

Original Article

Enhanced oxidative stress by lead toxicity retards cell survival in primary thyroid cells

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Abstract: Lead (Pb²⁺) has been appreciated as toxic to mammalian cells, which causes impaired signal transductions and mitochondrial functions. Previous reports have showed that lead toxicity may induce DNA damage, genetic mutations, and chromosome aberrations in many organs or cells. However, how lead toxicity affects thyroid development has not been well elucidated. In present study, we demonstrate that lead toxicity may induce oxidative stress in primary thyroid cells. We found that lead treatment could induce ROS accumulation in thyroid cells, which may be caused by the impairment of oxidative defense systems. The accumulated ROS in thyroid cells further activated apoptotic cascades and accelerated cell death. Our findings reveal that lead toxicity may affect thyroid cell survival by impairing their oxidative defense system and causing oxidative stress. Our work may provide a better understanding of molecular mechanisms in apoptosis of thyroid cells exposed to lead toxicity, and potential targets for heavy metal toxicity in thyroid diseases.

Keywords: Lead toxicity, reactive oxygen species, apoptosis, oxidative defense genes, thyroid cells

Introduction

Various environmental pollutants, including metals can cause toxicological effects on mammals especially human beings [1]. Heavy metals are one of the most important toxic elements due to its impact on human health through the food chain. Among these heavy metals, lead (Pb) is a highly toxic ingredient, which is released from smelting, burning of fossil fuels and municipal wastes, refining of metals, and cigarette smoking [2]. Now, it's well accepted that lead (Pb) has many toxic influences on the health of humans [3, 4]. For example, previous reports showed that lower lead levels may result in the toxicity on the developing central nervous system. In addition, lead also may lead to cognitive and behavioral toxicity and impaired attention span and executive functions [5, 6]. Moreover, lead toxicity has been thought to have carcinogenic effects in clinical cases and animal models [7, 8]. Thus, lead is a potential etiological factor in many human diseases.

Among all of the impairments of heavy metals in mammalian cells, oxidative stress is a promi-

nent feature in many heavy metals-related disorders [9, 10]. Previous findings have shown that heavy metals have the ability to produce reactive oxygen species (ROS), resulting in DNA damage, lipid peroxidation, depletion of protein sulfhydryls and other effects [11-13]. The excess ROS will be potential to interact with many cellular components, causing significant damage to membranes and other cellular structures. So, the major toxicity of heavy metals, such as lead, comes from the generation of excessive ROS. Importantly, it's of urgency to determine how lead toxicity cause ROS accumulation and its downstream molecular pathways.

Previous reports have showed that lead toxicity may induce DNA damage, genetic mutations, and chromosome aberrations in many organs or cells. For instance, Kermani *et al.* showed that lead toxicity may greatly reduce the developmental gene expression and the percentage of differentiated cells of bone marrow mesenchymal stem cells [14]. Also, other reports showed that lead toxicity may induce apoptosis in rat brain [15], rat testis [16], rat fibroblasts [17], rat lung [18], and rat and mouse retinal

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rod cells [19]. In vertebrates, normal thyroid function is essential for development, growth, and metabolic homeostasis [20]. Defects in any step of thyroid development (such as specification, proliferation, migration, growth, organization, differentiation, and survival) may result in a congenital abnormal and impaired hormonogenesis, leading to variable degrees of hypothyroidism [21-23]. The thyroid is sensitive and may be a potential organ for lead accumulation in humans. However, how lead toxicity affects thyroid development has not been well elucidated.

Here, we demonstrate that lead toxicity may induce oxidative stress in primary thyroid cells. Under oxidative conditions, the mitochondria functions of thyroid cells were impaired, and the oxidative defense system was disrupted. Therefore, the viability of thyroid cells was dramatically reduced by lead (Pb) treatment. All these data reveals that lead toxicity may affect thyroid cell survival by impairing their oxidative defense system and causing oxidative stress. Our work may provide a better understanding of molecular mechanisms in apoptosis of thyroid cells exposed to lead toxicity, and potential targets for heavy metal toxicity in thyroid diseases.

