Letter to the Editor

Cytotoxic Responses and Apoptosis in Rat Kidney Epithelial Cells Exposed to Lead*

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The toxic effects of lead on normal rat kidney epithelial cells (NRK cells) may occur via various pathways. However, the role of intrinsic mitochondrial pathway in Lead-induced apoptosis in NRK cells has not been investigated. The purpose of our study was to investigate cytotoxic responses and cell apoptosis mediated by lead in NRK cells. NRK cells were treated with different concentrations of Lead acetate for 12 h to determine the cytotoxicity of lead. Mitochondrial transmembrane potential was also analyzed using a fluorescence spectrophotometer. Moreover, the activities of caspase-3 and caspase-9 were detected in the presence of lead. Finally, the lead-induced cell apoptosis was evaluated by flow cytometry in the present of caspase inhibitors Z-VAD-FMK and Ac-LEHD-FMK, respectively. The results would contribute to clarify the role of Lead in proliferation and apoptosis of NRK cells, and help to understand the underlying mechanism responsible for lead-induced cell apoptosis.

Lead is one of the most important heavy metal pollutant factors. It exists ubiquitously in the environment and has caused global contaminations of air, water and soil. Lead may exert toxic effects on several organ systems, but those in the kidney are the most insidious[1]. Moreover, most of the acute and chronic toxic effects of Lead occur primarily in the renal proximal tubules of the kidney. After glomerular filtration, Lead is reabsorbed by renal tubules, which results in Lead accumulation in renal tubular epithelial cells and toxic effects on the kidney[2-3]. Depending on the amount of exposure, Lead can induce cell apoptosis or necrosis in a number of cell types *in vitro* systems, such as rat fibroblasts, rat pheochromocytoma cells (PC12), and rat retinal rod cells[4]. The normal rat kidney (NRK) epithelial cell line has been developed as a useful tool to study the mechanism of renal toxicity of environmental chemicals. However, whether Lead could induce apoptosis in NRK cells remains unknown.

Given that the kidney is the critical organ after long-term occupational or environmental exposure to Lead, the research on how Lead induces cytotoxicity and apoptosis in renal cells has become a topic of interest worldwide. Previous study has reported that Lead acetate could induce apoptosis of human kidney 2 (HK2) cells by stimulating DNA damage[5]. Meanwhile, Lead acetate increased lipid peroxidation and reactive oxygen species, and induced oxidative stress which is known as a mediator of apoptosis in cells or animals[2]. In Lead-induced apoptosis, it is thought that the mitochondria are most pertinent in mediating apoptosis. Lead depolarizes rod cell mitochondria, resulting in cytochrome C release, caspase activation and apoptosis[4]. Caspases are the most important proteases in the apoptotic process, and the activation of caspases is a key point in the implementation of cell apoptosis[6], For example, caspase-8 and caspase-9 play an important role in the NRK cell apoptosis induced by cadmium[7]. However, the role of intrinsic mitochondrial pathway in Lead-induced apoptosis in NRK cells has not been investigated.

The present study aims to investigate the effects of Lead on cell proliferation, mitochondrial transmembrane potential, apoptosis, activities of caspase-3 and caspase-9 in NRK cells. We aimed to reveal the possible signaling pathway responsible for the cytotoxicity of Lead in NRK cells and to provide the basic understanding for illustrating the mechanism of Lead-induced apoptosis in NRK cells.

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Normal rat kidney epithelial cell line (NRK) (CRL-6509™, ATCC, VA, USA) was cultured and maintained with Dulbecco's modified eagle medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA), 2% L-Glutamine, 1% penicillin and streptomycin at 37 °C in a humidified incubator with 5% CO_2. NRK cells were seeded in 96-well plates at a density of 1×10^5 cells per well. After 24 h, the cells were treated with Lead acetate (Sigma, USA) ranging from 0.5 to 2 μmol/L for 12 h, with the cells incubated in fresh medium without lead acetate as the control. Cell viability was conducted using the MTT assay by measuring the absorbance at wavelength of 560 nm using a microplate reader (Sunrise, Austria).

NRK cells were treated with various concentrations of Lead acetate for 12 h, they were incubated with 10 μg/mL of rhodamine123 (Rh123) (Nanjing Keygen Biotech, China) for 30 min in the dark at 37 °C and then were washed with ice-cold phosphate buffered saline (PBS), twice and centrifuged at 1500 rpm for 10 min. Finally the mitochondrial membrane potential (ΔΨm) was determined by a spectrofluorimeter (F-4500, Hitachi, Japan) at an excitation wavelength of 505 nm and emission wavelength of 534 nm.

