Biological Trace Element Research (2020) 198:644–653
https://doi.org/10.1007/s12011-020-02094-y

Selenium Prevents Lead-Induced Necroptosis by Restoring Antioxidant Functions and Blocking MAPK/NF-κB Pathway in Chicken Lymphocytes

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Received: 26 November 2019 / Accepted: 24 February 2020 / Published online: 12 April 2020

Abstract
Recent studies have identified a new existence of a genetically programmed and regulated cell death characterized by necrotic cell death morphology, termed necroptosis. Lead (Pb) is a ubiquitously distributed environmental pollutant that is highly toxic to animals and human beings. However, no detailed report has been conducted on the necroptosis in lymphocytes caused by Pb. Selenium (Se), a trace element in the body, has been shown to exert cytoprotective effect in numerous pathological injury caused by heavy metals. Here, lymphocytes isolated from chicken spleen were divided into four groups, control group, Se group, Pb group, and Pb + Se co-treatment group to investigate the potential mechanism in the necroptosis triggered by Pb and in the antagonistic effect of Se on Pb toxicity. Flow cytometry analysis and AO/EB staining showed Pb caused typical necrosis characteristics in the lymphocytes. The expression of RIP1, RIP3, and MLKL was increased, whereas the level of caspase 8 was declined in Pb group, which proved the occurrence of necroptosis. Meanwhile, Pb exposure disrupted the antioxidant enzyme (SOD, GSH-Px, and CAT) balance, promoted the expression of MAPK/NF-κB pathway factors (ERK, JNK, p38, NF-κB, and TNF-α), and activated HSPs (HSP27, HSP40, HSP60, HSP70, and HSP90). However, those Pb-induced changes were significantly alleviated in Se + Pb group. Our study revealed that Pb could trigger lymphocyte necroptosis through MAPK/NF-κB pathway activated by oxidative stress and that Se could antagonize Pb-induced necroptosis in chicken lymphocytes.

Keywords Lead · Selenium · Necroptosis · Lymphocytes · Chicken

Introduction

Lead (Pb) is a kind of heavy metal that is widely distributed in nature [1]. Under natural conditions, Pb is regarded as a low-level element, and the mean environmental concentration in soil is 16 ppm in the USA [2]. Due to excessive fossil fuel combustion, sewage discharge, and other industrial consumption, Pb has been viewed as a persistent global environmental pollution, thus threatening the health of humans and animals [3, 4]. In Paraguay, Lebanon, and Russia, the average total Pb concentration of the purchased enamel decorative paints reached 24,500 ppm (ppm, dry weight), which is 270 times higher than the limit Pb concentration threshold (90 ppm) in the USA [5]. The negative effects of Pb on living have received widespread attentions. Pb can spur diverse form of toxicity, such as reproductive and neurological dysfunction, anemia, and cancer [6–8]. Meanwhile, a large number of researchers have proved that Pb causes damage to immune tissues and cells. For example, it inhibited the formation of neutrophil extracellular traps in chicken neutrophils stimulated with phorbol myristate acetate (PMA) [9, 10]. One mechanism of action of Pb toxicity is to induce the occurrence of oxidative stress in organs. It has been reported that Pb caused oxidative stress and increased interleukin (IL-2, IL-4, IL-6, IL-12β, and IL17) and interferon gamma (IFN-γ), thus inducing immune damage in chicken bursa of Fabricius [11].

Additionally, oxidative stress is an initiator of numerous inflammatory signaling pathways in cells and mediates multiple cell death patterns, including necroptosis [12–14]. Necroptosis is a regulated form of cell death and generally manifests with morphological features of necrosis [15, 16]. This special death mode is mainly driven by the activation

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of TNF-α involving receptor-interacting serine-threonine kinase 1 (RIP1), RIP3, and mixed lineage kinase domain-like (MLKL) [17, 18]. It has been reported that the oxidative stress induced by hyperoxia exposure caused the pulmonary necroptosis in rat via the activation of RIP1, RIP3, and MLKL [19]. Meanwhile, aspartate-specific cysteine protease (caspase 8) plays a crucial role in the necroptosis activation, and its expression inhibition can change the cell death direction from apoptosis to necroptosis [20]. Recently, toxicological studies have reported that necroptosis can be induced by many harmful substances. For example, human lung epithelial and endothelial cells exposed to cigarette smoke exhibits damaged mitochondria and triggers mitophagy-driven necroptosis [21]. Cadmium (Cd) exposure mediates necroptosis and hepatic injury in hepatocytes of Institute of Cancer Research (ICR) mice [22, 23].