Materials and methods

Chemical and materials

For cell culture, DMEM and Fetal Bovine Serum (FBS) for primary mouse thyroid cell culture were purchased from GIBCO Invitrogen (Carlsbad, CA, USA), as well as antibiotics. The Hoechst kit was from Beyotime Biotechnology Co. (Haimen, Jiangsu, China). The ROS probe for reactive oxygen species detection was from Invitrogen (Carlsbad, CA, USA). In the western blot assays, all of the antibodies were of high quality and confirmed. The cleaved-caspase 3, and PGC1- α antibodies were from Cell Signaling Technology (Danvers, MA, USA). The Bax, Bcl-2, SOD1, SOD2 and catalase antibodies were from Abcam (Cambridge, UK). And anti-GAPDH was obtained from Millipore (Billerica, MA, USA). Other chemicals were of the highest purity available.

Primary mouse thyroid cell culture

Studies were approved by the Research Ethics Committee of the 2nd Affiliated Hospital of

Harbin Medical University. Mouse thyroid cells were prepared from C57BL/6 mice. Tissue was minced and digested in a 50-ml falcon tube with type II collagenase (Worthington Biochem, Lakewood, NJ) at a concentration of 5 mg/ml (units per vial between 18,000 and 20,000) for 3 h at 37°C. After 3 h, 10 ml medium (DMEM, 4.0 mM L-glutamine and sodium pyruvate, 10% FBS, and 1% antibiotic antimycotic solution) was added to the tube, and the product was strained through a 70- μ m cell strainer (BD Falcon, San Jose, CA) into another 50-ml falcon tube. The tube was centrifuged at 1000 rpm for 5 min. The supernatant was carefully removed from the pellet, and the pellet was washed with another 10 ml medium. Again, the tube was spun at 1000 rpm for 5 min. The medium was removed from the pellet. The pellet was suspended in 10 ml medium, and the cells were plated in an incubator at 37°C with 5% CO₂. For Pb²⁺ treatment, the final concentration (10 μ M) of Pb²⁺ was applied to thyroid cells for 0 to 24 h. Equivalent PBS was used as internal controls. Notably, thyroid cells were plated in 6-well plates at 1.0 \times 10⁵ cells/mL for Hoechst staining and ROS staining, and 1.0 \times 10⁶ cells/mL for Western blots and real-time PCR assay.

Hoechst staining and ROS staining

For the preparation of Hoechst/ROS staining, thyroid cells were plated with 1.0 \times 10⁵ cells/mL in 6-well plates. After Pb²⁺ treatment, thyroid cells were directly stained with Hoechst kit from Beyotime for Hoechst staining and ROS probe for ROS staining as instructions. The cell counting was carried out through the use of National Institutes of Health software ImageJ.

Assay of western blot

To detect the protein level alternations by Pb²⁺ treatment, immunoblot was carried out as standard procedures. Briefly, the cultured thyroid cells were sonicated with PBS buffer containing 1% Triton X-100 and protease inhibitors, and protein was extracted. The cell lysate supernatants were harvested by centrifugation at 10,000 rpm for 10 min at 4°C, and the supernatants were isolated. Protein contents were evaluated, and measured using BCA Protein Assay kit. Same amount of proteins were loaded into SDS-PAGEs. Finally, proteins were detected by Super Signal® enhanced chemiluminescence development (ECL) (Thermo Scientific Pierce)

