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Disturbances of energetic metabolism in rat epididymal epithelial cells as a consequence of chronic lead intoxication

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Abstract Adult male Wistar rats were intoxicated with 1% lead acetate (PbAc) administered in drinking water for nine months, which amounts to a period five times longer than the duration of one spermatogenesis. There were mitochondrial ultrastructure disorders of epididymal epithelial cells observed in PbAc-treated rats; also a significant lead-induced decrease in ATP concentration in epididymal epithelial cells (by 32%, $P < 0.05$), Adenylate Energy Charge value (AEC) (by 8%, $P < 0.05$) and an increase in ADP (28.5%, $P < 0.05$), AMP (27%, $P < 0.05$) and adenosine (by 56%, $P < 0.05$). The results were measured using high performance liquid chromatography (HPLC) and detected even at low lead concentrations in whole blood (M:7.03 μg/dL; Q1–Q3: 2.99–7.65). The function of mitochondria in cultured epididymal epithelial cells of control and PbAc-treated animals were evaluated using fluorophores: Mitotracker Green FM and JC-1. After incubation with Mitotracker Green FM, we observed active mitochondria producing bright green fluorescence in the cytoplasm of cultured epididymal epithelial cells, both in the control group and the Pb-treated animals. Incubation of cultured epididymal epithelial cells of animals from both groups produced red-orange fluorescence with the mitochondrial JC-1 probe indicating mitochondria with high membrane potential ($\Delta\Psi m > 80$–100 mV) and green fluorescence in the mitochondria with low membrane potential ($\Delta\Psi m < 80$ mV). The results showed that a chronic low-level exposure to lead, even without severe clinical symptoms of contamination, disrupted the ultrastructure and energy metabolism of mitochondria in epididymal epithelial cells.

Keywords Lead · Epididymis · Energy metabolism · Mitochondrial probe

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Introduction

Studies of the influence of compounds and toxic factors on the male reproductive system usually focus mainly on the gonad—the place of development and differentiation of spermatozoa. However, spermatozoa do not constitute fully mature reproductive cells when they leave the testis. During epididymal transport, which lasts for 10–14 days, they undergo a process of epididymal maturation in the caput of the epididymis which results in their acquiring the ability

to move and fertilize the egg (Bedford 1994; Jaiswal and Majumder 1998; Jones 1999a, b). During this process spermatozoa undergo morphological, biochemical and functional modifications facilitated by structural and enzymatic proteins, and glycoproteins synthesized by epididymal epithelial cells (Briz et al. 1995; Sivashanmugam and Rajalakshmi 1997; Yeung et al. 2000). Spermatozoa become mature and able to fertilize the ovum when they reach the central part of the corpus or the proximal part of the cauda epididymis. The epididymal cauda provides storage and viability to spermatozoa (Bedford 1994; Briz et al.1995). During their relatively long stay in the epididymis, spermatozoa may be affected by many harmful substances which includes environmental lead.

Despite its limited emission and various preventive activities, lead is an element that is still present in the environment. Being non-biodegradable, it is added to already existing deposits when it is newly emitted. Exposure to lead might occur via contaminated soil, water or industrial processes (CDC 2003, 2004). It is now thought that chronic exposure to a relatively low lead concentration, usually subclinical or without symptoms, is at present the prevailing form of environmental contamination. Its negative effects on the male reproductive system may become visible after many years, hence the problem is global and concerns the entire environment; any research that aims to explain the toxicity of lead seems to be well justified.

The negative impact of lead on reproductive processes is already known (Thoreux-Manlay et al. 1995a, b; Alexander et al. 1996; Foster et al. 1998; Gandley et al. 1999); however, the mechanism of its activity in the male reproductive system is not yet fully understood (Alexander et al. 1996; Bataineh et al. 1998; Robins et al. 1997). What is more, the studies of its influence on spermatogenesis, endocrinological functions and supplementary glands are often inconclusive (Apostoli et al. 1998). A decrease in the number of spermatozoa without any effect on their morphology or mobility was observed in workers with occupational exposure to lead, even if its level in blood was lower than what was considered acceptable—50 μg/dL. A correlation between those abnormalities and the length of exposure was clearly shown (Alexander et al. 1996). Some authors showed a tendency for lead to concentrate in accessory sex glands of the rat reproductive system and a decrease in the gland mass; however, there were no perceptible disruptions of epidydimis function or the number of spermatozoa present in the epididymal lumen (Thoreux-Manlay et al. 1995b). Batra et al. (2001) observed histological changes in the basal membrane, epithelium disruption and vacuolization of epididymal epithelial cells in male rats after a three month exposure to lead. However, after a chronic rabbits Pb exposure, a dose-dependent decrease of ejaculate volume, a decrease in spermatozoa number in ejaculate, a decrease in the quantity of motile spermatozoa, and an increase in the number of spermatozoa with malfunctioning morphology were observed. The examination of the gonads of these animals showed an inhibition of spermiation (Moorman et al. 1998).

