissue samples of testis, epididymis and liver were dried at 60 °C and combusted at 450 °C (24 h). Thereafter, the combusted samples were dissolved in hot solution of 1 M nitric acid. All the treatments were done twice. The samples were transferred into 50 ml volumetric flasks and adjusted with the deionized water to this volume. The blood samples were treated with Triton X-100 and extracted into methyl isobutyl ketone (Sigma/Aldrich, Germany). The appropriately diluted and digested tissue samples were analyzed using Solaar 969 atomic absorption spectrophotometer AAS (Hewlett Packard, USA).

Internal quality control used three standards prepared by the Heavy Metal Toxicology Central Laboratory of Work Medicine Institute in Lodz, Poland. This Laboratory's lead assay results in blood are under supervision of the Blood Lead Laboratory Reference System (BLLRS) conducted by CDC – American Center for Disease Control in Atlanta. The quality control was also

carried out with certified Nycomed lead standards. Control assays were carried out every 10 samples.

Electron Paramagnetic Resonance (EPR)

For EPR studies the tissues (*ca*. 200 mg) of caput and cauda epididymis, testis and liver were taken from control and lead-treated animals. They were subjected to lyophilization (lyophilizer LGA05). Then the tissues were ground in small porcelain evaporators, and after a lapse of 1 h, placed in 20 mg portions, in quartz tubes 5 mm diameter. The tube with the studied tissue was put into the resonance chamber of an electron paramagnetic resonance spectrometer (EPR spectrometer, type SE/X 2544 (Radiopan, Poland) with resonance chamber RCX 660) and its microwave spectrum was recorded. Measuring conditions were: frequency 9.45 GHz, power 3 mW, modulation 100 kHz 0.2 mT^{-1}, time constant 1 s, scan rate 20 mT 4 min^{-1}. Diphenylpicrylhydrazyl (DPPH, Sigma/Aldrich, Germany) was employed as a standard to determine the *g* coefficient and the concentration of unpaired spins. Measurements were made after the lapse of 1 h upon termination of the lyophilization process, when the EPR signal occurs at maximum. Details of the EPR method were described in earlier paper (Gonet 1994).

Determination of lipid peroxide content

The content of lipid peroxides in homogenates of testis, caput and cauda epididymis was colorimetrically measured using the thiobarbituric acid reaction method (Satoh 1978).

Separation of ascorbic acid and uric acid in plasma by High Performance Liquid Chromatography (HPLC)

The blood was heparinized and immediately centrifuged ($5000 \times g$ for 5 min, at +4 °C). Plasma was mixed with an equal amount of 10% metaphosphoric acid (Sigma/Aldrich, Germany) containing 1 mM EDTA (Sigma). The samples were centrifuged ($10{,}000 \times g$ for 10 min, at +4 °C) and the supernatant was analyzed with an isocratic HPLC method at +21 °C on a LiChrospher 100 RP-18 125×4 mm column (Merck, Germany) using 0.1 M phosphate buffer (pH = 3) as mobile phase (Muto *et al*. 1997). The detection of the ascorbic and uric acid was conducted at 243 and 285 nm, respectively.

Determination of total antioxidant status

Measurement of plasma total antioxidant status (TAS) was carried out with the Randox kit (Germany). The measurements were conducted at +37 °C and 600 nm wavelength (spectrophotometer Lambda 40 P, Perkin Elmer, USA). A_1 absorption was measured by adding 20 μl plasma to 1 ml phosphate buffer (80 mM, pH 7.4) containing 6.1 nM metmyoglobin and 610 nM ABTS. A_2 absorption was measured exactly 180 s after adding 0.2 ml phosphate buffer (80 mM, pH 7.4) containing 20 nM H_2O_2. TAS was calculated according to the formula:

$$\text{TAS [mM]} = \frac{\text{Concentration of standard}}{(\Delta\text{A blank} - \Delta\text{A standard})} \times (\Delta\text{A blank} - \Delta\text{A sample})$$

Statistical analysis

Results are expressed as median (M), lower and upper quartile (Q25–Q75). Non-parametric Mann–Whitney *U*-test for variables with distributions different from normal (Shapiro–Wilk's test) and Student *t*-test for normally distributed variables were used to check significance of differences between study and control groups. The value of $P < 0.05$ was considered to indicate statistically significant differences. Calculations were done using the software package Statistica 6.1.