Se is a widely recognized trace element which can antagonize the toxicity of heavy metals and reduce their contents in organs [24]. Xu et al. proved that Se and/or Pb could change the ion profiles in liver of chicken, indicating that Se protects against the hepatotoxicity effects of Pb [25]. Meanwhile, it has been found that dietary Se supplementation reduces Pb-induced oxidative stress, apoptosis, and mitochondrial damage in chicken kidney tissue [26]. In addition, the analysis of serum indicates Se alleviates the Th1/Th2 imbalance induced by Pb in the peripheral blood lymphocytes of chickens [27, 28]. Lymphocytes are one of the important components of the immune system in vivo, capable of immune response and elimination of invasive viruses and mutant cells [29]. In the present study, we isolated the lymphocytes from the spleen of chicken; examined the changes in necroptosis, antioxidant enzymes, mitogen-activated protein kinase/noncanonical nuclear factor-κB (MAPK/NF-κB) pathway-related genes, and heat-shock proteins (HSPs) after Pb, Se, and Pb + Se exposure, respectively; and identified the mechanism of cytotoxicity in chicken lymphocytes resulting from Pb exposure. The results of this study enrich our understanding of Se mitigating Pb damage to the immune system.

Materials and Methods

Ethics Statement and Animals

All procedures conducted in the present study were authorized by the Institutional Animal Care and Use Committee of the Northeast Agricultural University. Eighty layer chickens (90 days) were purchased from local chicken farm. We acclimated these chickens in a special henhouse (25 °C, 12 h light/12 dark cycle) for a week. Drinking water was taken from tap water, and food was provided twice a day.

Isolation and Treatment of Lymphocytes

All eighty chickens were experimented on, and each group was performed on 5 samples of lymphocyte pooled from 5 chickens ($n = 5$). The chickens were euthanized; their spleen tissue was extracted and placed in a petri dish containing PBS in aseptic environment. The lymphocytes were obtained from a chicken spleen lymphocyte separation kit (BeiJing Solarbio Biological Manufacture CO., China). After separation of lymphocytes, cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and diluted to a density of 1×10^6 cells/mL. The lymphocytes were then incubated with Pb (30 μM $(CH3OO)_2Pb$), Se (1 μM Na_2SeO_3), and Pb + Se (30 μM $(CH3OO)_2Pb$ + 1 μM Na_2SeO_3) for 24 h, respectively.

AO/EB Staining

Acridine orange/ethidium bromide (AO/EB) dual staining was performed to analyze cell necroptosis processes of lymphocytes. In brief, after treatment, cells in the culture medium were obtained by centrifugation at 800 *g*/min and rinsed with PBS. Subsequently each group cells were stained with AO/EB for 5 min. The necroptosis fluorescence images were visualized by employing fluorescence microscopy (Thermo Fisher Scientific, USA).

Flow Cytometry Analysis

We used Annexin V-FITC/PI detection kit (KeyGEN BioTECH, China) to quantify cell death. According to the manufacturer's instructions, after 24-h incubation, lymphocytes were treated with 5 μL of Annexin V-FITC and 5 μL of PI in 500 μL binding buffer for 5 min in the dark. Then, the populations of apoptotic, necrotic, and live cells were quantified via flow cytometric analysis.

Biochemical Assays

After 24 h of Pb, Se, and Pb + Se treatment, respectively, the activities of antioxidant enzymes (glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT), and inducible nitric oxide synthase (iNOS)) and contents of oxidative stress markers (malonic dialdehyde (MDA) and nitric oxide (NO)) observed in lymphocytes were detected using detection kits (Nanjing Jiancheng Bioengineering Institute, People's Republic of China) according to the manufacturer's instructions.

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from lymphocytes in the different processing groups using TRIzol reagent. The reverse transcription of mRNA was performed via First-Strand cDNA Synthesis Kit and BioRT Master HiSensi cDNA First Strand Synthesis kit (Bioer technology Co.Ltd., Hangzhou, China), and qRT-PCR was carried out using LineGene 9600 Plus (Bioer technology Co.Ltd., Hangzhou, China) and BioRT Real Time RT-PCR Kit (Bioer technology Co.Ltd., Hangzhou, China). The primers of gene for necroptosis (RIP1, RIP3, MLKL, and caspase 8), MAPK/NF-κB pathway (extracellular signal-regulated kinase (ERK), c-Jun amino terminal kinase (JNK), p38 mitogen-activated protein kinase (p38), NF-κB, and tumor necrosis factor alpha (TNF-α)), and HSPs (HSP27, HSP40, HSP60, HSP70, and HSP90) along with housekeeping gene GAPDH are shown in Table 1.

Western Blot

Western blotting was used to measure the presence of protein in lymphocytes after Pb, Se, and Pb + Se exposure. In brief, the total protein was obtained by RIPA supplemented with 1% PMSF. Supernatants were harvested by centrifugation at 4 °C, 12,000×*g*. The protein concentration was detected by using bicinchoninic acid assay. Protein extracts of each group were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions on 12% gels. Subsequently, the separated proteins were transferred to nitrocellulose membranes in Tris-glycine buffer containing 20% methanol using a tank transfer for 60 min at 200 mA. Then, nitrocellulose membrane was blocked for 2 h with 5% bovine serum albumin (BSA) (diluted in TBST) at 25 °C and was incubated for a night with antibodies used in the present study including MLKL (1:200, produced by our lab), RIP1 (1:200, produced by our lab), RIP3 (1:200, produced by our lab), caspase 8 (1:100, produced by our lab), TNF-α (1:500, Santa Cruz, CA, USA), NF-κB (1:500, Santa Cruz, CA, USA), ERK (1:1000, Santa Cruz, CA, USA), JNK (1:2000, Santa Cruz, CA, USA), p38 (1:1000, Santa Cruz, CA, USA), HSP60 (1:1500, produced by our lab), HSP70 (1:1500, produced by our lab), HSP90 (1:1500, produced by our lab), and GAPDH (1:1500, Sigma, USA).