The cells were plated in 6 well culture plates at approximately 1×10^5 cells/mL. After 12 h treatment, NRK cells were harvested and washed three times with PBS. Apoptosis was determined by Hoechst 33342 staining (Nanjing Keygen Biotech, China). In other experiment, NRK cells were pretreat with 20 μmol/L caspase inhibitor Z-VAD-FMK and 10 μmol/L caspase-9 inhibitor Ac-LEHD-FMK (Beyotime Institute of Biotechnology, China), incubated for 1 h, and then exposed to 0.5 μmol/L Lead acetate for 12 h. The apoptosis rate was detected following the Annexin-V-FITC/PI kit instruction by flow cytometry (BD FACSCalibur, USA).

2×10^4 cells per mL were seeded in the 6-well plates and cultured as described above. The medium was replaced with serum-free medium containing with different concentrations of lead acetate. After 12 h treatment, cells were collected to detect the activities of caspase-3 and -9 by using caspase assay kits (Abcam, Hong Kong) according to the manufacturer's instructions.

Cell treatments were performed in triplicate and repeated at least three times in separate experiments. All data were presented as mean±SD, and significant differences (P<0.05) and extremely significant differences (P<0.01) were determined among various groups by ANOVA and Tukey post-test using SPSS 12.0 statistical software.

Viability of NRK cells was progressively decreased by an increasing concentration of Lead acetate, and each lead acetate-treated group was significantly different from the control group (P<0.05 or P<0.01, Figure 1A). The MTT assay indicated that Lead acetate could inhibit NRK cell proliferation in a dose-dependent manner, which was related to the occurrence of apoptosis. In mammals, the change of mitochondrial membrane permeability is the first step in the mitochondrial apoptotic pathway, which

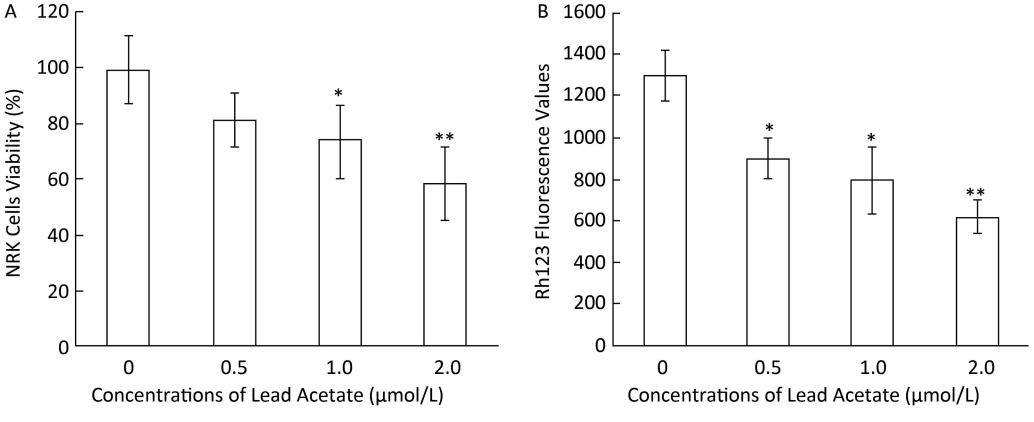

Figure 1. The toxic effects of lead acetate at different concentrations on NRK cells. (A) Lead acetate suppressed the relative NRK cells viabilities. The bars denote NRK cells viabilities. Vertical bars represent SD in four independent experiments. (B) Lead acetate decreased mitochondrial transmembrane potential (ΔΨm) of NRK cells. The bars denote ΔΨm of NRK cells. Vertical bars represent SD in three independent experiments. $^*P$<0.05 compared with controls, $^{**}P$<0.01 compared with controls. SD, standard deviation.

causes the loss of ΔΨm. The change of ΔΨm is considered as an important index in the occurrence of apoptosis[8]. Rh123 is a fluorescent indicator of mitochondrial membrane potential, and accumulates in mitochondrial in viable cells. When cells are damaged by chemical insult, the ΔΨm decreases, and therefore the accumulation of Rh123 in the mitochondria is reduced. As shown in Figure 1B, after 12 h exposure, the ΔΨm of Lead acetate treated-cells decreased significantly (P<0.05 or P<0.01) in a dose-dependent manner compared to the control group. These results demonstrated that lead acetate caused the loss of ΔΨm in NRK cells, which could further lead to the damage of mitochondria and finally induces apoptosis.