Results

Blood and tissue lead concentrations

After 9 months of exposure the lead-treated animals had significantly higher lead concentration in both the studied parts of epididymis: caput and cauda, testis, liver and blood, when compared to the control group (Figure 1a).

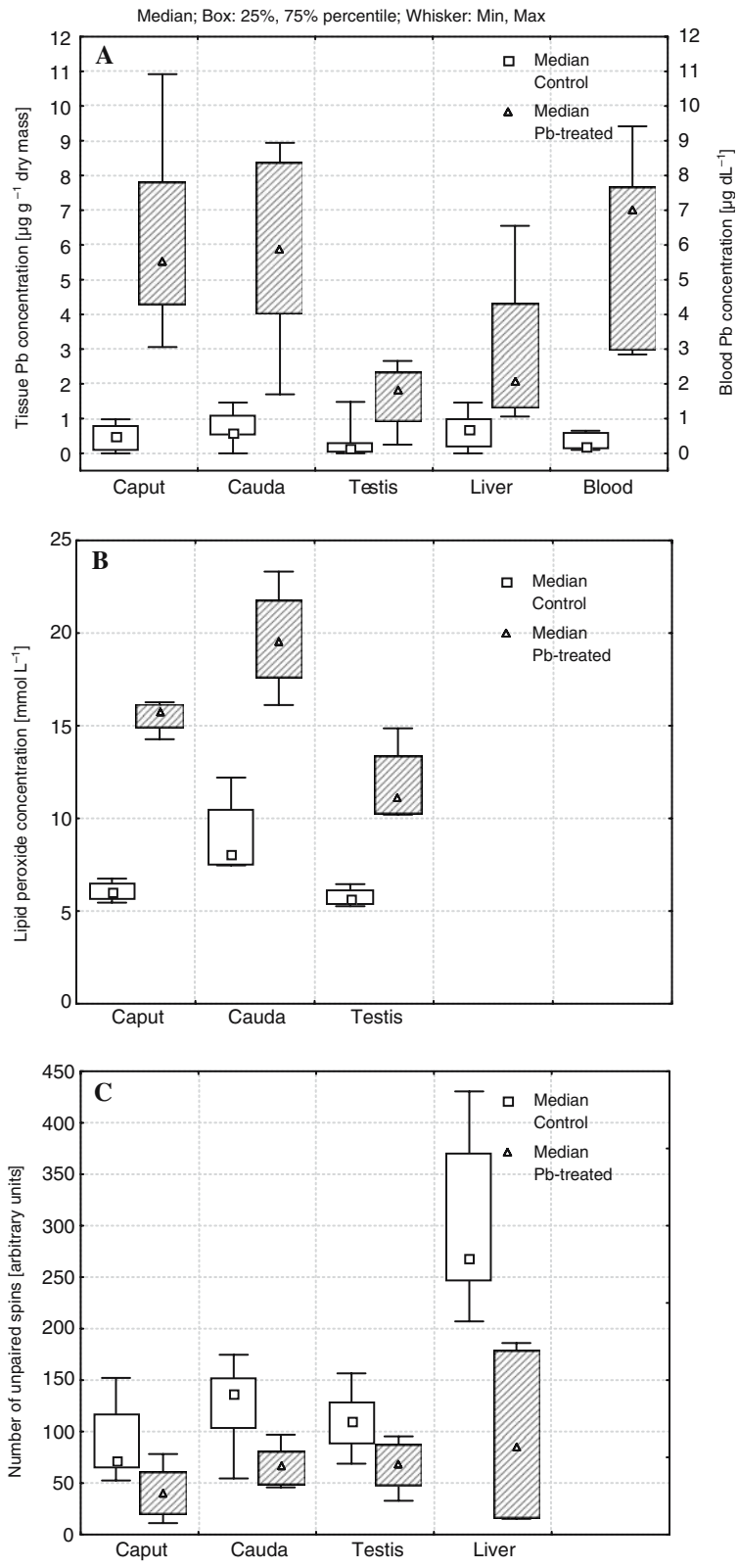
Median; Box: 25%, 75% percentile; Whisker: Min, Max
A
Median
Control
Median
Pb-treated
Tissue Pb concentration [µg g⁻¹ dry mass]
Blood Pb concentration [µg dL⁻¹]
Caput
Cauda
Testis
Liver
Blood
B
Lipid peroxide concentration [mmol L⁻¹]
C
Number of unpaired spins [arbitrary units]

