

Low blood Pb levels could adversely affect the pro-oxidant balance in rat epididymides

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ABSTRACT

Introduction: The impairment of male fertility, identified in epidemiological studies in recent decades, may be associated with increasing exposure to environmental factors, among which heavy metals, especially lead (Pb), have a unique role due to their prevalence and toxicity. The aim of the study was to determine the effect of Pb on the epididymis, an organ in which spermatozoa reach maturity and acquire the ability to fertilize an egg. In particular, it was investigated whether low blood Pb levels could adversely affect the pro-oxidant/antioxidant balance in rat epididymides.

Materials and methods: The intensity of oxidative stress was measured by changes in lipid peroxidation (by chemiluminescence method), as well as in protein expression and the activity of superoxide dismutase (SOD) and catalase (CAT). Additionally, it was investigated whether the toxic effects of Pb could be alleviated by the simultaneous administration of the antioxidants – tocopherol (vitamin E) or its water-soluble counterpart Trolox.

Results: Chronic exposure to Pb resulted in increased lipid peroxidation in the epididymis (manifested by a marked intensification

in chemiluminescence from the caput and cauda epididymis). Significantly, when Pb-treated rats were also supplemented with Trolox or tocopherol, the increase in chemiluminescence intensity was significantly lower, which indicated the beneficial effect of these antioxidants on reduction of Pb-induced oxidative stress. However, a direct beneficial effect of supplementation on the activity and expression of SOD and CAT was not demonstrated. The activity and expression of SOD was not affected by exposure to Pb or to simultaneous administration of antioxidants. The use of tocopherol and Trolox had also no beneficial effect on the Pb-induced reduction in the activity and expression of CAT.

Conclusions: The environmental Pb-exposure leading to metal accumulation in whole blood at concentrations below the “threshold level” (5 µg/dL) may cause disruption in the prooxidant/antioxidant balance in rat epididymides containing epididymal spermatozoa.

Keywords: lead (Pb); oxidative stress; epididymis; tocopherol; Trolox; antioxidant enzymes; lipid peroxidation.

INTRODUCTION

Lead (Pb) pollution still poses a significant risk to human health worldwide, despite various measures implemented in recent decades to curb the use of this heavy metal [1, 2, 3]. Its growing accumulation in the environment is associated with its non-degradability and its ongoing considerable release into the environment, currently estimated at 1.6 million metric tons per year [4]. Populations living in major agglomerations are particularly exposed to the adverse effects of Pb. Most notably, it affects pregnant women and children [5, 6]; however, people working in the production of lead-containing paints, varnishes, batteries, wires, cables, enamel paints, glass and crystals, type metal and solders are also affected [7].

Currently, many researchers propose an ever lower “safe threshold concentration” of this element. Although in 1991 the American Center for Disease Control [8] lowered the safe threshold concentration of Pb-B to 10 µg/dL [9], followed by 5 µg/dL for children and pregnant women [10], a lot of

data indicates that even lower concentrations pose a threat to health [8, 11, 12]. Some researchers argue that there is no such thing as “the safe Pb level”. This means that at each concentration of Pb in the body, we may expect irregularities in the biochemical processes in many organs [13, 14].

Lead can accumulate in most tissues of the human body, including the liver, kidneys and bones [15]. Accumulation begins as early as in the prenatal period since Pb is able to cross the blood-placenta barrier. Lead also has an adverse effect on the hematopoietic system [16, 17, 18], the immune system [19, 20], as well as the central and the peripheral nervous system [21, 22, 23, 24, 25, 26]. It impairs the brain [19, 23, 27], kidneys [15, 28], and liver [20, 25, 29]. Exposure to Pb can also be considered a potential factor responsible for deteriorating male fertility in the world [30, 31]. Oxidative stress can cause sperm impairment and, therefore, fertility disorders. People occupationally exposed to Pb exhibit a relationship between elevated blood Pb levels and a decreased number of spermatozoa with decreased mobility and abnormal morphology [32, 33]. On a global scale, approx. 15–20% of couples are infertile, with

male infertility responsible for 25–50% of cases [30, 31, 34, 35]. The mechanism of Pb toxicity in the male reproductive system is not yet fully understood [19].

The epididymis is an important organ for male fertility. The proximal (caput) part of the epididymis is responsible for the production of a microenvironment in which the sperm mature and acquire the ability to move forward, survive in the male and female genital tract, recognize and bind to the oocyte cell membrane [36]. The distal (cauda) section, on the other hand, maintains the viability of the sperm and inhibits their motility locally. These processes rely on the interaction between proteins secreted by epithelial cells into the lumen of the epididymis and sperm [37].

It is also known that epididymal epithelial cells can accumulate Pb. Therefore, it may be assumed that Pb has an ability to move through blood vessels to the epithelial cells of the epididymis as well as to the epididymis lumen by crossing the blood epididymis barrier. Lead can also damage the ultrastructure of epididymis epithelial cells in animals. Lead increases oxidative stress by inducing the production of reactive oxygen species (ROS) in various tissues, including in the male reproductive system [19, 24, 38], as shown in the testis and sperm [32, 34, 35]. An excess of ROS can lead to spermatozoa damage and, consequently, male infertility [30, 39]. Lead can also cause dose-related disturbances of the prooxidative and antioxidative balance. It was demonstrated that Pb toxicity can induce oxidative stress by generating hydroperoxides, singlet O_2 and H_2O_2 , or by causing a deficiency of antioxidants. The process of ROS generation by Pb in tissues occurs, among others, through an increased lipid peroxidation [5]. Phospholipids can act as substrates for lipid peroxidation in cells of animals with Pb toxicity. In the epididymis of PbAc-intoxicated rats, Wiszniewska et al. manifested an increased concentration of phosphatidylcholine (lecithin), kephalin, sphingomyelin and lysolecithin [40]. In turn, Pb has the ability to form strong bonds with phosphatidylcholine of cell membranes. As a result of lipid peroxidation, the physical properties of the membranes can change causing an increase in their permeability and even loss of integrity [41, 42]. This is particularly adverse for spermatozoa present in the epididymis as the antioxidant defence against the negative effects of ROS may not be sufficient [43]. In the epididymis, a very important protective function against oxidative stress is played by antioxidant enzymes synthesized by the epididymal epithelial cells, primarily superoxide dismutase (SOD) and catalase (CAT). It has been demonstrated [44] that Pb not only increases lipid peroxidation but also reduces SOD and CAT activity due to its high affinity for sulfhydryl groups ($-SH$) and metal-cofactors in antioxidant enzymes. Superoxide dismutase and CAT, along with glutathione peroxidase (GPX), constitute the first line of defence against ROS. Superoxide dismutase dismutates superoxide anion radical ($O_2^{\bullet-}$), CAT decomposes hydrogen peroxide (H_2O_2) and GPX breaks down lipid peroxides into less toxic molecules. The toxic activity of even low doses of Pb may be manifested by inhibiting the activity of enzymes taking part in the synthesis of haem as well as of glutathione and enzymes containing thiol groups (SOD, CAT, GPX).