Statistical Analysis

Each experiment was performed at least three independent times. The statistical significance of the data was applied with one-way analysis of variance using GraphPad Prism software 5. For all experiments, samples with different superscript letters represented statistically significant differences ($P < 0.05$).

Result

Effect of Pb and Se on the Cell Death

Firstly, the rate of death lymphocyte was analyzed by flow cytometry. As is shown in Fig. 1 a, the percentage of necrotic cells in the control group and Se group were 1.42% and 1.57%, respectively. Se alone had no effect on the necrosis rate of lymphocytes. However, the necrosis rate in the Pb group was 36.94% and obviously increased compared with control group. Meanwhile, in the Pb + Se group, the necrosis

Table 1 Gene-special primers used in the real-time quantitative reverse transcription PCR

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
MLKL	CACCGATACCAGGAGCCAGT	AGCCAGCAAGTCCACGATCT
RIP1	TGCCTTCTGTTCCTCACTGG	CCTGCGTAGGTATTGCAGCTT
RIP3	ACATCCTTCGCTCACAGCAA	ACCTGTGCTGCCTTCTCTCC
Caspase8	CACTGTGCCAACACTTGGAG	CATTTGCTTCCCTGCATTCT
TNF-α	CAGGACAGCCTATGCCAACA	ACAGCCAAGTCAACGCTCCT
NF-κB	TGGACCTGAAGCGACCTCTGC	ACAGGATCAAGCCTCCGTGT
ERK	TGGTACAGGGCTCCTGAAAT	GGAAAGATGGGTCTGTTGGA
JNK	ATTACGCCTTCTGCCTTGTG	AAAGCGCTGCATAAATGCTT
P38	GCATCCATCTTCGTCGTCAT	TCATCTACAGCAACCCAGAGG
HSP27	ACACGAGGAGAAACAGGATGA	ACTGGATGGCTGGCTTGG
HSP40	GGGCATTCAACAGCATAGA	TTCACATCCCCAAGTTTAGG
HSP60	AGCCAAAGGGCAGAAATG	TACAGCAACAACCTGAAGACC
HSP70	CGGGCAAGTTTGACCTAA	TTGGCTCCCACCCTATCTCT
HSP90	CAGTTGGTGGTGCACGATG	AGGTGGCATCTCCTCGGT
GAPDH	GAAGGCTGGGGCTCATCTG	CAGTTGGTGGTGCACGATG