Then, we used a Hoechst 33342 staining to determine whether Lead acetate-treated NRK cells undergo apoptosis. As shown in Figure 2A, the nuclei in apoptotic cells showed more intense blue fluorescence compared to the nuclei in normal cells (as denoted by the arrow). Viable cells number was significantly reduced with increasing concentrations of Lead acetate, and a larger number of apoptotic and necrotic cells appeared in the NRK cell treatment groups.

Apoptosis plays an essential role in maintaining homeostasis by removing redundant and abnormal cells. Apoptosis is mainly mediated by three major pathways including mitochondrial apoptotic pathway, the death receptor pathway, and endoplasmic reticulum pathway[9]. The mitochondrial pathway has caspase-dependent and caspase-independent mechanisms. Cysteinyl aspartate-specific proteinase (caspase) play an important role in regulating apoptosis[10]. In the caspase-dependent mitochondrial pathway, changes of mitochondrial membrane potential and permeability are indispensable in the progress of apoptosis. In a variety of adverse factors, the mitochondrial permeability transition pore (MPTP) located in mitochondrial membrane is irreversibly opened, which induced the collapse of ΔΨm, the uncoupling of oxidative phosphorylation, and the release of cytochrome C from mitochondria into the cytoplasm. Subsequently, cytochrome C activated caspase-9, caspase-3, caspase-7 etc, initiating the caspase cascade, thereby inducing apoptosis[9]. It has been reported that caspase-3 is involved in the apoptosis of human renal mesangial cells induced by Lead acetate[11].

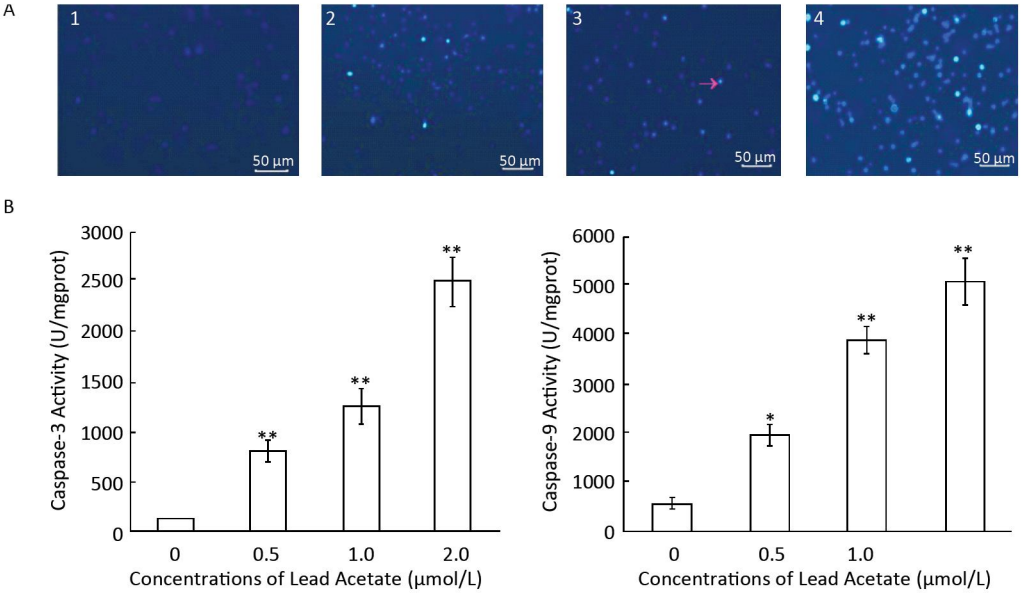

Figure 2. Lead acetate induced apoptosis and increased caspase-3 and caspase-9 activity in NRK cells. (A) Apoptotic cells with more intense blue fluorescence (as denoted by the arrow) in the nuclei by Hoechest 33342 staining increased with increasing concentrations of Lead acetate (1, 2, 3, 4 indicates 0, 0.5, 1.0, and 2.0 μmol/L of Lead acetate respectively). (B) Caspase-3 and caspase-9 activities were increased in NRK cells treated with various concentrations of Lead acetate. The bars denote the relative enzyme activity unit. Vertical bars represent SD in three independent experiments. $^{*}P$<0.05 compared with controls, $^{**}P$<0.01 compared with controls. SD, standard deviation.