A significant role in the protection against the negative effects of ROS is played by antioxidants, including vitamins [45, 46, 47]. Vitamin E (which mainly contains α -tocopherol) and Trolox (a water-soluble derivative of vitamin E) are referred to as non-enzymatic ROS inhibitors. Alpha-tocopherol acts mainly in the lipid phase of cell membranes [48], whereas Trolox acts predominantly in the aqueous phase [49, 50].

Vitamin E contains 4 types of tocopherols, the main being α -tocopherol – accumulated in the cell membrane, endoplasmic reticulum membrane and in mitochondria. Alpha-tocopherol has a protective effect on the stability of cell membranes [27] as it stops the chain reaction of lipid peroxidation depending on the dose. It scavenges 3 types of ROS: peroxides, hydrogen peroxide and hydroxyl radical [30]. Consequently, membrane lipids are protected against peroxidative damage by reducing the level of lipid peroxides and increasing SOD and CAT activity. This protective action was demonstrated with respect to erythrocytes, spermatozoa [32] as well as other tissues, e.g., the testes [51, 52].

Trolox, a water-soluble tocopherol analogue which lacks a side chain. This chain would allow attachment to the cell membrane however, according to some authors, it has no effect on the antioxidant activity of Trolox [53]. Some authors claim that in terms of providing protection against oxidative stress to cells, Trolox is more efficient than the same dose of α -tocopherol [48]. There is no consensus on whether simultaneous administration of both antioxidants (vitamin E and Trolox), shows a more efficient antioxidant action. Some authors confirm this synergistic effect [54] – others do not [38]. Nevertheless, numerous studies indicate that the administration of vitamin E may have a protective effect on the male reproductive system exposed to Pb [55].

Given the reports on deteriorating semen parameters [56, 57, 58], it seems crucial to investigate the effects of toxic agents widespread in the environment, such as Pb, on the epididymis which is the organ in which spermatozoa reach maturity and acquire forward motility and ability to fertilise an egg. The aim of this paper is to examine if environmental Pb exposure leading to metal accumulation in whole blood at concentrations below the “threshold level” ($5 \mu g/dL$) may cause disruption in the prooxidant/antioxidant balance in rat epididymides containing epididymal spermatozoa. We report oxidative stress intensity as well as the activity and protein expression of the most important antioxidant enzymes.

MATERIALS AND METHODS

Animals

Animals were treated in compliance with international standards of animal care to minimize the suffering and the number of animals used. We received the approval of the Local Ethical Committee (No. 15/2007). The experiments were carried out on 48 sexually mature male Wistar rats, weighing between 410–560 g each, living in a room with a controlled temperature, under the day/night 12/12 regimen. In the study, the animals were divided into the following groups: **Pb group** – rats receiving 0.1% solution of lead acetate (PbAc) in drinking water,

Polskie Odczynniki Chemiczne, Poland, ad libitum, $n = 8$; **Pb + Trl group** – rats receiving PbAc plus Trolox (water-soluble tocopherol analogue, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma/Aldrich, Poland), at a dose of 2.8 mg/kg bm/day, $n = 8$; **Pb + E group** – rats receiving PbAc plus vitamin E in drinking water (vitamin E, oral solution, Medana, Poland) at a dose of 2.8 mg/kg bm/day, $n = 8$.

The control animals were divided in the respective groups: **control group** – rats receiving distilled water, $n = 8$; **Trl group** – rats receiving distilled water with Trolox at a dose 2.8 mg/kg bm/day, $n = 8$; **E group** – rats receiving vitamin E at a dose of 2.8 mg/kg bm/day, $n = 8$.

The experiment lasted 6 months. Lead was administered orally to imitate environmental exposure. Our previous experiments showed that 0.1% PbAc in drinking water resulted in a whole blood concentration below 10 µg/dL [21], the threshold value considered safe for people [59]. Water and dietary intakes of the treated rats were the same as in the control group.

After the experiment, the rats were killed under Thiopental (120 mg/kg i.p., Biochemie GmbH, Austria) anaesthesia. The control and experimental rats were then weighed, blood was sampled, and the epididymides containing epididymal spermatozoa were placed in liquid nitrogen. The sperm were not rinsed out of the epididymides because the research model we used in all our previous studies did not use rinsed sperm. However, the sperm plasma and sperm did not show high enzymatic activity. The samples were stored at -80°C for further determination of enzyme activity and Western blot analyses. The epididymides were fixed in Bouin fluid for further light microscopic studies.

Atomic Absorption Spectroscopy lead determination

Lead content was analysed by graphite furnace atomic absorption spectrometry (GFAAS) with a Perkin Elmer 4100 ZL spectrometer, using Zeeman correction. Samples of epididymal tissue containing epididymal spermatozoa were digested with 1 mL of 65% HNO_3 at 120°C for 16 h in a closed Teflon container. After cooling, samples were treated with 1 mL 30% H_2O_2 and digested for the next 24 h in the same conditions. The obtained solution was diluted with deionised water to 10 mL and analysed by GFAAS together with blank and control samples. Whole blood samples were deproteinised with 65% HNO_3 and further analysed as described above. The detection limit was 0.3 µg/dL.

Light microscope examination

For morphological examinations, the serial slides were stained using the periodic acid-Schiff (PAS method), and the nuclei of cells were stained with acid hematoxylin [60].