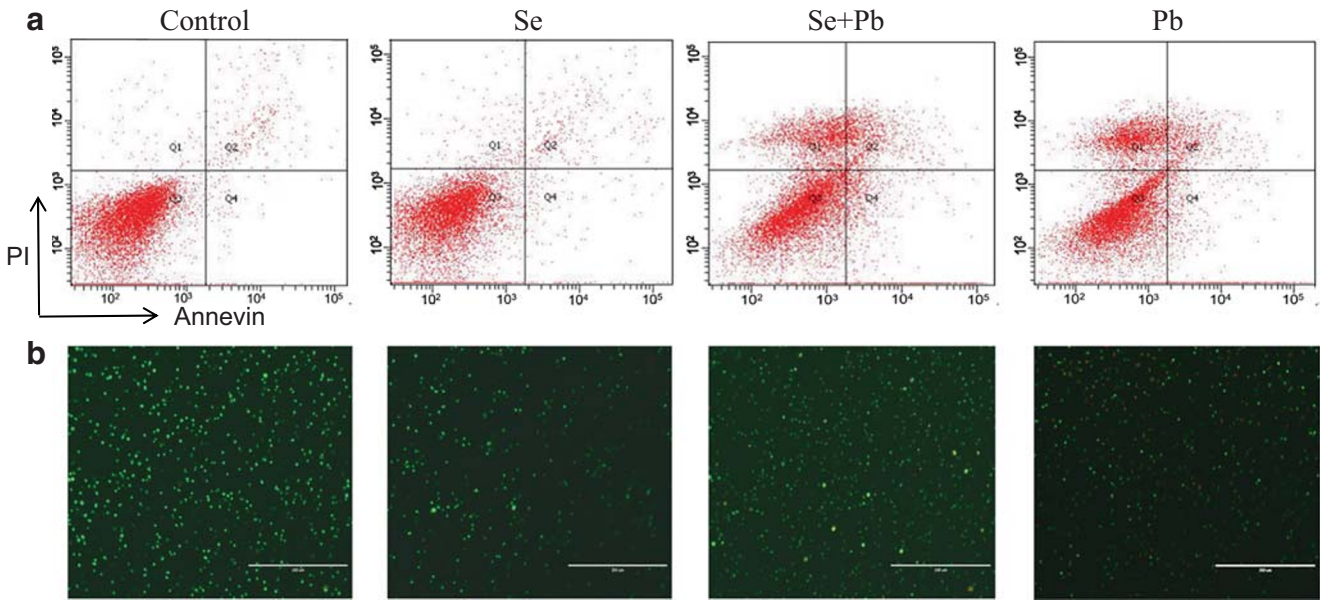

Fig. 1 Measurement of lymphocyte necroptosis isolated from the spleen of chickens. Lymphocytes were treated with Se, Pb, and Se + Pb for 24 h. **a** The percentage of necroptosis was measured by flow cytometric. The necrosis in part Q1, survival in part Q4. **b** The detection of necrosis by fluorescence microscopy. The green fluorescence is normal cell nucleus, and the red fluorescence is membranolysis of necrosis

rate was 23.64% and abated obviously than the Pb group. Additionally, AO/EB staining results showed that part cells treated with Pb appeared red, indicating the emergence of necrosis. No morphological changes were observed in the control and Se-treated cells, and the necrosis situation in the Pb + Se co-treatment group was lighter than the Pb group (Fig. 1b).

Effect of Pb and Se on the Necroptosis-Related Genes in Lymphocyte

The necrosis quantitative results of mRNA and protein are shown in Fig. 2. As the results shown in the Pb group (Fig. 2a), RIP1, RIP3, and MLKL mRNA expression quantity had increased severally than the control group, while caspase 8 mRNA expression was decreased. Comparing Pb + Se co-treatment group with Pb group, the mRNA expression quantity of RIP1, RIP3, and MLKL was decreased, respectively, and caspase 8 mRNA was increased. The necroptosis-related protein expression trend was similar to mRNA expression, and the results were shown in Fig. 2 b ($P < 0.05$). When caspase 8 decreased, that usually heightened necroptosis. Therefore, we concluded that Pb significantly activated necroptosis through MLKL activation and that this effect could be alleviated by Se, compared with control group.

Effect of Pb and Se on the Antioxidant Function in Lymphocyte

We next measured effects of Pb and Se on antioxidant capacities of chicken lymphocyte. As is shown in Fig. 3, we found that Se had no effect on lymphocyte antioxidant enzyme activities (GPH-Px, SOD, and CAT) and oxidative stress markers (MDA, iNOS, and NO). However, the activities of GSH-Px, SOD, and CAT were decreased, and contents of MDA, iNOS, and NO were increased in the Pb group cells, compared with control cells. Interestingly, we also found that the inhibition of Pb on the antioxidant function of lymphocyte could be resisted by the addition of Se ($P < 0.05$).

Effect of Pb and Se on MAPK/NF-κB Pathway

Subsequently, the mRNA and protein expressions of MAPK pathway-related genes (ERK, JNK, and P38) and NF-κB pathway key genes (NF-κB and TNF-α) were examined (Fig. 4a, b). The result showed that both Pb + Se co-treatment and Pb generally enhanced the expression of ERK, JNK, P38 NF-κB, and TNF-α in lymphocyte, compared with the cells without treatment ($P < 0.05$), but the expressions of these genes in Pb + Se group were lower than Pb group.

Effect of Pb and Se on HSPs' Expression in Lymphocyte

As HSPs can participate in the response of various compounds including drugs and heavy metal, we determined whether these stress genes play a rule in lymphocyte after Pb and Se treatment. As is shown in Fig. 5a, HSP27, HSP40, HSP60, HSP70, and HSP90 mRNA levels were markedly promoted by Pb relative to those of the control cells, and though Se could not change HSPs' gene