In our study, the activities of caspase-3 and -9 in Lead acetate-treated groups increased significantly, compared with the control group, which suggests that Lead acetate could activate the caspase-3 pathways (Figure 2B). Moreover, the activate caspase-9 pathways were statistically increased in NRK cells treated with Lead acetate in a dose-dependent manner, compared with the control group (Figure 2B). These results suggested that Lead acetate could activate caspase-3 and caspase-9 in a dose-dependent manner.

To further verify Lead acetate causes apoptosis of NRK cells via caspase-3 or caspase-9 signaling pathways, after pretreatment with a non-selective caspase inhibitor Z-VAD-FMK and a caspase-9-specific inhibitor Ac-LEHD-FMK, cells were stained with Annexin V-FITC/PI and flow cytometry was performed to show that both inhibitors effectively reduced apoptosis rates and significantly increased the number of living NRK cells treated with a low dose (0.5 μmol/L) of Lead acetate. The apoptosis rates reduced from 18.6% to 8.7% and 12.0% with the presence of inhibitor Z-VAD-FMK and Ac-LEHD-FMK (Figure 3). These results further indicate that Lead acetate-induced apoptosis was closely associated with the activation of caspases. However, caspase inhibitors cannot completely block the Lead acetate-induced apoptosis. These results suggest that there are caspase-independent pathways in the Lead acetate-induced apoptotic process. Recently, researchers found that there is another kind of cell death called 'proptosis' which is induced by caspase-1, which can also weaken cell function[12]. Whether caspase-1 is involved in Lead acetate-induced apoptotic process remains further exploration. Further our studies will focus on gene or protein expression changes of NRK cells induced by Lead from the aspects of molecular biology.

In summary, our findings imply that Lead acetate could inhibit the proliferation of NRK cells and destroy the cell membrane integrity, which were achieved by inducing apoptosis in renal tubular epithelial cells (NRK) via caspase-3 and -9 dependent mitochondrial pathways.

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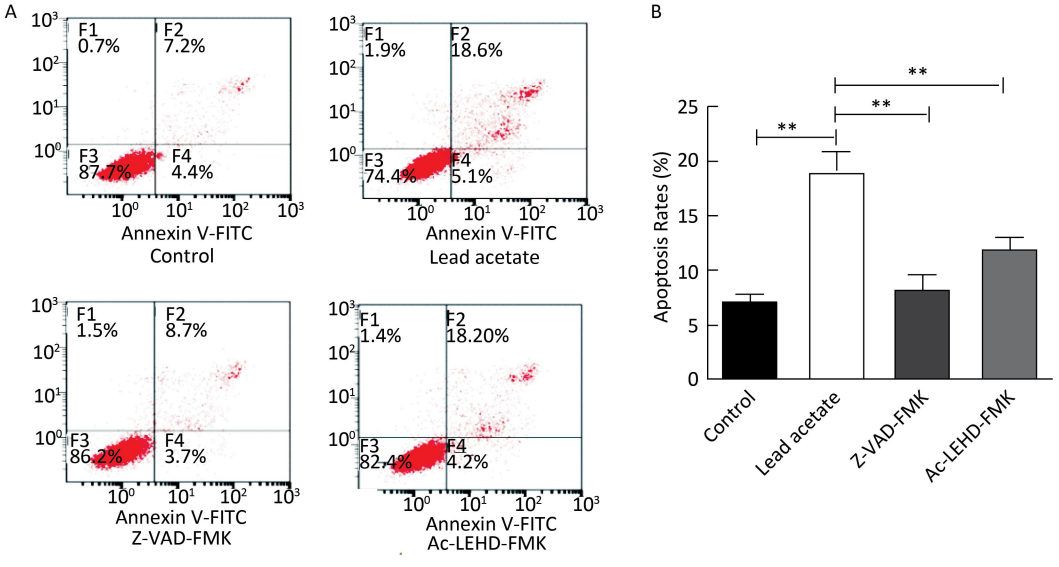

Figure 3. Blockage of caspase-3 and caspase-9 pathways reduced Lead acetate-induced apoptosis. (A) Z-VAD-FMK and Ac-LEHD-FMK inhibitor blocked cells apoptosis was detected by the Annexin V-FITC/PI assay. (B) The bars denote apoptosis rates, vertical bars represent SD in three independent experiments. **means significant difference (P<0.01).

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