Determination of tocopherol levels in plasma using high-performance liquid chromatography

The blood was collected in test tubes with ethylenediaminetetraacetic acid (EDTA) and then centrifuged ($850 \times G$ for 10 min, at a temperature of $+4^{\circ}\text{C}$). Prior to further analysis, the plasma was stored at -80°C under dark conditions in Eppendorf plastic test tubes. Plasma in the volume of 150 µL was mixed with the same amount of ethanol containing 0.5 mg/L trans- β -apo-8'-carotenal (internal standard) and 100 mg/L

di-tert-butyl-4-methylphenol (antioxidant) and then vortexed using a vortex mixer for 30 s. The whole procedure was conducted at a temperature of 0°C . Then, 600 µL of hexane (Polskie Odczynniki Chemiczne, Poland) was added and the whole sample was again mixed for 2 min [61]. Having centrifuged the sample again ($1000 \times G$ for 5 min at a temperature of 0°C), 480 µL of the upper layer of the solution was transferred to a glass tube and evaporated to dryness in an N_2 environment. The precipitate was dissolved in 100 µL of ethanol by stirring intensely for 30 s. Each time, the temperature was 0°C and the samples were protected against light. Reversed phase chromatographic separation was conducted using Agilent 1200 chromatograph (Agilent, Palo Alto, CA, USA) equipped with diode array detector, a thermostated column with a thermostated auto sampler fitted with 201TP54 column (250 x 4.6 mm) and a precolumn 201GK54 (Vydac, Poland). Isocratic separation of tocopherols was conducted at a temperature of $+21^{\circ}\text{C}$. The mobile phase was methanol-tetrahydrofuran-acetonitrile (Sigma-Aldrich, Poland), at a volume-to-volume ratio: 88.5:7.5:5.0 at a flow rate of 1.5 mL/min. The assessment of the tocopherol level was conducted at a wavelength of 295 nm, and an internal standard at a wavelength of 450 nm. The volume of the sample was 20 µL, the measurement duration was 10 min. Calibration was done using an alcoholic solution of α -tocopherol (Sigma-Aldrich, Poland) instead of ethanol [60, 61, 62].

Oxidative stress assay

Lipid peroxidation measurement by chemiluminescence

In order to perform chemiluminescence (CL), epididymides containing epididymal spermatozoa were excised and 0.5 g samples were homogenized in 4.5 mL of physiological saline. Chemiluminescence intensity was measured using a M12FQC 51 photomultiplier with a S20 cathode sensitive in the 200–850 nm range, operating with the Zeiss K-200 recorder. A 5 mL sample was placed in a cuvette (53 mm in diameter) mounted on the front of a photomultiplier and kept at room temperature. The intensity of light was monitored as a function of time, $I = f(t)$, and light sums – CL (the integrated relative intensity equal to the area under the kinetic curve of CL) were measured over 10 min and corrected for background signal. All the procedures for CL measurement were performed in darkness.

Enzyme analysis

Homogenization protocol

Frozen epididymides containing epididymal spermatozoa were taken from liquid nitrogen and placed in a thermobox (-21°C). A small fragment of the tissue was placed in a metal homogenisator (previously cooled in a container with liquid nitrogen) and poured on 2–3 times with liquid nitrogen; then it was fragmented with a few blows of a hammer (4–5 times) against a metal mandrel (previously cooled in a container with liquid nitrogen). The pulverized and frozen sample (volume equal to approx. 1 mg of protein) was placed with a cooled spoon in an Eppendorf tube containing 500 µL of buffer (according to commercial enzyme assay kit procedure) previously cooled to a temperature of 4°C . After being briefly vortexed, the sample was homogenised for about 15 s using a knife homogenisator. The extract mixtures

were then centrifuged (3000 x G for 10 min, at 4°C) and the supernatants were stored at -80°C for enzyme assays.

Enzyme activities

Superoxide dismutase activity was measured using the Superoxide Dismutase Assay Kit (Cayman, USA), according to manufacturer's procedure using a Lambda40 (Perkin-Elmer) spectrophotometer. Catalase activity was measured using the Bioxytech Catalase-520™ Assay Kit (OxisResearch, Poland), according to the manufacturer's procedure.

Western blotting analysis of enzyme protein expression

The homogenization of the epididymis containing epididymal spermatozoa samples was carried out using a ristocetin-induced platelet aggregation buffer (pH 7.4) that consisted of 20 mM Tris; 0.25 mM NaCl; 11 mM EDTA; 0.5% NP-40, 50 mM sodium fluoride and protease, phosphatase inhibitors (Sigma, Poland) [63]. Bradford Protein Assay (Sigma, Poland) was used to determine total protein levels. Homogenates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and examined for the expression of SOD and CAT as specified by Baud et al. [63]; 12% gel (SDS-PAGE) and Mini Protean Tetra Cell System (Bio-Rad, Poland) were used to separate the extracted proteins (20 µg/well). Fractionated proteins were transferred to a 0.2 µm PVDF membrane (Bio-Rad, Poland). The membranes were then blocked with 3% bovine serum albumin (BSA) in buffer for 1 h at room temperature. The protein expression of the epididymis was detected using antibodies against SOD – sheep pAb (Calbiochem, USA) in a 1:1000 dilution, and sAb bovine anti sheep IgG HRP (Santa Cruz Biotech, Poland); CAT – rabbit pAb (Abcam, USA) in dilution 1:1000 and sAb bovine anti rabbit IgG HRP (Santa Cruz Biotech, USA). The membranes were developed with the ECL Advance Western Blotting Detection Kit (Amersham Life Sciences, UK) and bands were visualized using the Gel DOC-It Imaging system.

Protein measurement

Protein concentration was determined with the bicinchoninic acid (BCA) method using a commercial Pierce™ BCA Protein Assay Kit (Thermo Scientific™, USA).

Statistical analysis

The statistica 10.0 software package was used to perform a statistical analysis of the results. Arithmetical mean and standard deviation (±SD) were calculated for each of the studied parameters. The distribution of the results for individual variables was determined by the Shapiro-Wilk W-test. Because the majority of the distributions were not normal, the non-parametric Mann-Whitney U-test and Kruskal-Wallis ANOVA was used for further analyses and differences were considered statistically significant at $p < 0.05$.

RESULTS

Morphological and biochemical studies were conducted on the epididymides of the control group, the group intoxicated with

0.1% PbAc solution, and the group with PbAc supplemented with antioxidants: Trolox or vitamin E for 6 months (the duration of spermatogenesis in rats).