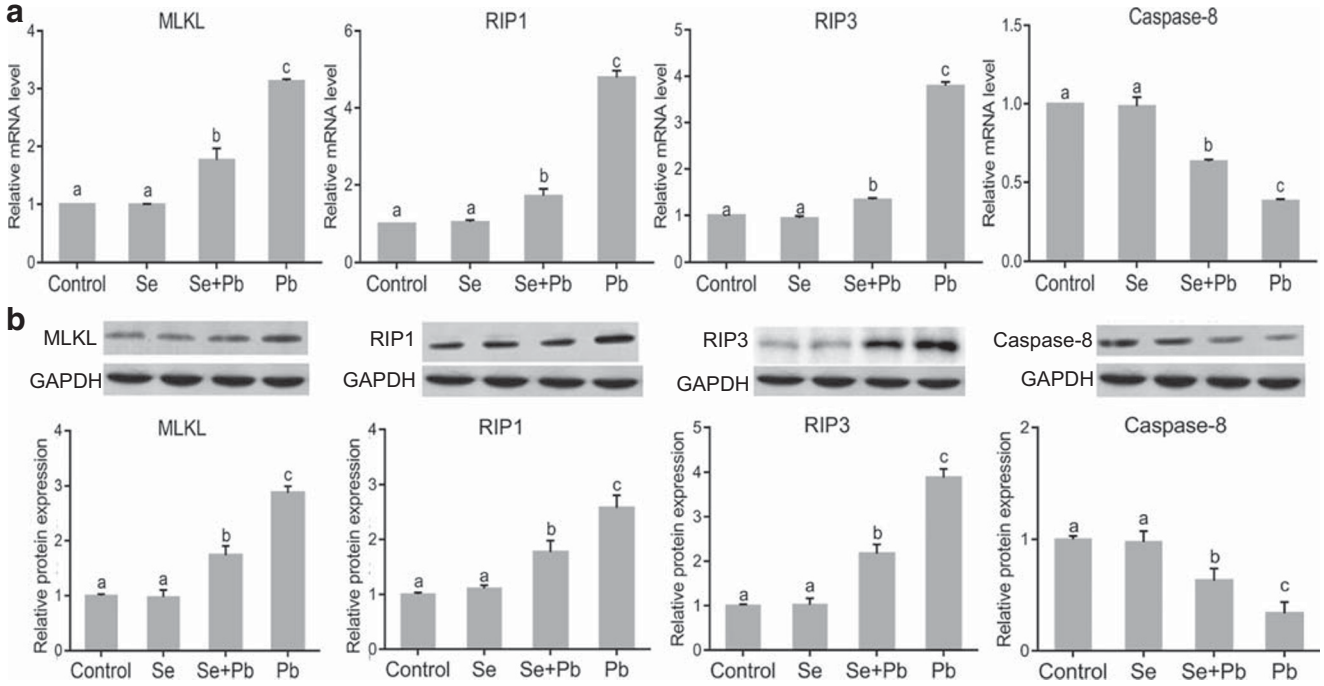

Fig. 2 Measurement of necroptosis-related genes. Lymphocytes were treated with Se, Pb, and Se + Pb for 24 h, respectively. **a** The mRNA expression of MLKL, RIP1, RIP3, and Caspase8 detected by qRT-PCR. **b** The protein expression of MLKL, RIP1, RIP3, and Caspase8 detected by Western blot. The data are presented as the mean ± SD ($n = 3$). Bars with different letters indicate significant difference from the corresponding normal ($P < 0.05$)