The number of spermatozoa in the cauda of epididymis in lead-intoxicated Sprague–Dawley rats was found to decrease, similarly to the reduction in percentage of spermatozoa with normal motility. A higher percentage of spermatozoa with symptoms of premature acrosome reaction and a decreased ability to fertilize the oocyte have also been reported. Significantly increased reactive oxygen species (ROS) generation was also shown, which resulted in higher spermatozoa chemiluminescence (CL) (Hsu et al. 1997, 1998).

Some evidences suggest that Pb-exposure enhances intracellular ROS production and lipid peroxidation which may lead to tissue damage (Valverde et al. 2001; Kruk 1998; Ercal et al. 2001; Tandon et al. 2002). Our previous study also showed enhanced ROS concentration in homogenates of epididymis in lead-intoxicated rats (Marchlewicz et al. 2004, 2007). It has been shown that Pb-induced disruption of the balance between pro-oxidants and antioxidants in Pb-containing tissues contributes to tissue injury through oxidative damage to lipids, proteins and DNA (Kumar et al. 2002).

The results of our previous experimental studies unequivocally proved that lead caused more damage to the epididymis than to the testis (Marchlewicz et al. 1993; Piasecka et al. 1995, 1996; Wenda-Różewicka et al. 1996). Our previous studies showed that the male gonad is strongly protected by the blood-testis barrier. The only testis cells that contained electron dense deposits of lead (as confirmed by the analysis in transmission electron microscope with link analytical side-units for the X-ray microanalysis) were the

macrophages of the interstitial tissue (Wenda-Różewicka et al. 1996). Our study showed that the blood-epididymis barrier is not so selective. The lead concentration was higher in the epididymis than in the testis (Marchlewicz et al. 1993). We could track the route of lead from blood vessels via myocytes that surround the duct of the epididymis, to the epithelial cells and even further, into the lumen of the duct where the lead ions could have had direct contact with spermatozoa. Lead deposits in the cytoplasm of epididymal epithelial cells were usually covered with a membrane which may have limited their toxic effect on the cell (Piasecka et al. 1995).

Our electron microscopic studies showed the presence of electron-dense deposits in vacuoles in the cytoplasm of the epididymal epithelial cells in Pb-treated rats. The Golgi apparatus was enlarged and distended in these cells (Piasecka et al. 1995). Also many spermatozoa with ultrastructural changes of mitochondria were observed in the lumen of the epididymis of PbAc-treated rats (Piasecka et al. 1996).

Thus, it seemed interesting to examine whether Pb causes damage to mitochondrial ultrastructure of epididymal epithelial cells and whether it has an influence on the energy metabolism of these cells.

Materials and methods

The studies were carried out on sexually mature 3-month-old male Wistar rats. The animals were kept in a room with a controlled temperature under a LD 12/12 regime. The animals of the experimental group ($n = 8$) were allowed to drink ad libitum a 1% solution of lead acetate [$Pb(CH_3COO)_2 \times 3H_2O$] for nine months (duration of five spermatogeneses). The control group ($n = 8$) of rats were given distilled water. Our experiments were approved by the Local Ethics Committee. After the time of treatment animals were sacrificed under thiopental anesthesia (120 mg kg^{-1} b.w., i.p., Biochemie GmbH, Austria). Biochemical and molecular studies were performed in the isolated and in the cultured epididymal epithelial cells of both control and PbAc-intoxicated rats.

Ultrastructural studies

For electron microscopic studies, fragments of the caput (zones 1–3) and cauda epididymides (zones 6A and 6B) (Reid and Cleland (1957) of the control and PbAc-exposed rats were fixed in 0.25 mol/L glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4 for 2 h at 4°C, postfixed in 0.04 mol/L OsO_4. This method of fixation ensured that the tissues were not damaged, which was indicated by the correct morphology of control animals cells.