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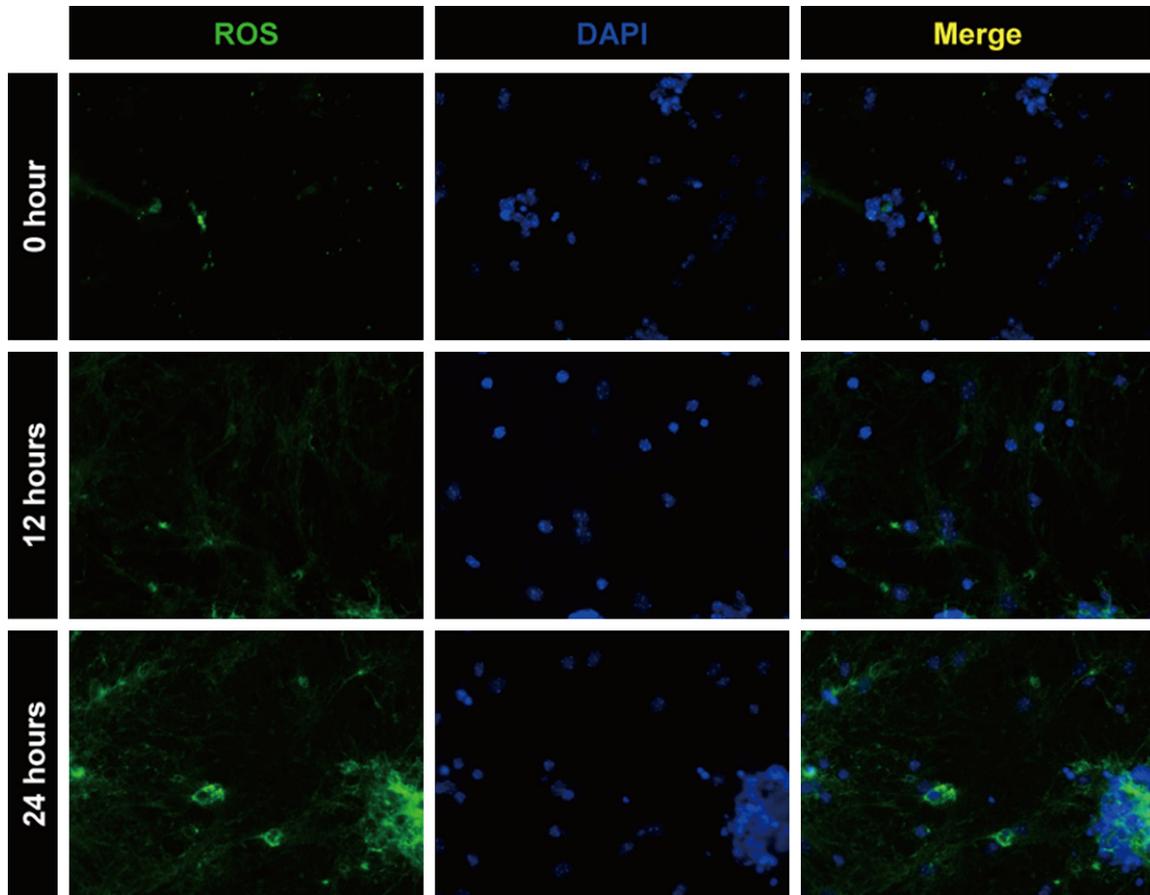


Figure 1. Lead toxicity induces ROS accumulation in cultured thyroid cells. Reactive oxygen species (ROS) probe staining showed that the accumulated ROS was observed by Pb²⁺ treatment (10 μ M for 0 to 24 h) in cultured thyroid cells. Green fluorescence indicated ROS accumulation, and blue fluorescence indicating DAPI.

reagent and exposed to films (Kodak). The protein level quantification was carried out by ImageJ.

Assay of real-time PCR

Total RNA were isolated from cultured cells for reverse transcriptions. Quantitative real-time PCR was performed using Bio-Rad iQ5 system, and the PCR products were analyzed using Bio-Rad proprietary iQ5 software (Hercules, CA, USA). The relative expression levels were analyzed using GAPDH as internal control. Primer sequences for SYBR Green probes of target genes were as follows: *Pgc1- α* : CTCCTGTG-GATGAAGACGG and GCAAATCACAATCACAGG; *Sod1*: CAAGCGGTGAACCGATTGTG and TGAG-GTCCTGCACTGGTAC; *Sod2*: GCCTGCACTGAAG-TTCAATG and ATCTGTAAGCGACCTTGCTC; *Catalase*: ACCCTTTATACCAGTTGGC and GCAT-GCACATGGGGCCATCA.