Body mass and epididymis mass in rats

No essential differences were recorded between body mass and the mass of the epididymis containing epididymal spermatozoa in animals drinking 0.1% PbAc for 6 months (with or without antioxidants) and the animals of the respective control groups (Tab. 1).

TABLE 1. Body mass and the mass of the epididymis containing epididymal spermatozoa of lead-treated or lead-treated and vitamin E or Trolox-supplemented rats

Experimental conditions	Epididymis (g)	Body (g)
Control	0.79 ± 0.14	525.00 ± 38.34
Pb	0.69 ± 0.08	448.57 ± 40.18
Trl	0.76 ± 0.10	493.33 ± 59.89
Pb + Trl	0.70 ± 0.10	450.00 ± 48.65
E	0.78 ± 0.10	520.00 ± 37.42
Pb + E	0.66 ± 0.09	471.25 ± 51.39

The results are expressed as mean ±SD. No statistically significant differences between the studied groups were found (Mann-Whitney U-test).

Blood and epididymis lead concentrations

After 6 months of exposure, the Pb-treated animals had a significantly higher Pb concentration in whole blood and both studied parts of the epididymis: the caput and cauda, when compared to the control group (Tab. 2).

TABLE 2. Lead concentration in whole blood and in the caput and cauda of the epididymis containing epididymal spermatozoa of lead-treated or lead-treated and vitamin E or Trolox-supplemented rats

Experimental conditions	Blood (µg/dL)	Epididymis (µg/g dry mass)	
		caput	cauda
Control	0.30 ± 0.25	0.34 ± 0.24	0.45 ± 0.21
Pb	2.17 ± 0.77 ^{α*}	3.05 ± 1.50 ^{a**}	3.97 ± 1.09 ^{A**}
Trl	0.31 ± 0.25	0.35 ± 0.19	0.46 ± 0.15
Pb + Trl	2.21 ± 0.77 ^{β*}	3.70 ± 1.10 ^{b**}	3.66 ± 1.56 ^{B**}
E	0.30 ± 1.12	0.42 ± 0.32	0.47 ± 0.18
Pb + E	2.89 ± 1.14 ^{γ**}	3.34 ± 1.84 ^{dc**}	3.81 ± 1.54 ^{C**}

The results are expressed as mean ±SD. Statistically significant differences versus: α – blood Control vs. Pb group; β – blood Trl vs. Pb + Trl group; γ – blood E vs. Pb + E group; a – caput Control group vs. Pb group; b – caput Trl vs. Pb + Trl group; c – caput E vs. Pb + E group; A – cauda Control vs. Pb group; B – cauda Trl vs. Pb + Trl group; C – cauda E vs. Pb + E group.

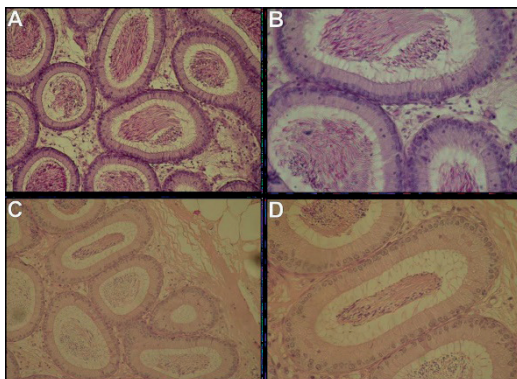
* $p < 0.01$; ** $p < 0.001$ (Mann-Whitney U-test)

Long-term administration of Pb to rats resulted in a marked increase in Pb concentration in whole blood as compared to the control (7-times, $p < 0.005$), as well as in the caput and cauda epididymis as compared to the control (in both cases at least 8-times, $p < 0.005$). In the group of rats intoxicated with Pb plus Trolox, an increase in Pb concentration in whole blood, as

compared to the control, was also found (7-times, $p < 0.005$), as well as in the caput and cauda epididymis (10 times and 8 times higher levels, respectively, $p < 0.005$ for both). In the group of rats receiving Pb with vitamin E, the concentration of Pb in whole blood was significantly higher than that of the control group (9 times higher, $p < 0.005$); in the cauda epididymis it was 8 times higher ($p < 0.05$) and in the caput epididymis 10 times higher ($p < 0.005$). In Pb-treated rats supplemented with vitamin E, whole blood Pb concentration was found to be significantly higher (9 times higher, $p < 0.005$); in the caput epididymis and cauda epididymis, it was approx. 8 times higher ($p < 0.005$), as compared to the rats receiving only vitamin E. Lead concentration in the blood and tissues of Pb + E rats, and of Pb + Trl rats, showed no statistically significant differences. There was no evidence of statistically significant differences in Pb concentration between the caput epididymis and cauda epididymis in any of the groups under analysis (Tab. 2).

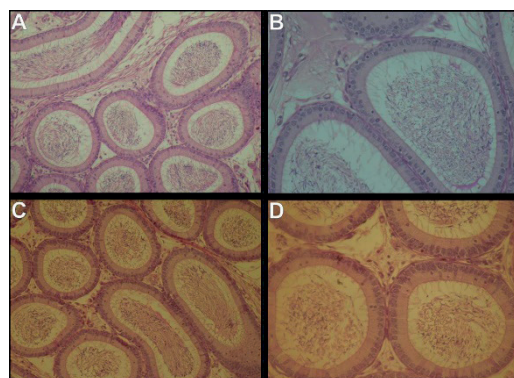
Morphological studies

The morphology of the epididymal epithelial cells of the caput (Fig. 1, 2) and cauda (Fig. 3, 4) of the epididymides of the rats receiving Pb (with or without antioxidants) was similar to the control group.