Then the tissues were dehydrated in increasing concentrations (20–95%) of ethyl alcohol and then acetone, embedded in a Spurr low-viscosity embedding kit (Polysciences, Inc. Warrington) (Piasecka et al. 1995; Wenda-Różewicka et al. 1996). Ultrathin sections were prepared with Reichert OmU2 ultramicrotome (Leica Aktiengesellschaft, Vienna, Austria), contrasted with uranyl acetate and lead citrate (Sigma), and analysed under JEM-1200 EX (JEOL Ltd., Tokyo, Japan) transmission electron microscope at 80 kV.

Isolation of epididymal epithelial cells and tissue cultures

Epididymides from control and PbAc-treated rats were dissected free of fat and connective tissue. Their fragments were subjected to multi-step enzymatic isolation (0.25% trypsin, 0.1% collagenase). This procedure yielded small segments of epididymal duct deprived of smooth muscle cells, fibroblasts and spermatozoa. Thereafter the isolated fragments of epididymal epithelium were cultured or frozen at –70°C until further analysis.

Cultured epididymal epithelial cells from the control or PbAc-treated rats were incubated in a modified Dulbecco's medium containing sodium pyruvate and glutamine (GIBCO BRL, UK), 5% fetal calf serum (FCS, GIBCO, USA), antibiotics—4,000 U Penicillin/100 mL with 4 mg Streptomycin/100 mL of medium (Sigma/Aldrich, Germany) and dihydrotestosterone (DHT—5α-androstan-17β-ol-3-one, Sigma/Aldrich, Germany) with a final concentration 0.1 nmol/L. All the cultures were incubated for 3 days in a humidified incubator with 5% CO_2 at 34°C (MiniGalaxy A, RS Biotech, UK).

Lead concentration in blood and tissues

Lead content was measured in the testis, caput and cauda epididymis, and in whole blood by Behari method (1981). Tissue samples of testis and epididymis were dried at 60°C and combusted at 450°C

(24 h). Thereafter, the combusted samples were dissolved in a hot solution of 1 M nitric acid (Sigma/Aldrich, Germany). All the treatments were done twice. The samples were transferred into 50 mL volumetric flasks and adjusted with 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 samples were analyzed using a Solaar 969 AAS, atomic absorption spectrophotometer (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 the supervision of the Blood Lead Laboratory Reference System (BLLRS) conducted by CDC—American Center for Disease Control in Atlanta. Quality control was also carried out with certified Nycomed lead standards. Control assays were carried out every 10 samples.

HPLC method separation of purines in epididymal epithelial cells

After isolation, the epididymal epithelial cells were washed with PBS buffer (Sigma/Aldrich, Germany) at +4°C and centrifuged (500G) for 10 min at +4°C. The centrifuged cells were suspended in 200 μL PBS and deproteinized with 200 μL 1.3 mol/L $HClO_4$. The samples were centrifuged (14,000G) for 10 min at +4°C. 200 μL of the supernatant was neutralized with 20 μL 3 M K_3PO_4 (micro select, Fluka, Poland) to achieve a pH range of 6.0–7.0. The samples were centrifuged again (in the same conditions) and the supernatant was frozen at −70°C until further analysis. The concentrations of purines (ATP-adenosine-5′-triphosphate, ADP-adenosine-5′-diphosphate, AMP-adenosine-5′-monophosphate, Ado-adenosine) were determined in the prepared samples using Smolenski et al. (1990) method.

For HPLC analysis, a Hewlett Packard chromatographic system (HP 1100, Austria) was used. The analytical column (100 × 4.6 mm) was packed with 3 mm Hypersil BDS-C_{18}. The following buffers were used: buffer A—KH_2PO_4, KCl, K_2HPO_4 (150 mM) adjusted to pH 6.0; buffer B—15% solution of acetonitrile in buffer A. Peaks were detected by absorption measurements at 254 nm wavelength.

AEC (Adenylate Energy Charge) was evaluated according to Atkinson (Atkinson and Walton 1967): $AEC = ([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP])$.

Fluorescence studies

Fluorescence studies were performed using two specific probes (Molecular Probes, Europe BV, Leiden, Holandia; Haugland 2002). Application of mitochondrial molecular probes facilitated the finding of active mitochondria (MitoTracker Green FM) and mitochondria with a normal inner mitochondrial membrane potential (JC-1) in the epididymal epithelial cells of the lead intoxicated rats. The initial solution, from which the incubation medium was prepared, was a solution containing 20 μg MitoTracker Green FM diluted in 1 mL DMSO (Dimethylsulfoxide, Sigma, Poland). The incubation medium, contained 0.2 μg fluorochrome in 1 mL Dulbecco's medium.

