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The accumulation and efflux of lead partly depend on ATP-dependent efflux pump–multidrug resistance protein 1 and glutathione in testis Sertoli cells

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HIGHLIGHTS

- Mrp1 lower-expression TM4-sh cells were used.
- In the presence of lead acetate, the amount of cumulative lead in TM4-sh was much higher than that in TM4.
- Switch to lead-free medium, the lead content in TM4-sh remain higher than that in TM4 cells at 1, 3, 6 and 9 h time points.
- Expression levels of mRNA and protein of *mrp1* gene in TM4 were significantly induced by lead.
- Lead excretion requires energy and partly be dependent on Mrp1 and GSH.

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ABSTRACT

Since lead accumulation is toxic to cells, its excretion is crucial for organisms to survive the toxicity. In this study, mouse testis sertoli (TM4) and Mrp1 lower-expression TM4-sh cells were used to explore the lead accumulation characteristics, and the role of ATP-dependent efflux pump–multidrug resistance protein 1 (Mrp1) in lead excretion. TM4 cells possess Mrp-like transport activity. The expression levels of *mrp1* mRNA and Mrp1 increased after lead treatments at first and then decreased. The maximum difference of relative mRNA expression reached 10 times. In the presence of lead acetate, the amount of cumulative lead in TM4-sh was much higher than that in TM4. After the treatment with lead acetate at 10–40 μM for 12 h or 24 h, the differences were about 2–8 times. After with the switch to lead-free medium, the cellular lead content in TM4-sh remains higher than that in TM4 cells at 1,3, 6, and 9 h time points ($P < 0.01$). Energy inhibitor sodium azide, Mrp inhibitors MK571 and glutathione (GSH) biosynthesis inhibitor BSO could block lead efflux from TM4 cells significantly. These results indicate that lead excretion may be mediated by Mrp1 and GSH in TM4 cells. Mrp1 could be one of the important intervention points for lead detoxification.

Abbreviations: Pb, lead; TM4, mouse testis Sertoli cells; Mrp1, multidrug resistance protein 1; TM4-sh, Mrp1lower-expression TM4 cells; ABC, ATP-binding cassette; GSH, glutathione; GST, glutathione-*S*-transferase; EA, ethacrvnic acid; BSO, Lbuthionine–sulfoximine; RT-qPCR, real-time fluorescence quantitative polymerase chain reaction; MK571, (*E*)-3-[[[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl][[3-(dimethylamino)-3-oxopropyl]thio]methyl]thio]-propanoic acid; PBS, phosphate-buffered saline; CCK8, Cell counting Kit-8.

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1. Introduction

Lead (Pb) is a toxic heavy metal and people can be exposed to it due to its presence in our living environment. Lead is mostly accumulated in bone and the kidney, next in the testis. The deposition of lead in the testis explains the sensitivity of the reproductive system to lead toxicity (Batra et al., 1998). So, the consequence of lead accumulation in male reproductive system is far reaching and is of great interest. Studies (Alexander et al., 1996; Sokol and Berman, 1991; Wang et al., 2006) have shown that lead was correlated with reduced human semen quality. When the animals were exposed to lead acetate, serum testosterone level, and sperm concentration and production rate were significantly suppressed. The seminiferous tubules became thin and disappearance of most spermatids and spermatozoa were also observed as compared with control. Additionally, shrunken cells with pyknotic nuclei appeared in parts of Sertoli and Leydig cells, demonstrating cellular destruction.

As a ubiquitous environmental pollutant, lead can gain entry into cells (Bridges and Zalups, 2005; Chiu et al., 2009) through simple diffusion, membrane carriers or ion channels, and in turn, can accumulate in tissues and cells. The mechanisms involved in the detoxification of heavy metals could be: reducing uptake, increasing efflux from cells and compartmentalization in specific organelles (Oh et al., 2009; Rosen, 2002). After organisms exposured to heavy metals, metal binding proteins such as metallothionein (MT), GSH and so on are often induced to chelate intracellular free metal and to confine heavy metals within internal organelles. At the same time, protein transporters promoting the discharge of heavy metals also are induced to accelerate the excretion of intracellular heavy metals. Both efflux and sequestration are the most efficient detoxification ways.

Multidrug-resistance associated proteins (Mrps) belong to the subfamily C of ATP binding cassette (ABC) superfamily. They are best known for their contributions to chemoresistance through the efflux of anticancer drugs from cancer cells (Fletcher et al., 2010). In fact, Mrps can transport not only natural product drugs but also organic anions (Wortelboer et al., 2008; Zhou et al., 2008). Up to now, nine MRP transporters have been identified with typical tissue/cell distrition and substrate specificity (Zhou et al., 2008). Glutathione S-conjugate metabolites and xenobiotics could be exported from the cells by Mrp1 (Cole and Deeley, 2006). Recently, the possible relationship between Mrp1 and the resistance to toxicants including heavy metals, such as arsenate, cadmium and mercury, have been studied with great enthusiasm (Aleo et al., 2005; Leslie et al., 2004; Long et al., 2011a; Oh et al., 2009). But the effect of Mrp1 on lead efflux from cells is not clear. Sertoli cells, the somatic constituents that extend from the base to the apex of the seminiferous epithelium, are considered the "supporting cells" of the testis and interact directly with developing germ cells throughout spermatogenesis (Mruk and Cheng, 2004). Sertoli cells are obliged to synthesize, secrete, and efficiently deliver products that are essential for the growth and differentiation of developing germ cells, which create an impermeable and immunological barrier (Buganim et al., 2012). The mouse Sertoli cell line,TM4 was originated from the immature testis of normal 11- to 13-day-old BALB/c mice and characterized originally as Sertoli cells based on their morphology, hormone responsiveness, and metabolism of steroids (Gomez et al., 2012). Since its generation, this cell line has been used in numerous studies with results similar to those obtained using primary cultures of Sertoli cells (Gomez et al., 2012; Zhang et al., 2012). It was reported that Mrp1/MRP1 is present at high concentrations in testes and is localized to the Leydig and Sertoli cells in humans and mice (Bart et al., 2004; Setchell, 2009). Moreover, Mrp1 is also highly expressed in TM4 cells (Robillard et al., 2012). So, in this study, TM4 cells were used as subjects to explore the lead accumulation characteristics, and to address whether Mrp1 plays a critical role in the lead detoxification.