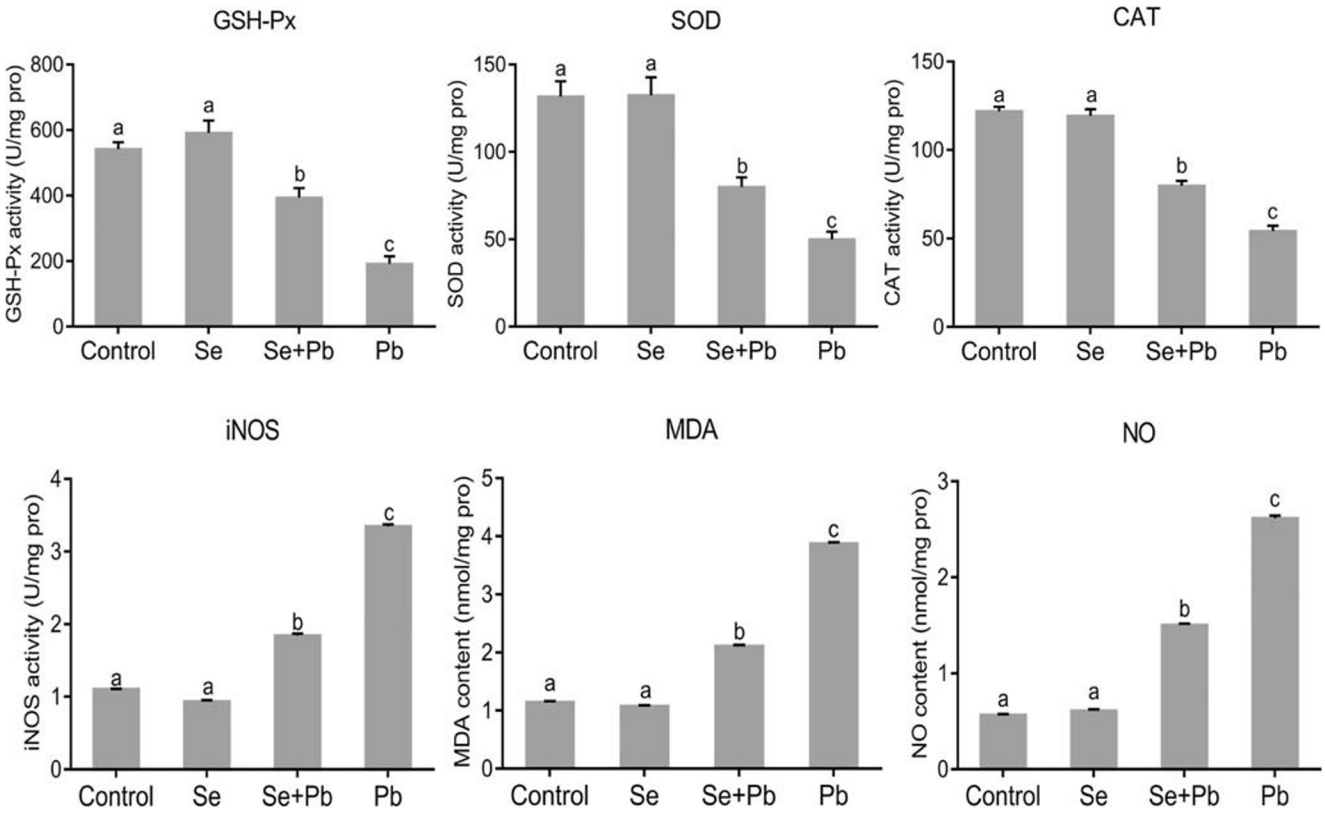

Fig. 3 Measurement of activities of antioxidant enzymes and the content of oxidative stress biomarkers. Lymphocytes were treated with Se, Pb, and Se + Pb for 24 h, respectively. The data are presented as the mean ± SD ($n = 3$). Bars with different letters indicate significant difference from the corresponding normal ($P < 0.05$)

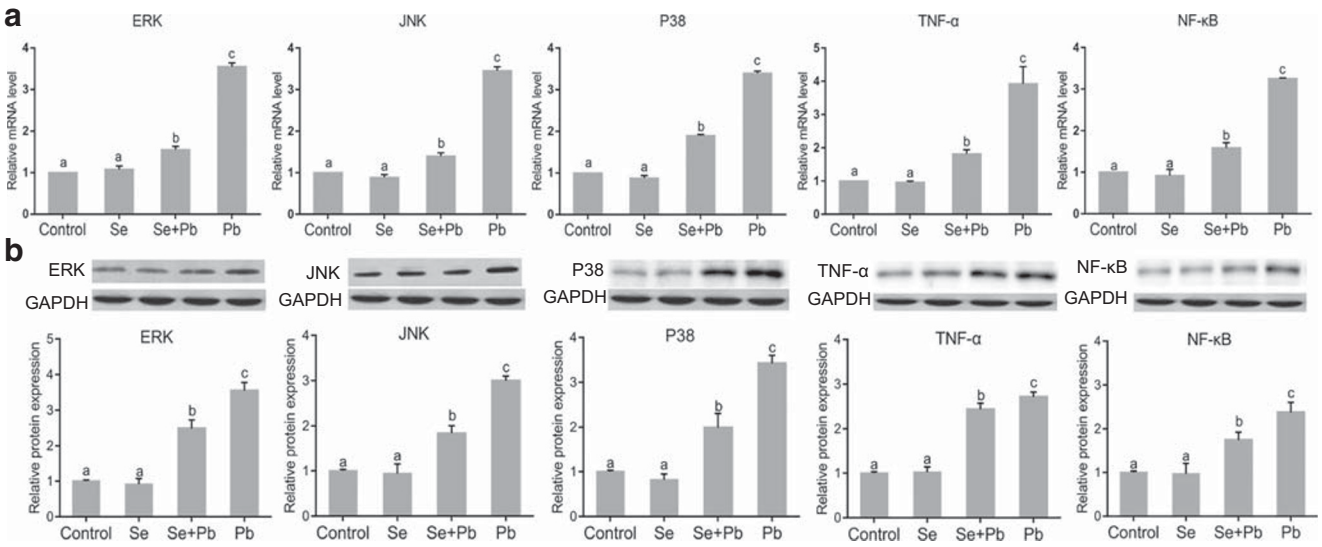

Fig. 4 Measurement of related genes in MAPK/NF-κB pathway. Lymphocytes were treated with Se, Pb, and Se + Pb for 24 h, respectively. **a** The mRNA expression of ERK, JNK, p38, TNF-α, and NF-κB detected by qRT-PCR. **b** The protein expression of ERK, JNK, p38, TNF-α, and NF-κB detected by Western blot. The data are presented as the mean ± SD ($n = 3$). Bars with different letters indicate significant difference from the corresponding normal ($P < 0.05$)

expression, it evidently reduced the promotion of Pb on their expression ($P < 0.05$). At the same time, our detection of HSP60, HSP70, and HSP90 at protein level was similar to the qRT-PCR results (Fig. 5B).