Figure 1. Box-and-whisker plots of the presented data in control and lead acetate treated rats: (a) Lead content in tissues and blood; (b) Lipid peroxides content in tissues; (c) Number of unpaired spins (arbitrary units: $X{\cdot}10^{16}g^{-1}$ dry mass) in tissues.

Concentration of lipid peroxides in homogenates of testis and epididymis

In control rats the concentration of lipid peroxides in homogenates of cauda epidydymis was 35% higher than in caput epidydymis and 43% higher than in testis. The differences of lipid peroxides content between the group exposed to PbAc and control group in caput epidydymis, cauda epidydymis and testis were 162%, 125% and 97% respectively (Figure 1b).

Concentration of ascorbyl free radical in the lyophilized tissues

All the EPR signals obtained from tissues display the same shape and location ($\Delta B_{pp} = 0{,}8$ mT, g = 2.005) and they differ only with respect to amplitude, which denotes different concentration of unpaired spins. The number of unpaired spins in the studied tissues calculated in arbitrary units $X \cdot 10^{16}g^{-1}$ of dry mass is given in Figure 1C. After 9 months of exposure to PbAc a significant decrease in EPR amplitude from the studied tissues: caput and cauda epididymis, testis and liver, was noted.

Concentration of L-ascorbic and uric acid in plasma

The concentration of plasma L-ascorbic acid in control rats was 38.48 ± 8.25 μmol l^{-1}, while in the rats exposed for 9 months to PbAc plasma concentrations of L-ascorbic acid were significantly lower: 27.62 ± 3.58 μmol l^{-1} ($P < 0.05$; Student *t*-test). Plasma uric acid concentrations in the exposed rats were significantly higher: 118.85 ± 33.9 μmol l^{-1} when compared to the animals from the control group: 64.53 ± 14.47 μmol l^{-1} ($P < 0.05$; Student *t*-test).

Total antioxidant status

In control animals the median value of total antioxidant status (TAS) was 0.51 mmol l^{-1} (Q25–Q75: 0.49–0.55). After 9 months of intoxication with AcPb the serum TAS value was significantly lower: median 0.39 mmol l^{-1} (Q25–Q75: 0.35–0.42; $P < 0.05$ Mann–Whitney *U*-test).

Discussion

Some authors have reported elevation of oxidative stress indicators as a result of exposure to lead. Oxidative stress may be a result of excessive ROS generation or failure of the cellular antioxidant system (Kruk 1998; Hsu *et al.* 1998a). Lipid peroxidation was increased in the brain of animals exposed *in vivo* to lead (Adonaylo & Oteiza 1999). Valverde *et al.* (2001) found induction of lipid peroxidation and increased ROS level in tissues of mice after intoxication with PbAc for 1 h. Their findings are in agreement with results obtained by several authors (Yang *et al.* 1999; Adonaylo & Oteiza 1999) suggesting induction of PbAc cytotoxicity by indirect mechanisms i.e. in part by oxidative stress. Our current investigations showed significantly increased concentration of lipid peroxides in testis and epididymis of lead-exposed rats. Our most recent results revealed significantly increased intensity of chemiluminescence (CL) in testicular, epididymal (Marchlewicz *et al.* 2004) and liver homogenates (unpublished data) from rats chronically exposed to PbAc, most likely resulting from increased lipid peroxidation. These data are consistent with results obtained by Hsu *et al.* (1998a) who revealed elevated intensity of CL from epididymal spermatozoa in AcPb-intoxicated rats. The latter resulted from increased generation of ROS in these cells (Hsu *et al.* 1997, 1998a). Intensification of lipid peroxidation caused by lead may affect the composition of cytoplasmic, mitochondrial and acrosomal membranes. It is known that on the way from caput to cauda epididymis the sperm membranes become richer in unsaturated fatty acids and therefore they show tendency to instability. It promotes acrosomal reaction but at the same time the membranes become more prone to peroxidative damage (Aitken *et al.* 1999; Batra *et al.* 1998).