Statistical analysis

All statistical analysis was performed by Image software. Quantitative data were showed in $\bar{x} \pm s$ using ANOVA tests for comparisons. The value 0.05 (*), 0.01 (**), and 0.001 (***) was analyzed as the level of significance for the statistic tests.

Results

Lead toxicity induces ROS accumulation in cultured thyroid cells

Previous reports have showed that heavy metals toxicity may induce the production of ROS, and further mediate cell death in various cell lines [11, 12, 17]. To determine whether lead toxicity could induce oxidative stress in thyroid cells, we carried out ROS staining firstly. Images showed that lead toxicity indeed induced ROS

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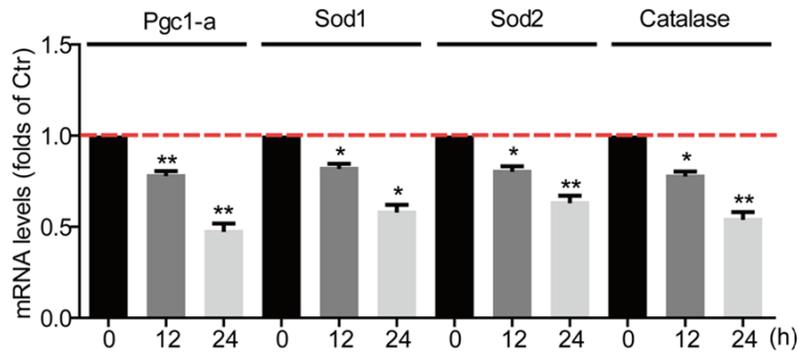


Figure 2. Lead toxicity reduces mRNA levels of oxidative defense genes in thyroid cells. Real-time PCR results showed that the decreased transcription levels of well-known oxidative defense genes, *Pgc1- α* , *Sod1*, *Sod2* and *Catalase*, are observed by Pb^{2+} treatment (10 μ M for 0 to 24 h) in cultured thyroid cells. Results are averages of three independent experiments. Data represent mean \pm SEM. * $P < 0.05$, and ** $P < 0.01$.

generation in the cell bodies of cultured thyroid cells in a time dependent manner, as shown in increasing fluorescence intensity (**Figure 1**). We noted that the accumulation of ROS in cultured thyroid cells exposed to 10 μ M lead for 24 h was higher than those in 12 h exposure. Therefore, our data clearly showed that ROS accumulation has occurred in thyroid cells exposed to lead toxicity.

Lead toxicity impairs expressions of oxidative defense genes in thyroid cells

Since lead treatment could induce ROS accumulation in thyroid cells, we wonder how the oxidative defense system responded to the lead toxicity. Thus, we examined the gene expressions of oxidative defense genes in lead-treated thyroid cells. Real-time PCR results showed that the mRNA levels of *Pgc1- α* gene decreased significantly when cells treated by 10 μ M lead for 12 and 24 h, and the levels were 79% and 48.3% of control untreated cells for 12 and 24 h, respectively (**Figure 2**). Moreover, the mRNA levels of *Sod1* and *Sod2* in the treated groups showed decreased trend compared to those in the control, and the values decreased by 59% and 64% for 24 h treatment (**Figure 2**). After treatment for 12 and 24 h, it was also found that the mRNA levels of *Cat* gene were less in the treated groups than those in the control group, with the values decreasing by 78.7% and 55% compared to the control, respectively (**Figure 2**). All these data indicates that lead-treatment may downregulate oxidative defense gene transcriptions.

Based on the results of mRNA analysis, we next examined the protein levels of these oxidative defense genes (PGC-1 α , SOD1, SOD2 and Catalase). Biochemical results showed that protein levels of PGC-1 α , SOD1, SOD2 and Catalase decreased by about 35%, 55.7%, 69.3% and 55% after 24 h treatment compared to those in the controls, respectively (**Figure 3A** and **3B**). The changes in these oxidative defensive proteins were highly consistent with the quantitative changes of

mRNA expression levels. Therefore, all these findings suggest that lead toxicity may induce the generation of excessive ROS, at least partly by inhibiting the expression of oxidative defense genes.