Mito Tracker Green FM stains active mitochondria with a bright green fluorescence, regardless of mitochondrial membrane potential ($\Delta\Psi m$) (wavelength 516 nm).

A lipophilic cationic fluorochrome JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyane iodide) exhibits potential-dependent accumulation in mitochondria and displays two colors of fluorescence: green and red-orange. A mitochondrial membrane potential ($\Delta\Psi m$) above 80–100 mV causes a red-orange fluorescence (maximum emission at 590 nm) due to the reversible formation of JC-1 aggregates in polarized mitochondria. A green fluorescence is characteristic for the monomeric form of the dye which accumulates only in mitochondria with low mitochondrial membrane potential ($\Delta\Psi m$ <80 mV) (induction at 490 nm, max emission at 510–527 nm). Incubation medium containing JC-1 at the final concentration of 10 mg/mL medium was prepared from the initial solution containing 1 mg JC-1/1 mL DMSO.

The epididymal epithelial cells were incubated with each of the mentioned fluorophores (after 3 days of culture, when the cells were attached to the bottom and possessed typical in vivo-like features) at 37°C, atmosphere 95% air, 5% CO_2 for 15 min. After the completion of incubation, the preparations were examined under a fluorescence microscope

(Axioskop, Carl Zeiss, Germany) using filter 09 (Filter set 09-487909-0000; Carl Zeiss, Germany).

Statistical analysis

Results are expressed as mean and standard deviation ($X \pm$ SD). 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 the significance of differences between PbAc-intoxicated and control groups. A 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 nine months of exposure the PbAc-treated animals had significantly higher lead concentrations in both studied parts of the epididymis: caput (6.16 ± 2.58 μg/g dry mass) versus control (0.47 ± 0.38 μg/g dry mass), cauda (5.9 ± 2.61 μg/g dry mass) versus control (0.74 ± 0.49 μg/g dry mass), testis (1.62 ± 0.85 μg/g dry mass) versus control (0.33 ± 0.29 μg/g dry mass), and whole blood (5.89 ± 2.63 μg/dL), versus control (0.3 ± 0.25 μg/dL) (Table 1).

Ultrastructural studies

The ultrastructural studies of epididymal epithelial cells of the PbAc-treated animals showed the presence of vacuoles in the cytoplasm filled with various amounts of electron-dense deposits, and distended and enlarged Golgi apparatus (Fig. 1a, b). The studies also showed disruptions of mitochondrial ultrastructure. A dilution of the mitochondrial matrix was observed as well as inner mitochondrial membrane ruptures and a lack of mitochondrial cristae (Fig. 2b). The presence of membranes with a concentric myelin-like arrangement was observed in the mitochondria of many epididymal epithelial cells of the Pb-treated animals (Fig. 2b). In the epididymal epithelial cells of control rats, we observed mitochondria with correct ultrastructure (Fig. 2a).

Adenine nucleotide and adenosine concentrations in isolated epididymal epithelial cells

A significant lead-induced decrease in ATP concentration (by 32%, $P < 0.05$) and Adenylate Energy Charge value (by 8%, $P < 0.05$) were observed together with an increase in ADP (by 28%, $P < 0.05$), AMP (27%, $P < 0.05$) and adenosine (by 56%, $P < 0.05$) concentrations in isolated epididymal epithelial cells of PbAc-intoxicated rats versus the control rats (Table 2).

Fluorescence studies

After incubation of epididymal epithelial cells with a Mitotracker Green FM probe after 3-days of culture, a bright green fluorescence was observed in the cytoplasm of most caput and cauda epididymal epithelial cells of the control rats (Fig. 3a). It indicated the presence of active mitochondria in the cytoplasm of these cells. A similar fluorescence was observed in the cytoplasm of the cultured cells of PbAc-intoxicated rats (Fig. 3b).

After incubation of caput and cauda epididymal epithelial cells of both control and Pb-treated rats with JC-1, the mitochondria of the cultured cells demonstrated an intense red-orange fluorescence coming from JC-1 aggregates. In some cells there were visible areas of the cytoplasm demonstrating only green fluorescence coming from JC-1 monomers (Fig. 4a, b).