In the present study, lyophilized tissues of lead-intoxicated animals revealed also a considerable decrease of the ESR signal amplitude. The range

of the ESR signal amplitude depends on the concentration of ascorbyl free radical in the lyophilized tissue (Gonet 1994). The decrease in ESR signal amplitude seen in the experiment indirectly indicates a decrease in the tissue content of L-ascorbic acid. Significant decrease in content of L-ascorbic acid and increased content of uric acid were demonstrated using the direct HPLC method in the plasma of exposed rats. Changes in the concentration of L-ascorbic acid in plasma may be caused by utilization of this compound in the process of elimination of ROS generated during exposure to Pb. Halliwell and Gutteridge (1999) in studies on oxidative stress revealed the following order of consumption of antioxidants: ascorbate and thiol groups in proteins, bilirubin, uric acid and alpha-tocopherol. Higher plasma concentrations of uric acid in PbAc-exposed rats may be the result of excessive purine catabolism caused by disturbance of energy metabolism. It was found that ATP levels in erythrocytes (Baranowska-Bosiacka & Hlynczak 2003) and tissues [unpublished data] decreased after treatment with lead. ATP catabolism leads (through ADP, AMP, adenosine, inosine, hypoxanthine and xanthine) to increased synthesis of uric acid (Baranowska-Bosiacka *et al*. 2004).

The role of ascorbic acid in the reduction of the oxidative stress observed after lead intoxication has been confirmed by the lowering of the stress markers in animals treated simultaneously with L-ascorbic acid (Hsu *et al*. 1998b; Patra *et al*. 2001; Upasani *et al*. 2001; Marchlewicz *et al*. 2004). Our earlier studies revealed significantly lower CL intensity in homogenates of testis and epididymis from rats simultaneously treated with PbAc and L-ascorbic acid (at a dose of 500 mg l^{-1} in drinking water) when compared to a group of rats treated with PbAc only (Marchlewicz *et al*. 2004).

In our experiment the plasma total antioxidant status (TAS) of the intoxicated animals was significantly lower than in the control group. Serum antioxidant ability depends on concentrations of L-ascorbic acid, albumin, bilirubin and uric acid. The latter compound binds copper and iron ions, prevents spreading of the free radical reactions and scavenges peroxyl $LOO^{\bullet}$ radicals and singlet oxygen that may initiate lipid peroxidation. Ascorbic acid has multiple antioxidant properties, including the ability to regenerate uric acid. A reactive urate anion radical, a result of reaction with $^{\bullet}OH$, is reduced by ascorbate (Sevanian *et al*. 1991). The changes in plasma TAS level may be the result of endogenous protection mechanisms against free radical toxicity.

In summary, our studies indicated that lead, even at low levels (blood Pb level below 10 $\mu g\ dl^{-1}$) causes disturbances in metabolism of male rat reproductive system organs. The observed decrease of L-ascorbic acid level and increase in lipid peroxide concentration in the tissues of male reproductive system may confirm the presence of imbalance between ROS generation and removal, caused by exposure to lead. It also proves that the element exerts at least part of its toxic effects in male reproductive system of rats through the triggering of oxidative stress in these tissues. Moreover, the studies indicated in conjunction with other studies (Hsu *et al*. 1998b; Patra *et al*. 2001; Upasani *et al*. 2001) that ascorbic acid, through its antioxidant properties, is involved in the reduction of lead toxicity.

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