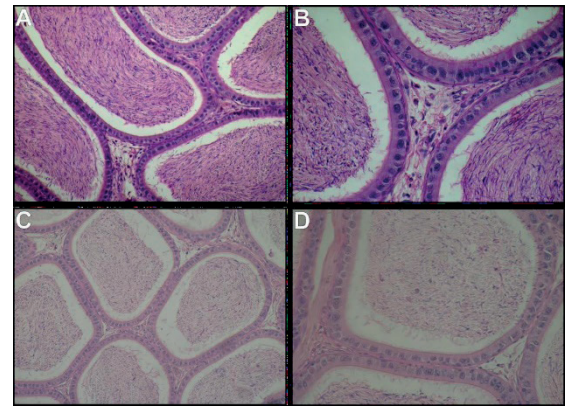
PAS – A, C x 320; B, D x 670

FIGURE 1. Cross-section of the caput epididymis duct of the control group rats (A, B) and rats receiving lead acetate (Pb group; C, D)



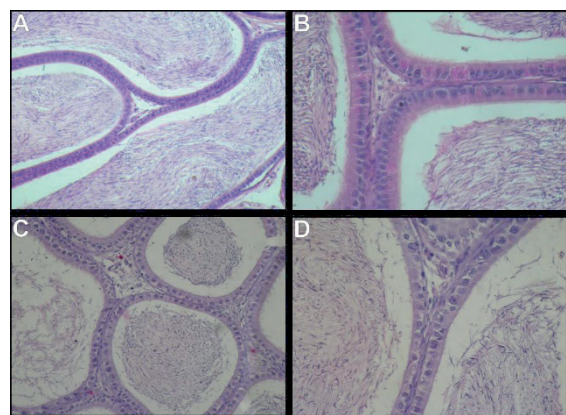
PAS – A, C x 320; B, D x 670

FIGURE 2. Cross-section of the caput epididymis duct of the rats receiving lead with Trolox (Pb + Trl group; A, B), and rats receiving lead and vitamin E (Pb + E group; C, D)



PAS – A, C x 320; B, D x 670

FIGURE 3. Cross-section of the cauda epididymis duct of the control group rats (A, B) and rats receiving lead acetate (Pb group; C, D)



PAS – A, C x 320; B, D x 670

FIGURE 4. Cross section of the cauda epididymis duct of the rats receiving lead with Trolox (Pb + Trl group; A, B), and rats receiving lead and vitamin E (Pb + E group; C, D)

Alpha- and gamma-tocopherol concentration in plasma

In the plasma of the control group, α - and γ -tocopherols were identified; the concentration of α -tocopherol was found to be higher. In the group of rats receiving Pb, a lower concentration of these compounds in plasma, as compared to the control group, was found (by 10 and 60%, respectively, $p < 0.05$). In the group of rats intoxicated with Pb with Trolox, a decrease in concentration of plasma α - and γ -tocopherols was found, as compared to the control, by 15% and 40%, respectively ($p < 0.05$). Concentrations of α - and γ -tocopherol were found to be statistically significantly lower in the group of rats intoxicated with Pb with an addition of Trolox in comparison with the group of rats receiving only Trolox (by 45%, $p < 0.005$ and 70%, $p < 0.05$, respectively).

In the group intoxicated with Pb with additional vitamin E, the concentration of α -tocopherol was higher than that of the control by 75% ($p < 0.05$) and, in comparison with the group supplemented only with vitamin E, the concentration was lower by approx. 20% ($p < 0.05$). However, the concentration of γ -tocopherol was decreased by approx. 65% in comparison with that of the control group ($p < 0.05$) and by approx. 96% when compared with the group receiving only vitamin E (Tab. 3).

TABLE 3. The concentration of tocopherols alpha and gamma (mg/L) in the plasma of lead-treated or lead-treated and vitamin E or Trolox-supplemented rats

Experimental conditions	Alpha-tocopherol	Gamma-tocopherol
	(mg/L) in plasma	
Control	7.42 ±1.90	0.27 ±0.09
Pb	6.68 ±1.55*	0.11 ±0.08*
Trl	11.50 ±0.40*	0.50 ±0.13**
Pb + Trl	6.29 ±0.57*^	0.15 ±0.03*^
E	16.05 ±0.34**	2.16 ±0.04**
Pb + E	13.04 ±4.34*&	0.09 ±0.03**#

The results are expressed as mean ±SD.

* p < 0.05 vs. control; ** p < 0.005 vs. control; ^ p < 0.005 vs. Trl; & p < 0.05 vs. E; # p < 0.005 vs. E (Mann-Whitney U-test)

Chemiluminescence from epididymis homogenates

In all analysed groups, higher CL sums were found in the cauda epididymis homogenates than in the caput homogenates. In the group of rats receiving only Pb, there was a statistically significant increase in the intensity of CL from the caput as well as the cauda epididymis homogenates as compared to the control (by 85%; p < 0.005 and by 40%; p < 0.005, respectively). Chemiluminescence from the cauda epididymis or caput homogenates of rats receiving Trolox or vitamin E did not show statistically significant differences compared to control. Chemiluminescence from epididymis homogenates of rats receiving Trolox with Pb was found to be higher than that of the control group by approx. 30% for the caput (p < 0.005). The CL value was markedly lower than that determined for the group receiving only Pb by approx. 30% for the caput epididymis and approx. 20% for the cauda. No significant differences in terms of CL intensity were found between the group receiving Pb with vitamin E and the control group, with respect to the caput epididymis and cauda. Moreover, CL obtained from the tissues of the Pb and vitamin E group was also found to be markedly lower than that of the group receiving only Pb – by approx. 45% for the caput epididymis and approx. 20% for the cauda. The intensity of CL in the cauda epididymis was higher in all the groups under study than that determined for the caput epididymis (p < 0.005 for all) – Table 4.

TABLE 4. Comparison of chemiluminescence sums from tissue homogenates of lead-treated or lead-treated and Trolox or vitamin E-supplemented rats

Group	Caput epididymis	Cauda epididymis
	arbitrary units	
Control	60.20 ±12.28	124.14 ±31.91#
Pb	111.43 ±18.32*	174.50 ±29.95*#
Trl	58.10 ±2.98	120.43 ±12.90#
Pb + Trl	74.10 ±11.97*^	137.00 ±11.12^#
E	50.86 ±5.89	110.86 ±4.79#
Pb + E	62.47 ±17.16&	137.25 ±36.69&#

The results are expressed as mean ±SD.