Table 1 Lead content in blood, testis and epididymis of control and lead acetate treated rats

Group $n = 8$	Blood (μg/dL)		Caput epididymis (μg/g dry mass)	Cauda epididymis (μg/g dry mass)	Testis (μg/g dry mass)
Control (K)	X ± SD	0.3 ± 0.25	0.47 ± 0.38	0.74 ± 0.49	0.33 ± 0.29
Pb-treated	X ± SD	5.89 ± 2.63 vs.K**	6.16 ± 2.58 vs. K***	5.90 ± 2.61 vs. K***	1.62 ± 0.85 vs. K**

X ± SD. mean ± SD., *n*= 8 animals per group, *vs*. versus, *K* control, Asterisks indicate statistically significant differences in Mann–Whitney *U* test: ** $P < 0.01$, *** $P < 0.001$

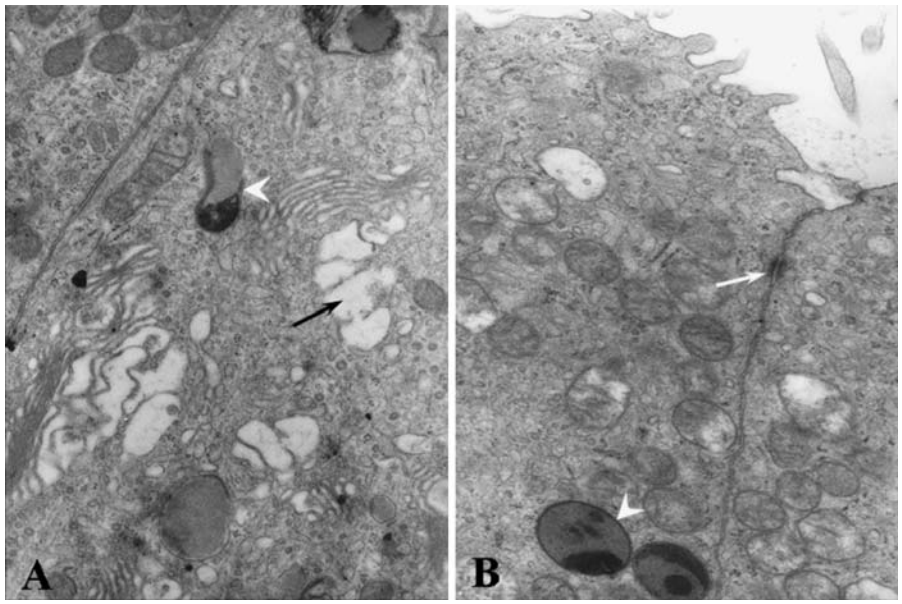

Fig. 1 The ultrastructure of caudal epididymal epithelial cells in rat treated with lead acetate for a period of five spermatogeneses. **a** Enlarged and distended cisternae in trans-Golgi region (*black arrow*). **a**, **b** *Grey vacuoles* with electron-dense deposits are present next to the cell membrane (*arrowheads*). **b** Normal intercellular junctions (*white arrows*) visible in the apical part of principal cells. **a** 15,000×, **b** 20,000×

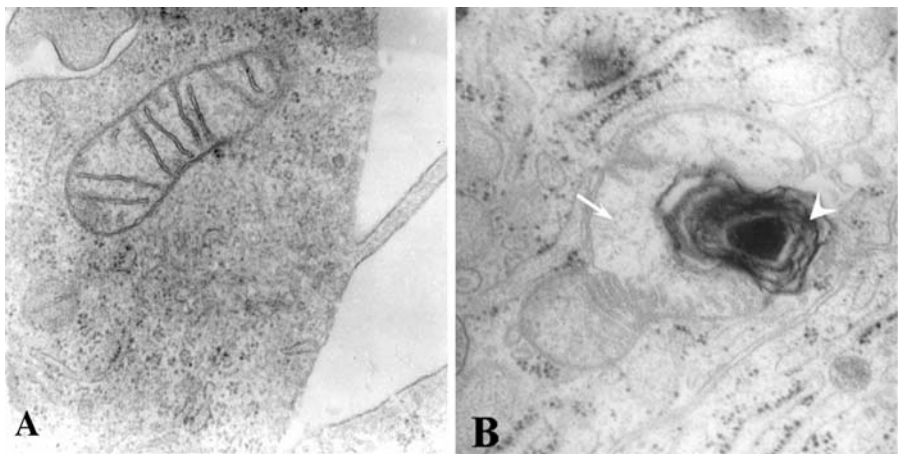

Fig. 2 **a** Mitochondrion with normal ultrastructure in cytoplasm of the principal cell of the epididymal epithelium in control rat. **b** Altered mitochondrium in cytoplasm of the epididymal epithelium cell in rat treated with lead acetate for a period of five spermatogeneses. Atrophy of the mitochondrial cristae (*white arrow*). The presence of lamellar, myelin-like structures with concentric membranes (*arrowhead*). **a** 40,000×, **b** 30,000×