Lead toxicity induces apoptosis in cultured thyroid cells

We have proved that lead treatment could induce excessive ROS accumulation in thyroid cells. Because ROS accumulation was a direct factor for apoptosis initiation, we next investigated whether the cell survival was affected by lead treatment in thyroid cells. To determine whether lead toxicity affects thyroid cell survival, we quantified the apoptosis of thyroid cells exposed to 10 μ M lead treatment for 12 and 24 h. By Hoechst staining and quantifications, we found that apoptosis percentages were 22.8% and 72.3% at 12 and 24 h, respectively (**Figure 4A** and **4B**).

To further analyze the effects of lead-induced apoptosis in thyroid cells, we examined the apoptotic cascade activations. Western blots results showed that the protein levels of cleaved caspase-3 (apoptosis marker) at 12 and 24 h were 1.72 and 3.76-folds compared to the control, respectively. These results confirmed that lead toxicity may induce apoptosis in thyroid cells, which may be related to the activation of caspase-3. Further, we found that Bax protein levels increased by 2.85 and 3.23 folds in thyroid cells exposed to 10 μ M lead for 12 and 24 h compared to those of the control

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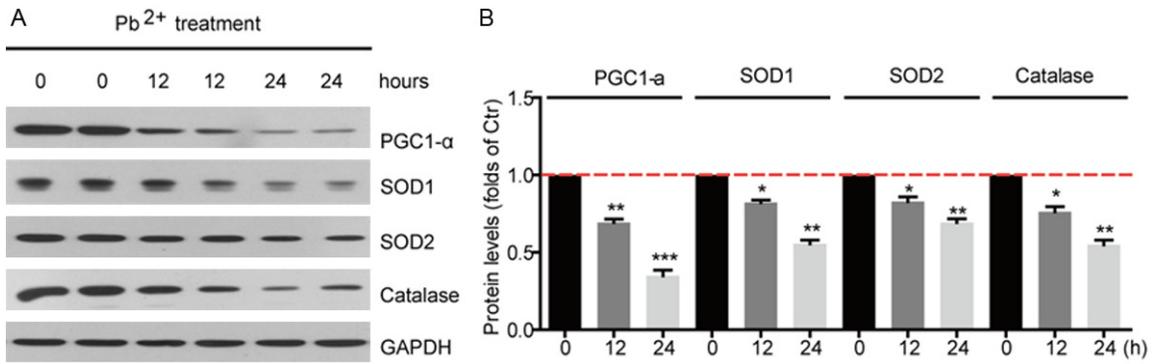


Figure 3. Lead toxicity reduces protein levels of oxidative defense genes in thyroid cells. Western blots (A) and quantifications (B) showed that the reduced protein levels of oxidative defense genes were observed, including PGC-1 α , SOD1, SOD2 and Catalase, by Pb²⁺ treatment (10 μ M for 0 to 24 h) in cultured thyroid cells. Results are averages of three independent experiments. Data represent mean \pm SEM. *P<0.05, **P<0.01 and ***P<0.001.

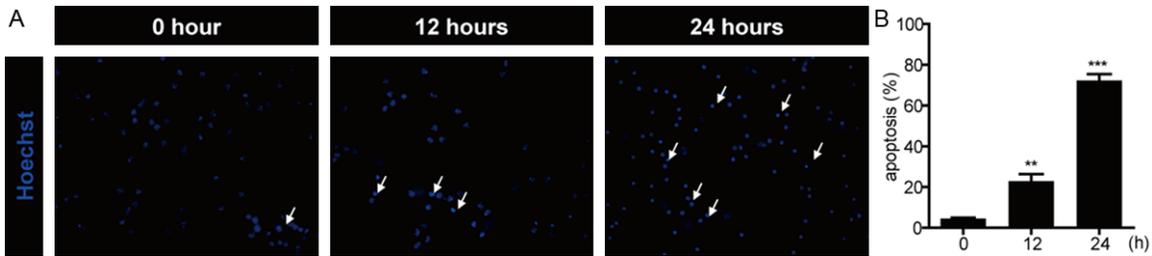


Figure 4. Lead toxicity induces apoptosis in cultured thyroid cells. Hoechst staining (A) and histograms (B) showed that the increased cell death (%) after Pb²⁺ treatment (10 μ M for 0 to 24 h) in cultured thyroid cells was found. White arrows show apoptotic cells. Data represent mean \pm SEM. **P<0.01 and ***P<0.001.