* p < 0.005 vs. control; ^ p < 0.005 vs. Trl; & p < 0.05 vs. E; &# p < 0.005 vs. E; # p < 0.005 caput vs. cauda (Mann-Whitney U-test)

Activity of antioxidative enzymes in the epididymis

Superoxide dismutase activity in the epididymis

In the homogenates of the epididymides containing epididymal spermatozoa of Pb-treated rats, as compared with control, a decrease in the activity of this enzyme was observed – by 26% for the caput and 44% of the cauda epididymis – this difference, however, was not statistically significant. The analysis of the tissues of rats receiving Pb with Trolox also showed an insignificant decrease in the activity of the enzyme in comparison with the group receiving only Trolox (by 10% in the caput and by 26% in the cauda). In Pb-treated rats supplemented with vitamin E, an insignificant decrease in this enzyme was found as compared to the group receiving only vitamin E (by 17% in the caput, and by approx. 36% in the cauda epididymis) – Table 5.

TABLE 5. Superoxide dismutase activity in the caput and cauda epididymis containing epididymal spermatozoa of the control, lead-treated or lead-treated and vitamin E or Trolox-supplemented rats

Group	Caput epididymis	Cauda epididymis
	U/mg of protein	
Control	0.87 ±0.39	1.03 ±0.71
Pb	0.64 ±0.12	0.57 ±0.26
Trl	0.72 ±0.78	0.84 ±0.07
Pb + Trl	0.65 ±0.32	0.62 ±0.57
E	0.82 ±0.58	1.02 ±0.75
Pb + E	0.68 ±0.52	0.65 ±0.21

The results are expressed as mean ±SD.

Catalase activity in epididymis

Catalase activity in epididymis homogenates was identified both in the control group as well as in the Pb-intoxicated group. Long-term administration of Pb resulted in a significant decrease in CAT activity in the caput and cauda of the epididymis (approx. by 70%, p < 0.005 for both tissues) in comparison with the control. In the group of rats intoxicated with Pb with the addition of Trolox, a decrease in the enzyme activity in the caput (approx. by 70%, p < 0.005) and the cauda (approx. by 78%, p < 0.005) as compared with the group receiving only Trolox was found. Furthermore, a significant decrease in CAT activity in the caput (83%, p < 0.005) and the cauda (approx. by 84%, p < 0.005) was found in rats receiving Pb and vitamin E as compared to the group of rats receiving only vitamin E. A statistically significantly lower activity of CAT was identified in the cauda than in the caput of rats receiving Pb (by 33%, p < 0.005) and in the group receiving Pb supplemented with Trolox (by 27%, p < 0.005) – Table 6.

Enzyme expression in the epididymis

Superoxide dismutase expression in epididymis

In epididymis homogenates of rats receiving Pb, there was a statistically insignificant decrease in enzyme protein expression, respectively by 15% in the caput, and by 17% in the cauda epididymis as compared to the control. Similarly, in the tissues of rats receiving Trolox with Pb, an insignificant decrease in

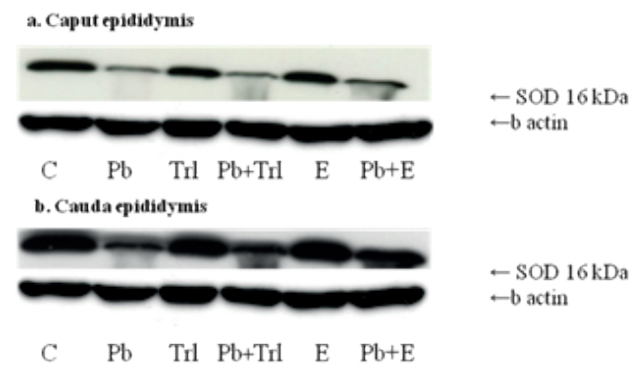
TABLE 6. Catalase activity in the caput and cauda epididymis containing epididymal spermatozoa of control, lead-treated or lead-treated and vitamin E or Trolox-supplemented rats

Group	Caput epididymis	Cauda epididymis
	U/mg of protein	
Control	69.7 ±0.23	64.78 ±0.55
Pb	22.68 ±6.25*	15.31 ±0.12*#
Trl	60.20 ±2.78	60.84 ±3.07
Pb +Trl	17.78 ±4.76^	13.00 ±3.54^#
E	62.25 ±5.98	62.94 ±4.75
Pb + E	11.39 ±5.87&	9.99 ±3.56&

The results are ex-pressed as mean ±SD.

* p < 0.005 vs. control; ^ p < 0.005 vs. Trl; & p < 0.005 vs. E; # p < 0.005 caput vs. cauda (Mann–Whitney U-test)

enzyme expression was identified in comparison with the group receiving only Trolox (approx. by 20% in the caput and cauda of the epididymis). In the group of rats treated with Pb and supplemented with vitamin E, the decrease in the expression of the enzyme was also insignificant (approx. by 25% in the caput and approx. by 20% in the cauda epididymis), as compared to the group receiving only vitamin E (Fig. 5 and Tab. 7).



The superoxide dismutase (SOD) was determined using Western blot analysis. Representative Western blots of enzyme protein normalized to β-actin in the caput and cauda were shown. Data represent the means ±SD for 6 independent experiments.

FIGURE 5. Superoxide dismutase protein expression in the caput (a) and the cauda (b) of rat epididymis**TABLE 7. Superoxide dismutase protein expression in caput and cauda epididymis of control, lead-treated or lead-treated and vitamin E or Trolox-supplemented rats**

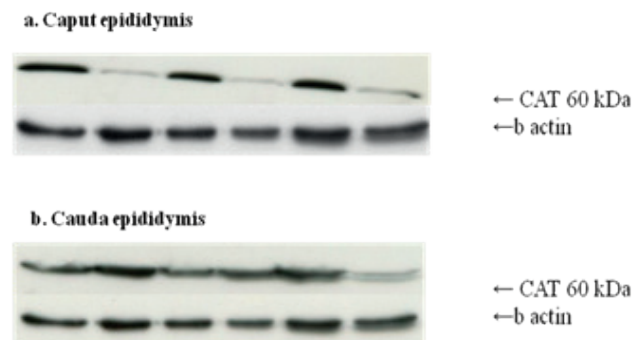
Group	Caput epididymis	Cauda epididymis
	relative expression (SOD protein/β-actin)	
Control	19.51 ±0.22	14.58 ±0.25#
Pb	16.62 ±0.65	12.11 ±1.35#
Trl	20.43 ±2.58	15.04 ±0.65#
Pb + Trl	16.32 ±0.36	12.09 ±1.24#
E	22.35 ±1.28	15.31 ±1.07#
Pb + E	16.75 ±2.15	12.05 ±2.52#