Discussion

The protective function of the epididymis for the spermatozoa has never been adequately appreciated and has never been so intensively researched as nowadays. Interest in this problem stems undoubtedly from a growing number of cases of male infertility. The factors which can cause damage to spermatozoa in the epididymis are not always the same as those that can disrupt the endocrinal and spermatogenic functions of the testis. The most frequently studied are negative environmental factors, ROS

Table 2 Content of adenine nucleotides (ATP, ADP, AMP) and adenosine (Ado) in isolated epididymal epithelial cells of control and lead acetate treated rats

Group $n = 8$	ATP [nmol/10^6 cells ($X \pm$ SE)]	ADP [nmol/10^6 cells ($X \pm$ SE)]	AMP [nmol/10^6 cells ($X \pm$ SE)]	AEC [nmol/10^6 cells ($X \pm$ SE)]	Ado [nmol/10^6 cells ($X \pm$ SE)]
Control (K)	3.79 ± 0.21	0.91 ± 0.10	0.11 ± 0.003	0.88 ± 0.02	0.32 ± 0.01
Pb-treated	2.57 ± 0.23*	1.17 ± 0.004*	0.14 ± 0.006*	0.81 ± 0.01*	0.5 ± 0.01*

$X \pm$ SD, mean ± SD; $n = 8$ animals per group. Asterisks indicate statistically significant differences in Student's t test: * $P < 0.05$

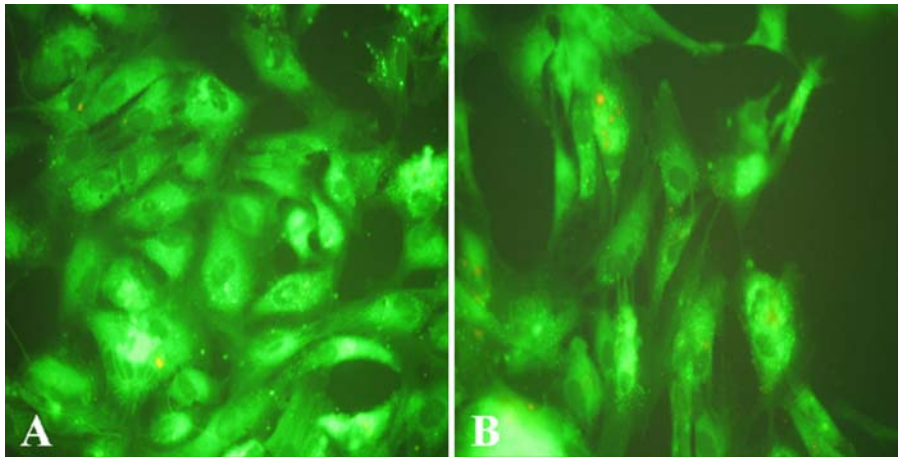

Fig. 3 An intense clear-yellow fluorescence in the cytoplasm after incubation with MitoTracker Green FM probe in cytoplasm of most cultured epithelial cells of the caput epididymis in the control rat (**a**) and in cytoplasm of some epididymal epithelial cells of rat treated with lead acetate for a period of five spermatogeneses (**b**). **a**, **b** 670×

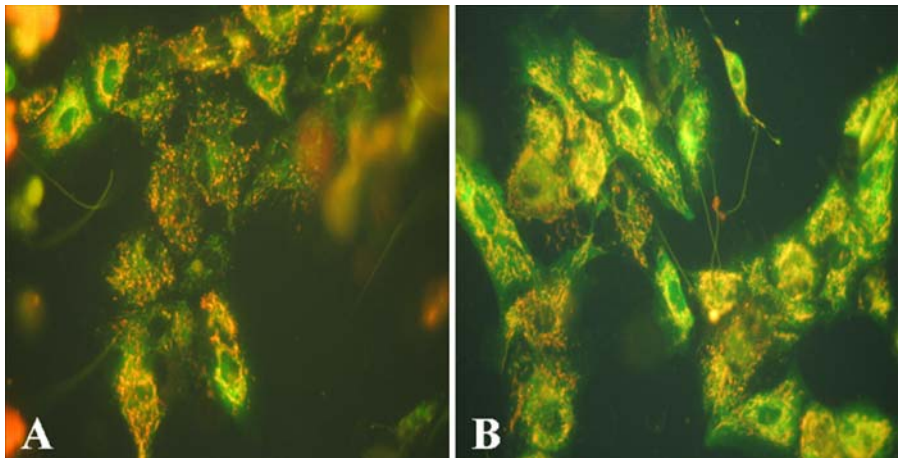

Fig. 4 An intense yellow-orange fluorescence after incubation with JC-1 probe within the cytoplasm of the cultured cauda epididymis epithelial cells in the control rat (**a**) and rat treated with lead acetate for a period of 5 spermatogeneses (**b**). Additional visible green fluorescence within the cytoplasm of some cells. **a**, **b** 670×

and autoimmunological reactions (Bohring and Krause 1999; Chen et al. 2003; Kumar et al. 2002; Latchoumycandane et al. 2002; McLachlan 2002; Ochsendorf 1999).