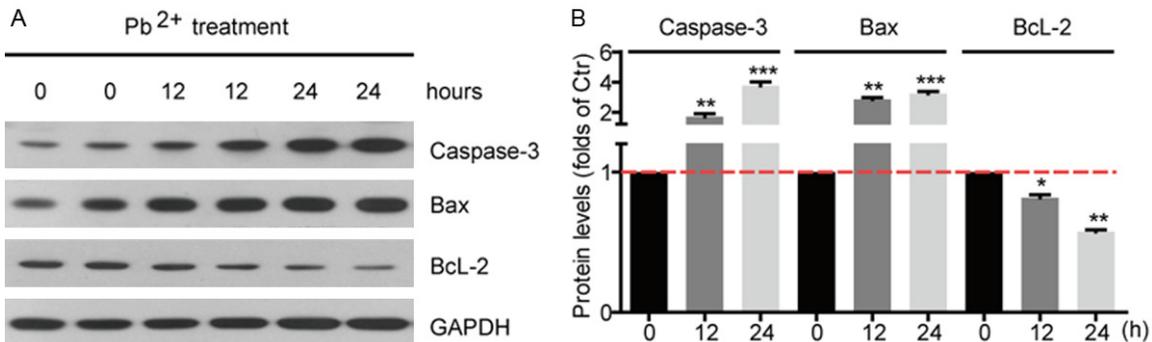


Figure 5. Lead toxicity activates apoptotic cascades in cultured thyroid cells. Western blots (A) and quantifications (B) showing the increased Caspase-3, Bax and decreased Bcl-2 protein levels by Pb²⁺ treatment (10 μ M for 0 to 24 h) in cultured thyroid cells, confirming robust apoptosis. Results are averages of three independent experiments. Data represent mean \pm SEM. *P<0.05, **P<0.01 and ***P<0.001.

values, respectively. Whereas the Bcl-2 protein levels significantly decreased to 81.7% and 57.3% of the control value exposed to 10 μ M lead for 12 and 24 h. The alternation of Bax/Bcl-2 support the notion that lead toxicity activate apoptotic pathways and inactivate anti-apoptotic pathways (Figure 5A and 5B). Taken together, all of these data clearly showed that

lead toxicity may dramatically induce apoptosis in cultured thyroid cells, which may be caused by excessive ROS accumulations.

Discussion

Nowadays, evidence is emerging that heavy metals, such as lead, has toxicity to mammali-

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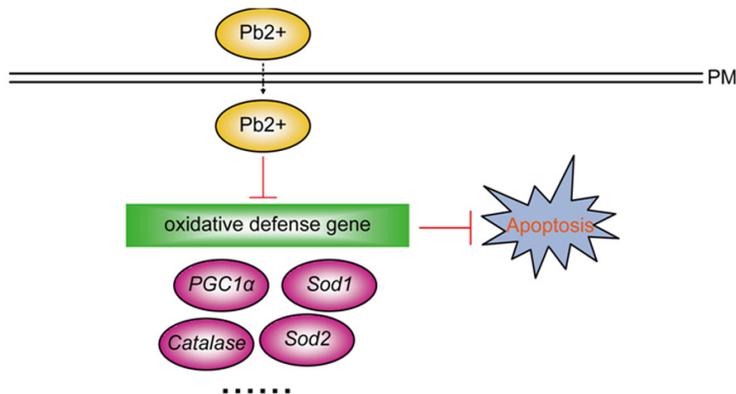


Figure 6. Schematic representation highlighting the mechanism of lead (Pb^{2+}) on thyroid cell apoptosis. Overloaded Pb^{2+} entry inactivates oxidative defense genes, such as PGC-1 α , SOD1, SOD2 and catalase in cultured thyroid cells, which subsequently leading to ROS accumulations and finally apoptosis.

an cells [24-26]. For example, it has been indicated that developing central nervous system is especially sensitive and vulnerable to lead toxicity [2, 27]. Although much progress has been made, how lead toxicity affects thyroid development has not been well elucidated. Here, the present study has designed to analyze if lead toxicity induce ROS accumulation, oxidative stress and apoptosis in cultured thyroid cells, for better understanding the molecular mechanisms of lead toxicity in humans. We found that lead treatment could induce ROS accumulation in thyroid cells, which may be caused by the impairment of oxidative defense systems. The accumulated ROS in thyroid cells further activated apoptotic cascades and accelerated cell death (Figure 6).