Densitometric analysis of superoxide dismutase (SOD) protein normalized to β-actin antibody in the caput and cauda epididymis were shown. The results are expressed as mean ±SD.

p < 0.005 caput vs. cauda (Mann–Whitney U-test)

Catalase expression in the epididymis

There was a significant decrease in the expression of the enzyme protein in the epididymides of rats receiving Pb, respectively by 35% (p < 0.005) in the caput and 25% (p < 0.005) in the cauda as compared to the control. In the tissues of rats receiving Pb with Trolox, there was a significant decrease in the expression of the enzyme in comparison to the group receiving only Trolox – by 38% (p < 0.005) in the caput and by 34% (p < 0.005) in the cauda. In the group of rats treated with Pb and supplemented with vitamin E, there was also a decrease in enzyme expression as compared to the group receiving only vitamin E by 33% (p < 0.005) in the caput and approx. by 29% (p < 0.005) in the cauda epididymis (Fig. 6 and Tab. 8).



The superoxide dismutase (SOD) was determined using Western blot analysis. Representative Western blots of enzyme protein normalized to β-actin in the caput and cauda were shown. Data represent the means ±SD for 6 independent experiments.

FIGURE 6. Catalase (CAT) protein expression in the caput (a) and the cauda (b) of the rat epididymis**TABLE 8. Catalase protein expression in the caput and cauda epididymis of control, lead-treated or lead-treated and vitamin E or Trolox-supplemented rats**

Group	Caput epididymis	Cauda epididymis
	relative expression (CAT protein/β-actin)	
Control	39.84 ±0.11	34.68 ±0.31#
Pb	25.99 ±0.16*	26.54 ±0.14*#
Trl	40.03 ±1.82	35.02 ±0.87#
Pb +Trl	24.64 ±0.66^	23.05 ±0.36^
E	38.25 ±0.36	33.78 ±0.54#
Pb + E	25.51 ±0.45&	23.95 ±0.62&

Densitometric analysis of CAT protein normalized to β-actin antibody in the caput and cauda of the epididymis were shown. The results are expressed as mean ±SD.

* p < 0.005 vs. control; ^ p < 0.005 vs. Trl; & p < 0.005 vs. E; # p < 0.005 caput vs. cauda (Mann–Whitney U-test)

DISCUSSION

Body mass and epididymis mass

In our experiment, we found no significant changes in the body mass or epididymis mass in rats intoxicated with 0.1% PbAc for 6 months, as well in those treated with Pb and an antioxidant (Trolox or vitamin E), as compared to respective control groups. Similarly, the results of other studies conducted on rats

intoxicated with 1% PbAc for a period of 6 months also did not show any differences in body mass or epididymis mass in comparison to the control and the group receiving Pb with Trolox or ascorbic acid [39]. Gautam and Flora did not identify changes in body mass of rats intoxicated with 0.5% PbAc for 3 weeks, as compared to control [25]. However, an experiment on 40-day old rats, mothers of which were given a single dose of 200 mg/kg bm PbAc on the 18th day of pregnancy [26], and a study on rats intoxicated with Pb in the prenatal period and during lactation [27], or sexually mature rats receiving a dose of 5 mg/kg bm or 15 mg/kg bm/i.p PbAc for 7 days or another study on rats intoxicated with 25 mg/kg bm PbAc for 30 days [64] showed a lower body mass of rats as compared with the control [65]. In rats intoxicated with PbAc at a dose of 8 mg/kg bm for a period of 14 days, a decreased mass of the testis and associated organs of the male reproductive system was also found [66].

Lead in whole blood (Pb-B)

Although we employed a 10 times lower concentration of PbAc in the present study than that used in the aforementioned studies, a significantly higher Pb concentration was identified in the blood of rats intoxicated with 0.1% PbAc for a period of 6 months, with/without Trolox or vitamin E, as compared to the values identified in the blood of the control group. Also, the concentration of Pb in the caput and cauda epididymis of rats receiving PbAc was significantly higher than in the control group. The administration of PbAc with Trolox or vitamin E did not decrease Pb concentration in the blood and tissues, in comparison to the group receiving only Pb. Similarly, previous studies revealed that Pb concentration in the blood and epididymis homogenates of rats intoxicated with 1% PbAc for 6 or 9 months was higher than that identified in the control group [38, 44], even in the case of rats receiving PbAc with Trolox [38].

Morphology of the epididymides

The present study did not identify any abnormalities with respect to the morphology of the epithelial cells of the epididymides of rats treated with PbAc alone, PbAc with Trolox or Pb with vitamin E under light microscope. Similar results were obtained by Marchlewicz et al. in rats intoxicated with a 1% PbAc solution given with drinking water for a period of 9 months [38]. In 1 study, rats intraperitoneally intoxicated with Pb for 8 weeks showed a marked decrease in the number of spermatozoa in epididymis and a decrease in the percentage of motile sperm [67]. Comparable results were obtained following the administration of per os 10 mg AcPb/kg bm for 35 days [68]. Wiszniewska et al. demonstrated abnormalities in the structure of mitochondria and the Golgi system as well as a large number of lamellar lipid structures in the cytoplasm of epididymal epithelial cells, which can originate from mitochondria damaged by Pb or from phagocytized abnormal spermatozoa [40].

Chemiluminescence and lipid peroxidation of the epididymides

For the purpose of the assessment of the effect of Pb on lipid peroxidation in rat epididymides, the present study applied the CL

method based on the determination of an ultra-weak emission of light. A greater emission of light from a given tissue is associated with an increased generation of lipid peroxides, which indicates the presence of oxidative stress [38]. In our study, we demonstrated CL from epididymis homogenates in all studied groups as well as in the control group. Moreover, in all groups of rats, the tissues of the cauda epididymis had greater CL than in the caput epididymis. This may be connected with a higher number of spermatozoa generating ROS present in the cauda than in the caput epididymis [68]. During spermatozoa transport through the epididymis, their cell membrane becomes more fluid, which is crucial for their mobility and for performing the acrosome reaction. The characteristics of the cell membrane stems from an increased amount of polyunsaturated fatty acids present in the membrane. Consequently, the cell membrane of a spermatozoon becomes more susceptible to peroxidative damage. According to Chabory et al., spermatozoa demonstrate a high sensitivity to oxidative stress connected with lipid peroxidation. This process may negatively affect sperm motility and cause DNA damage [69]. Damaged and morphologically or functionally abnormal spermatozoa may additionally contribute to an increased CL in the cauda epididymis, which is confirmed by the results obtained in our study.