Our previous studies, which were carried out on chronically lead-intoxicated rats, showed that this element accumulates in the epididymis to a significantly higher degree than in the testis (Marchlewicz

et al. 1993). These results were similar to those achieved by other authors (Johansson and Wide 1986; Quintanilla-Vega et al. 2000).

In our current study of rats subjected to chronic low-dose lead intoxication for nine months (duration of five spermatogeneses), the lead concentration in whole blood was significantly higher (7.03 μg/dL) than in the blood of rats from the control group (0.76 μg/dL). However, it fell within the scope considered acceptable in the general population (i.e. people with no occupational exposure to lead) which is 10 μg/dL of blood (CDC 1991). The lead content in the caput and cauda epididymis homogenates of PbAc-intoxicated animals was significantly higher than in the gonad, suggesting Pb-accumulation in this part of the male reproductive system.

Our previous (Piasecka et al. 1995) and current transmission electron microscopic (TEM) research has allowed us to evaluate the changes in epididymal epithelial cells of PbAc-intoxicated animals. The Golgi apparatus was enlarged and distended; a significant amount of electron-dense deposits were observed, as well as vacuoles and conglomerates including osmophilic material with a frequent presence of numerous membranes. Spermatozoa present in the lumen of epididymal ducts of PbAc-treated animals often demonstrated abnormal structure of axoneme, thick fibers and mitochondrial sheath. Some mitochondria lacked both membranes or had only the outer mitochondrial membrane (Piasecka et al. 1996, 1995). In our current TEM research, special attention is being paid to mitochondrial ultrastructures in the epididymal epithelial cells of lead-intoxicated rats. We observed the presence of mitochondria with an electron-lucent matrix, with damaged crests or mitochondria without crests at all. In some mitochondria the presence of osmophilic lamellar structures of myelin-like arrangement were observed. Similar lead-induced changes were observed in the structure of mitochondria in various types of cells by other authors (Batra et al. 1998; Bizarro et al. 2003; Buchheim et al. 1998; Szynaka et al. 1999). Szynaka et al. (1999) observed damage to the outer or inner mitochondrial membrane in the cells of the exocrine part of the pancreas of PbAc-treated Wistar rats.

Some authors observed lead-accumulation in the mitochondria of various organs cells like kidney, spleen, prostate, testis, epidydimis (Batra et al. 1998). Other authors observed lead-induced oedema of rabdomyocytes mitochondria (Buchheim et al. 1998) and Schwann cells (Tang et al. 1996). In a study of sexually mature PbAc-treated mice, Bizarro et al. (2003) observed the oedema of mitochondria in Sertoli cells, a lack of mitochondrial crests and the presence of electron-dense spherical inclusions in these organelles. The authors suggested that lead caused the increased ROS production leading to oxidative stress in the cells, which caused an increase in the membranes permeability, changed the Ca^{+2} concentration, caused mitochondrial oedema and rupture of the inner mitochondrial membrane as well as inhibition of ATP synthesis. Our previous studies PbAc-treated rats (Marchlewicz et al. 2004, 2007) also showed an increased concentration of ROS in the testis and epididymis homogenates, which could result from the above mentioned abnormalities in mitochondrial ultrastructures, and could to some degree disrupt the functioning of these organelles.

Incubation of the epididymal epithelial cells with a mitochondrial probe, once they had the features they used to possess in vivo, after three days of culture, allowed the evaluation of the influence of lead on the functional status of the mitochondria. The study with a MitoTracker Green FM probe showed that in most cultured epididymal epithelial cells of the rats that were exposed to PbAc for nine months, there was a bright green fluorescence visible in the cytoplasm indicating the presence of intact mitochondria (Haugland 2002), while the study with the JC-1 mitochondrial probe showed an intensive red-orange fluorescence which was visible in most cells of both studied groups. Some areas in the cytoplasm or the whole cytoplasm of some cells showed some green fluorescence caused by JC-1 monomers, which suggests a lack of mitochondria with an adequately high inner mitochondrial membrane potential. However, as similar images were observed under a fluorescent microscope in cells from the epididymis of both control and PbAc-treated rats, it was difficult to rule out the presence of some differences in the number of mitochondria with a low inner membrane potential and this will be the subject of our further studies.