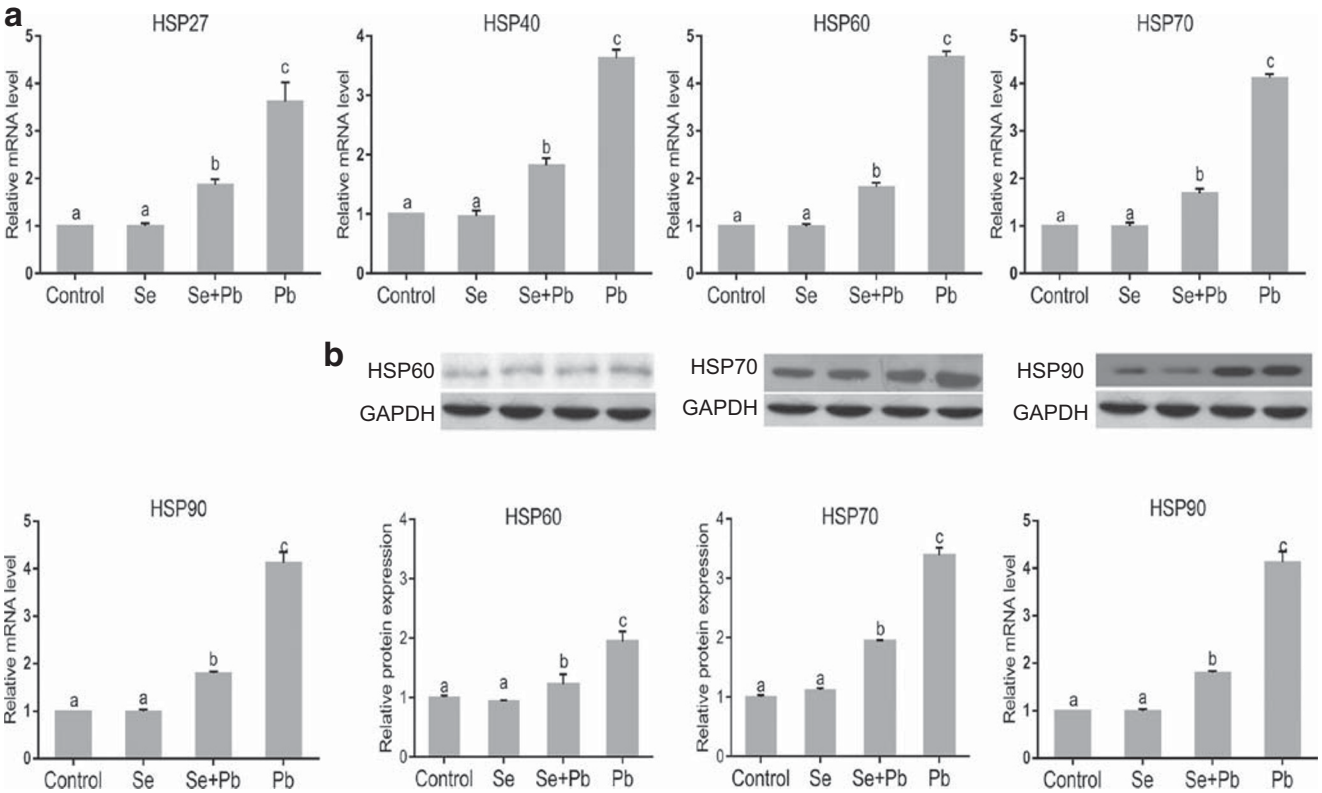

Fig. 5 Measurement of HSPs related genes. Lymphocytes were treated with Se, Pb and Se + Pb for 24 h, respectively. **a** The mRNA expression of HSP27, HSP40, HSP60, HSP70, and HSP90 detected by qRT-PCR. **b** The protein expression of HSP60, HSP70, and HSP90 detected by Western blot. The data are presented as the mean ± SD ($n = 3$). Bars with different letters indicate significant difference from the corresponding normal ($P < 0.05$)

Discussion

As a major heavy metal pollutant, Pb can be absorbed and bioaccumulated from environment to organs of animals and even humans through every step of the food chain [30]. Pb exposure results in decreases of phenoloxidase activity and O_2^- production, disturbs the developing of hepatopancreas and hemolymph, and causes the inhibition of nonspecific immune responses [31]. Analysis of blood lymphocytes in preschool children living in e-waste recycling areas shows that the percentages of $CD4^+$ central memory T cells and $CD8^+$ central memory T cells are increased as well as the percentage of NK cells is decreased with chronic Pb exposure. Numerous studies report that Se, as an essential trace element in the body, can antagonize the toxicity of heavy metal, including Pb [32]. The apoptosis of lymphocytes induced by Pb via the activation of mitochondrial pathway was partially attenuated by Se supplementation [33]. Our results showed that the treatment of Pb in vitro induced necroptosis of chicken spleen lymphocytes through activating RIP3/MLKL pathway gene and inhibiting caspase 8 expression. In addition, Pb exposure induced oxidative stress, decreased the activities of SOD, GSH-Px, and CAT, activated MAPK/NF-κB pathway, and promoted the expression of HSPs. The Se co-treatment reduced these negative effects of Pb on lymphocytes.