The present study also demonstrated markedly higher CL in the epididymis homogenates of rats intoxicated with PbAc as compared to the control. Significantly, the administration of PbAc with tocopherol caused the CL from the caput and cauda epididymis homogenates to return to control group levels. The administration of PbAc with Trolox showed no such effect in the caput but it did result in a significant decrease in CL in the cauda epididymis. Similar results were obtained in a study on rats receiving 1% PbAc for a period of 6 months where CL from testis tissue, caput and cauda epididymis was significantly higher than control – which indicated an increase in ROS generation in these tissues [38]. In contrast, CL from the caput and cauda epididymis of rats receiving PbAc + Trolox or PbAc + vitamin C were found to be markedly lower than in the group intoxicated with Pb alone [39]. Similarly, in another study, elevated CL was identified in the brains of rats, mothers of which were administered a single dose of lead citrate on the 18th day of pregnancy [26]. An experiment on mice intraperitoneally intoxicated with Pb showed an increased lipid peroxidation in testis tissue, a reduced number of spermatozoa and an increase in the percentage of abnormal forms of spermatozoa. In contrast, intraperitoneal simultaneous administration of vitamin E resulted in a decrease in lipid peroxidation, an increase in the number of spermatozoa and a decrease in the percentage of abnormal spermatozoa forms [70].

Tocopherol and Trolox supplementation and superoxide dismutase, catalase activity and expression

It is believed that antioxidants play a crucial role in the treatment of Pb poisoning [5, 25, 51, 52]. Therefore, in the present experiment, the rats were given Pb and tocopherol (in a dose equivalent to that applied to humans – 200 mg/day) or

Trolox at the same dose. In the course of this experiment, in the plasma of rats receiving Pb or Pb with Trolox, no significant changes in concentration of α -tocopherol were found. However, in the group of animals receiving tocopherol with Pb, there was a marked increase in α -tocopherol concentration as compared to the group receiving PbAc alone or PbAc + Trolox. Concentration of γ -tocopherol in plasma of rats intoxicated with Pb showed no significant changes in comparison to the results obtained for the control group. The plasma of rats receiving Trolox together with Pb showed no significant changes in terms of γ -tocopherol concentration as compared to the control group receiving only this element. Our study showed only an insignificant decrease in SOD activity in rat epididymis homogenates intoxicated with Pb, as compared to the control. Superoxide dismutase activity also did not show significant changes in the epididymis homogenates of rats receiving Pb with antioxidants, in comparison to the respective control group. Other authors also did not identify marked changes in SOD activity in erythrocytes of people exposed to Pb for a period of 20 years [71]. Casado et al. did not observe the effect of SOD added to erythrocytes of people exposed to Pb on decreasing hemolysis and CL intensity [24]. Another study on people occupationally exposed to Pb identified a decrease in SOD activity as well as an increase in lipid peroxidation in blood [72]. Reduced SOD activity in plasma has also been identified in rats receiving 15 mg PbAc/i.p. for 7 days [65] and in the testis of rats which were given 10 mg PbAc p.o. for 35 days [68]. Experiments on mice receiving 0.2% PbAc showed a decrease in SOD activity in the blood, a reduced haemoglobin level, an increased cell apoptosis and DNA damage in the liver. Reduced SOD activity in the liver was determined in rats intoxicated with PbAc at a dose of 200 mg/L for 3 weeks [73]. Reduced SOD activity was detected in the brains of rats whose mothers were given PbAc at a dose of 0.2% during lactation from day 1–23 [74], and in the brains of rats postnatally intoxicated with PbAc [5, 25]. Those authors that this may result from an excessive accumulation of hydrogen peroxide during oxidative stress. Other authors observed an increase in SOD activity in the kidneys of rats following a short-term exposure to PbAc at a dose of 25 mg/kg bm [61] and in the liver of mice receiving PbAc 3 times a week for a period of 3 weeks [29].

The results of our present study demonstrated a decrease in CAT activity in the epididymis of Pb treated animals, as compared to the control. Reduced CAT activity was also observed in the epididymis of rats receiving Pb with Trolox or tocopherol, in comparison to the respective control group. Similar results were obtained by other authors – reduced activity of this enzyme, as compared to the control, was identified in the kidneys, the liver and the brain of rats receiving 20 mg PbAc/kg bm/i.p. for 20 days and in the testis of rats given p.o. 10 mg PbAc for 35 days [68]. The offspring of mice who were given 0.2% PbAc in drinking water during lactation also showed reduced CAT activity in the brain. The authors speculate that oxidative stress may have caused a decrease in CAT activity and, consequently, accumulation of hydrogen peroxide which in turn led to intensified lipid peroxidation [75]. Reduced CAT

activity was found in the brains of rats intoxicated with 0.5% PbAc for 3 weeks [25]. Similarly, a decrease in CAT activity was found in the liver of mice and rats intoxicated with PbAc [29, 73]. People occupationally exposed to Pb also showed decreased CAT activity in erythrocytes [72, 76]. In turn, an increase in the activity of CAT was demonstrated by Conterato et al. in the kidneys of rats intoxicated with 5 mg PbAc/kg bm for 30 days [64]. In rats intoxicated with Pb during the prenatal period and lactation, there was a marked increase in the activity of CAT in the brain, as compared to the control group [27]. However, other authors did not identify significant changes in CAT activity following exposure to Pb [71].

CONCLUSIONS

Chronic exposure to Pb resulted in increased lipid peroxidation in the epididymis (manifested by a marked intensification in CL from the caput and cauda epididymis). Significantly, when Pb-treated rats were also supplemented with Trolox or tocopherol, the increase in chemoluminescence intensity was significantly lower, which indicated the beneficial effect of these antioxidants – a reduction in Pb-induced oxidative stress. However, a direct beneficial effect of supplementation on the activity and expression of SOD and CAT was not demonstrated. The activity and expression of SOD was not affected by exposure to Pb or to simultaneous administration of antioxidants. The use of tocopherol and Trolox had also no beneficial effect on the Pb-induced reduction in the activity and expression of CAT.

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