Other authors (Chavez et al. 1987, Chen et al. 2003) showed a lead-induced decrease of inner mitochondrial membrane potential. There have also been reports of a decrease in inner mitochondrial membrane potential by oxidative stress caused by

various factors. Armstrong et al. (1999) indicated a decrease in mitochondrial membrane potential value, a decrease in ATP concentration and inhibition of spermatozoa motility in spermatozoa exposed to ROS.

A good indicator of the energetic status of mitochondria in epididymal epithelial cells could also be the purine nucleotides concentration ATP, ADP, AMP and AEC value. Many occurrences and reactions in a cell require an energy supply. The value that AEC can take in a cell is between 0 and 1.0 (Atkinson and Walton 1967). The minimal value refers to a state when all the nucleotides are AMP, and the maximum value refers to a state when adenalyte nucleotides are in the form of ATP. In the homeostasis conditions, the AEC value is precisely controlled and is between 0.75 and 0.95. The value 1.0 suggests a high metabolic energy potential, whereas a value between 0.55 and 0.75 indicates the negative influence of a stress factor, causing energy expenditure for protective mechanisms. A value below 0.5 leads to the death of a cell (Kamp et al. 2003, 1996; Yun et al. 2000).

In the current study, we observed a significantly lower AEC value in the epididymal epithelial cells of PbAc-treated animals as compared to the control group; however, it fell within the scope of the values acceptable for homeostasis. It may be the reason why the fluorescences that was observed after the incubation of epididymal epithelial cells with Mitotracker Green FM and JC-1 were similar in both the control group and Pb-treated. However, we observed a significantly lower ATP concentration in epididymal epithelial cells of Pb-intoxicated animals. It could be the result of mitochondria ultrastructural damage observed during our current study, and it could indicate that Pb, even in a low blood concentration (M: 7.03 μg/dL) can negatively influence the energetic metabolism of epididymal epithelial cells mitochondria.

Other authors showed similar results in their studies on the influence of Pb^{+2} ions on the energetic processes in various cells (Baranowska-Bosiacka et al. 2004, Baranowska-Bosiacka and Hlynczak 2003; Grabowska and Guminska 1996; Yun et al. 2000). Yun et al. (2000) observed a significantly lower ATP concentration in cerebral cortex cells of rats exposed to chronic PbAc-intoxication. Baranowska-Bosiacka and Hlynczak (2003) showed a decrease in ATP concentration and AEC value in human erythrocytes incubated in vitro with lead ions. Erythrocytes with a decreased purine nucleotide value demonstrated changes in shape. A decrease in ATP concentration can disrupt the processes of phosphorylation of cytoskeleton proteins which usually condition the correct conformational isomerism of the spectrin-actin network, determining the physiological shape of blood cells (Belloni–Olivi et al. 1996).

Epididymal epithelial cells are metabolically active and therefore require appropriate ATP amounts for various processes, e.g., active transport of substances and ions which ensure optimal composition of the fluid for the spermatozoa in each region of the epididymis (Hinton and Palladino 1995; Hinton et al. 1995). Also lead excretion from the cell is a process that requires energy supplies (Dekaney et al. 1997). It was observed that spermatozoa can stimulate the secretory function of the epididymal epithelial cells through the ATP that spermatozoa release, affecting the composition of the fluid present in the lumen of the epididymal duct (Leung and Wong 1994). A decrease in ATP concentration under the influence of lead in epididymal epithelial cells and in spermatozoa might thus have manifold consequences.

Our current research also demonstrated a significantly higher adenosine concentration in epididymal epithelial cells of rats exposed to chronic Pb-intoxication. According to some literature data, in cells subjected to oxidative stress, an increased adenosine production was observed which can decrease oxidative stress through the activation of anti-oxidative enzymes in a cell (Maggirwar et al. 1994).

Conclusion

The observed decrease in ATP concentration in epididymal epithelial cells of lead-intoxicated rats indicates impaired energetic metabolism in these cells. This finding may suggest that chronic Pb-intoxication of mature male rats may result in metabolic changes in the epididymis and may be one of the reasons responsible for a reduced number and quality of spermatozoa present in the lumen of the epididymis in the intoxicated rats.

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