It has long been thought that necrotic cell death is only a passive process, not a regulated mechanism [34]. However, there is increasing evidence that a death form called necroptosis is similar to apoptosis in the body and is mediated by cellular processes [35, 36]. Classical necroptosis requires RIP1 for the activation of RIP1/RIP3 necrosome during Caspase8 inhibition by recruiting the downstream mediator MLKL [37]. Heavy metals have the ability to cause necroptosis. Zhang S et al. observed the formation of necrosome and occurrence of necroptosis in hepatocytes after cadmium chloride ($CdCl_2$) exposure [22]. In addition, alkynyl gold(I) complex enters the mitochondria and disrupts its normal function, which triggers necroptosis of colorectal adenocarcinoma Caco-2 cell line [38]. However, relatively little is known of the impacts of Pb exposure on necroptosis. The present study confirmed that exogenous Pb stimulation effectively inhibited the expression of Caspase8 and trigger RIP3-dependent necroptosis in lymphocytes. Se can antagonize Pb toxicity. Meanwhile, our previous research identified that Se deficiency cause the necroptosis in cardiomyocytes through inhibiting ring finger protein 11(RNF11) [39]. The result showed that Se reduced the percentage of necroptosis in lymphocytes isolated from spleen induced by Pb treatment.

Due to the ability to activate multiple inflammatory pathways, oxidative stress has been considered a driving force for RIP3-dependent necroptosis [40]. This complex biological process involves the excessive generation of reactive oxygen species (ROS) and the disturbance of antioxidant systems under adverse stimulation. SOD is believe to be the first line against oxidative stress by capturing superoxide anion (a kind of ROS) to convert into hydrogen peroxide (H_2O_2) [41]. Subsequently, GSH-Px and CAT are responsible for clearing H_2O_2 by binding to and interacting with peroxides and free radicals [42]. Wang et al. found high concentrations of Cd stimulation can inhibit the expression of SOD, CAT, and GSH-Px proteins and cause oxidative stress, thus causing lysosomal dysfunction in primary rat proximal tubular cells [43]. In this study, Pb damaged the activities of SOD, GSH-Px, and CAT and induced oxidative stress in lymphocytes. Se and a variety of Se-rich foods are potent antioxidants, which prevent the oxidative damage by enhancing and stabilizing endogenous antioxidant and biological activities [44]. Consequently, levels of antioxidant enzymes and oxidative stress in Pb-exposed lymphocytes were alleviated by Se treatment.

MAPK pathway is the important downstream inflammatory signaling pathway of oxidative stress and can be activated by the excessive ROS. MAPK pathways composed of ERK, p38, and JNK mediate various biological processes, including cell death, survival, growth, and differentiation. It has been reported that during the treatment of various cancers, cisplatin causes the acute kidney injury (AKI) via necroptosis regulated by the increase of ROS and the activation of ERK and p38 [45]. A study on colon cancer found that 2-methoxy-6-acetyl-7-methyljuglone (MAM) induced necroptosis by mitochondrial ROS elevation, mitochondrial depolarization, and ATP depletion and by activating the JNK pathway [46]. Additionally, hydrogen sulfide gas exposure evoked spleen necroptosis via MAPK pathway mediated by oxidative stress and energy metabolism dysfunction [47]. NF-κB pathway can directly induce the necroptosis formation by inducing TNF-α release and is sensitive to oxidative stress [48–50]. Extensive research suggests that inflammatory factor TNF-α triggered by NF-κB activation is a key controller in the process of necroptosis by modifying RIP1 to recruit RIP3 [51]. In the present study, the increased levels of ERK, JNK, p38, NF-κB, and TNF-α in lymphocytes indicate Pb may regulate necroptosis by evoking MAPK/NF-κB pathway. Dietary intake of the micronutrient Se is essential for normal immune functions and resistance of inflammation [52, 53]. We demonstrated that Pb-induced inflammatory pathway activation was markedly inhibited by co-treatment with Se. Meanwhile, HSP family (HSPs) has ability to respond to stimuli and activate inflammatory responses and necroptosis [54]. HSP60 is one of the most powerful methods of sending a “danger signal” and is involved in the ERK activation [55]. HSP70 can markedly stimulate IL-1β and TNF-α secretion of head kidney leukocytes through JNK, p38, and NF-κB pathways in a dose- and time-dependent manner [56, 57]. HSP90 is a novel MLKL-interacting protein and can regulate the stability of MLKL and RIP3, and the inhibition of HSP0 function decreases the TNF-

induced necroptosis [58]. Our results showed that Pb induced the high expression of HSPs, which could be restored by Se co-treatment. In view of these results, we identify that Se may partially alleviate the Pb-induced necroptosis by inhibiting HSPs' activation.

Overall, Se protects against Pb-induced necroptosis in leukocytes by restoring antioxidant function, blocking MAPK/NF-κB pathways, and inhibiting the HSPs' activity. These findings give a novel insight into the concrete mechanism of Pb-induced immunotoxicity and further expand the understanding of signaling pathways involved in Se-mediated immunoprotection.

Author's Contribution Shiwen Xu and Gang Sun conceived of and designed the experiments. Jiayong Zhang and Xiaofang Hao performed the experiments. Jiayong Zhang analyzed the data and wrote the paper. Shiwen Xu assisted in critically revising the manuscript.

Funding Information The research was supported by funds provided by the China Agriculture Research System-41-17 and the National Key Research and Development Program of China (No. 2016YFD0500501).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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