It has been widely accepted that ROS generation is significantly induced by heavy metals toxicity. Such changes might be associated to the disruption of cellular membrane integrity and mitochondrial dysfunction in the cells [27]. Excessive ROS may activate several defensive reactions and signal pathways, and further induce wide-ranging damage to proteins, nucleic acids and lipids, eventually leading to cell death [13]. Moreover, some reports showed that excessive ROS also was considered as stress factor, and caused apoptosis [12, 28]. All these findings showed that excessive ROS in cells will result in cellular and membrane damage, and further lead to biochemical changes and induce cell toxicity. Here, our results confirmed the lead toxicity on mitochondrial oxida-

tive defense systems. Moreover, our work figure out that lead toxicity could downregulate mitochondrial genes related to oxidative defense, which may enhance lead toxicity on developing thyroid cells.

Increased ROS has been shown to trigger oxidative stress in cells, which may lead to damage to proteins, nucleic acids and lipids of cell membrane, eventually result in cell death. Thus, cells will need to develop a series of defensive mechanisms avoiding harm from these biochemical and physiological changes. SOD and CAT are often

marked as effective defensive molecules when the cells and/or organ are exposed to heavy metals [12, 27]. Notably, our findings suggested that in thyroid cells, exposure to lead may inhibit the expression levels in SOD and CAT at tested concentration. Interestingly, peroxisome proliferative activated receptor- γ co-activator 1 α (PGC1- α) play important roles in avoiding harm from oxidant stress [29]. Our results indicated that the changes in PGC1- α showed a trend similar to those of SOD and CAT. The decreases in these oxidative defense genes/proteins may be responsible for cell death of thyroid cells by excessive ROS accumulation.

Apoptosis is complex and controlled at multiple pathways in the cells, and the extrinsic and intrinsic apoptosis are considered as two main pathways. The extrinsic apoptosis pathways that external signals interact with death receptors, and further activate caspase-8 and caspase-3/-7. The intrinsic apoptosis pathways that initiate cytochrome c release by the loss of mitochondrial transmembrane potential, and further activate caspase cascade [30]. In present study, lead toxicity resulted in an increase in Bax and cleaved caspase-3 protein levels in thyroid cells. Bax is a second regulator of cellular responsiveness, which located in the monomer form in the cytosol or is loosely bound to the outer mitochondrial membrane. The overexpression of Bcl-2 may inhibit apoptosis, and a predominance of Bax to Bcl-2 accelerates apoptosis upon apoptotic stimuli. Moreover, Bcl-2 and Bax interactions are considered as a

model where the cell's fate can be changed by changing the balance or ratio of Bax and Bcl-2 protein expression. Here, we found that enhanced Bax protein levels may help to activate caspase-dependent apoptosis pathway when thyroid cells are exposed to lead. Moreover, decreased protein levels of Bcl-2 were observed by lead treatment in a time-dependent manner. All the present findings suggested that lead toxicity may sustainable induce mitochondrial damage, and further produce the excessive ROS and promote cell death in thyroid cells.

Conclusion

In summary, the present study indicated that lead toxicity may induce oxidative stress in primary thyroid cells. Under oxidative conditions, the mitochondria functions of thyroid cells were impaired, and the oxidative defense system was disrupted. Therefore, the viability of thyroid cells was dramatically reduced by lead treatment. Our findings reveal that lead toxicity may affect thyroid cell survival by impairing their oxidative defense system and causing oxidative stress.

Disclosure of conflict of interest

None.

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