2. Materials and methods

2.1. Cells and chemicals

TM4 (CRL-1715) mouse Sertoli cells were purchased from ATCC. Cell culture reagents, enzymes for real-time fluorescence quantitative polymerase chain reaction (RT-qPCR), and Trizol were purchased from Life Technologies Corporation (Invitrogen, Grand Island, NY, USA). Dimethylsulfoxide (DMSO), puromycin, Calcein AM, SNARF-1 AM, MK571 and L-Buthionine-sulfoximine (BSO) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethacrvnic Acid (EA) was obtained from Enzo life sciences (Enzo,USA). The mouse monoclonal primary anti-Mrp1 and β-actin antibodies for Western blotting were bought from Abcam (Cambridge Science Park, UK). Cell counting Kit-8 (CCK8) was obtained from Beyotime Biotechnology (Haimen, China). Assay kits for reduced glutathione (GSH), glutathione-*S*-transferase (GST), adenosine triphosphate (ATP) and total protein were obtained from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). Lead acetate and other chemicals made in China were of the highest purity or analytical grade. Primers were all synthesized by Shanghai Sangon Biological Engineering Co., Ltd. (Shanghai, China).

2.2. Cell culture

TM4 cells were grown in a 1:1 mixture of Ham's F-12 medium and Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% horse serum, 2.5% fetal calf serum and 1% penicillin/streptomycin at 37 °C in an atmosphere containing 5% CO_2.

The expression of *mrp1* gene in TM4 cells were knocked down by a lentivirus vector mediated RNA interference (RNAi) technology. The efficiency and specificity was validated by quantitative real-time PCR and Western blotting. The resulted cell line with lowered-expression of Mrp1 was called TM4-sh. TM4-sh cells were grown in the same medium containing 1.5 μM puromycin.

2.3. Cell viability assay

TM4 cells were seeded at 1×10^5 cells/well in 96-well plates. Following incubation for overnight, the medium was removed and replaced with 100 μl fresh medium containing different concentrations of lead acetate (0, 10, 20, 40, 80, 100 μM) for 24 h. On completion of incubation, cell viability was determined using CCK8 according to the manufacturer's instructions. Ten microliters of CCK-8 reagent was added to each well and incubation was continued for 4 h. The absorbance (OD) of each well was read at 450 nm using microplate reader (BioTek, instruments, Inc., Winooski, VT, USA). Percentage survival rate was calculated using the following equation: survival rate (%) = $(OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100\%$. Each experiment was done in triplicates.

To evaluate whether MRPs, GSH, glutathione-*S*-transferase (GST), adenosine triphosphate (ATP) play any role in the transportation of lead, MRP specific inhibitors MK571, GSH biosynthesis inhibitor BSO, GST inhibitor EA and ATP inhibitor sodium azide (NaN_3) were used to inhibit MRP activity, GSH biosynthesis, GST activity and ATP biosynthesis, respectively. To select the doses, cells were exposed to these inhibitors for 24 h and their viabilities were also determined by CCK-8 assays, respectively.

2.4. Analysis of Mrp-like transport activity

Mrp1-mediated transport was studied using both calcein and 5-carboxyseminaptharhodafluor (SNARF-1) efflux assay as described by the studies (Weekes et al., 2013; Wortelboer et al., 2008). The cells were seeded at 5×10^5 cells/well in 24-well plates and incubated for 24 h. Then, the medium was replaced with 500 μl fresh medium containing fluorescent Mrp substrates (calcein AM or SNARF-1 AM) with either Mrp1 substrate inhibitor MK571 (0, 5, 10, 25, 50, 100 μM) or lead acetate (0, 5, 10, 20, 40, 80 μM), and cells were incubated at 37 °C for 30 min, respectively. After that, cells were kept on ice and washed three times with ice-cold PBS and finally lysated in PBS containing 0.1% TritonX-100. Fluorescence in lysates was determined using BioTek's high-performance multi-mode plate reader (Synergy 2) at 485 nm excitation and 530 nm (for calcein) or 615 nm (for SNARF) emission wave lengths. The data were presented as fluorescence units per 5×10^5 cells.

2.5. Detection of GSH, GST and ATP

The measurements of GSH, GST and ATP were undertaken according to the manufacturer's instructions. The enzyme activity of GST was expressed as units per milligram protein. The GSH and ATP levels were expressed as micromoles per gram protein. The protein content of the lysates was measured by the enhanced BCA protein assay kit according to the manufacturer's instructions.